# Isolation and characterization of a gene required for peroxisome biogenesis from the yeast *Pichia pastoris*

**Weiqiao Xie**

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The dissertation "Isolation and characterization of a gene required for peroxisome biogenesis from the yeast *Pichia pastoris*" by Weiqiao Xie has been examined and approved by the following Examination Committee:

James M. Cregg, Thesis advisor
Associate Professor

Matthew S. Sachs
Assistant Professor

Margaret Alic
Research Scientist

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## ABSTRACT

# Isolation and characterization of a gene required for peroxisome biogenesis from the yeast *P. pastoris*

Weiqiao Xie, M.S.

Supervising Professor: James M. Cregg

This thesis describes the cloning and analysis of *PER6*, a gene required for peroxisome biogenesis in *Pichia pastoris*. The gene was cloned by functional complementation of a *per6 P. pastoris* mutant strain that was one of a number of peroxisome-deficient mutants isolated in this laboratory. The complementing activity was localized to a small DNA fragment by subcloning and Northern filter hybridization analysis and the DNA sequence of the fragment was determined. The sequence revealed a 1296-bp open reading frame which potentially encodes a 432-amino acid protein of 49 kD. The gene was transcribed into a message of 1.4 kilobases that was constitutively expressed but induced several-fold in cells growing on methanol. A mutant strain with a deletion of a large portion of the open reading frame was constructed and used to genetically demonstrate that the cloned gene was identical to the defective gene in the originally isolated *per6* mutant. The predicted amino acid sequence of the *PER6* product revealed several interesting features, including a significant regional similarity to *PAF-1*, a gene known to be defective in some patients with Zellweger syndrome, a lethal human genetic disease caused by peroxisome deficiency. Finally, the *PER6* product was produced in *E. coli* and purified to serve as antigen for antibody production.

# CHAPTER 1
# INTRODUCTION

## 1.1 Functions of peroxisomes

The peroxisome is a single-membrane enclosed eukaryotic organelle that was first described in the early 1950s in mouse kidney cells (Rhodin, 1954). Initially given the morphological name microbody, the organelle was later characterized biochemically and given the functional name peroxisome (Baudhuin, et al., 1965). Among organelles, peroxisomes are unusual in that the metabolic reactions they contain vary depending upon organism, tissue and even environmental conditions. In particular, changes in the environment induce dramatic morphological and enzymatic responses in peroxisomes (van den Bosch, et al., 1992).

The unifying feature that defines peroxisomes is the presence of at least one hydrogen peroxide-generating oxidase as part of each metabolic pathway that involves the organelles. These oxidases use oxygen as the oxidizing reagent and produce hydrogen peroxide ($H_2O_2$) as the reducing product. Since $H_2O_2$ is reactive and toxic, enzymes exist in the peroxisome to rapidly destroy this product. Two types of peroxisomal $H_2O_2$ decomposition enzymes have been identified. The first is catalase, which performs a catalitical reaction that converts $H_2O_2$ into water and oxygen. The second enzyme type performs a peroxidative reaction that reduces $H_2O_2$ to water with the concomitant oxidation of a second substrate such as ethanol or formate or certain nitrites and quinones (Chance and Oshino, 1971). The peroxisomal location of these cellular respiration reactions compartmentalizes $H_2O_2$ production along with the peroxidative reactions that detoxify it and serves to limit the leakage of $H_2O_2$ into the cytosol (Chance and Oshino, 1971). As a result, the cell is able to perform essential

oxidative reactions without suffering damage from their damaging intermediate.

Peroxisomes have been extensively examined in animals (Borst, 1989), plants (Beevers, 1979), protozoans (Müller, et al., 1968; Opperdoes, 1987), and fungi (Veenhuis, et al., 1983). In this chapter, we briefly review the function and morphology of peroxisomes in selected organisms, as well as the current state of knowledge on peroxisome biogenesis.

### 1.1.1 Peroxisomes in humans and other mammals

In mammalian cells, peroxisomes are multifunctional organelles, hosting portions of a number of anabolic and catabolic pathways, many of which are involved in lipid metabolism. Peroxisomes are particularly abundant in liver and kidney cells where they are observed as round or oval structures that are 0.3 to 1.0 μm in diameter, often located close to the sites of lipid synthesis such as the endoplasmic reticulum (Böck, et al., 1980).

Important peroxisomal anabolic pathways include the biosynthesis of plasmalogen, cholesterol and bile acids, and the transamination of amino acids (Lazarow and Moser, 1989). Plasmalogens are a class of ether-linked lipids that are major components of the myelin sheath surrounding nerve cells. While the endoplasmic reticulum is the primary compartment for the synthesis of the more commonly found ester lipids, the first three reactions of ether lipid synthesis take place in peroxisomes (Hajra and Bishop, 1982; Hajra, et al., 1979). The peroxisomal enzymes are dihydroxyacetone phosphate (DHAP) acyltransferase, alkyl-DHAP synthase, and acyl/alkyl-DHAP reductase.

Bile acids are formed by the hydroxylation of the cholesterol ring structure and the shortening of its side chain via β-oxidation. Both of these reactions are peroxisomal (Hanson, et al., 1979; Krisans, et al., 1985; Monnens, et al., 1980; Pedersen and Gustafsson, 1980). For cholesterol synthesis from acetyl-coenzyme A (-CoA), several pathway enzymes including hydroxymethyl-glutaryl-CoA (HMG-CoA) reductase, the rate-limiting enzyme in the pathway, are found in peroxisomes (Brown and Goldstein, 1980; Keller,

et al., 1985; Keller, et al., 1986). Mammalian peroxisomes contain a thiolase which catalyzes the first step in cholesterol synthesis (Thompson and Krisans, 1990). Several additional cholesterol biosynthetic enzymes are found in the peroxisome as well, such as dihydrolanosterol oxidase, steroid-3-ketoreductase, steroid-14-reductase, and steroid-8-isomerase are in peroxisomes as well (Appelkvist, et al., 1990). Although these enzymes are also located in the endoplasmic reticulum, recent evidence suggests that, at least in humans, the peroxisomal pathway predominates in cholesterol biosynthesis (Hodge, et al., 1991). Thus, by controlling cholesterol biosynthesis, or degradation (through bile acid synthesis), or both, peroxisomes may play an important role in regulating cholesterol levels in humans (Thompson, et al., 1987).

Transamination of amino acids can also take place in the peroxisomes by way of enzymes that remove the amino group from the carbon skeleton (Hsieh and Tolbert, 1976). In human cells, such transaminases include serine:pyruvate aminotransferase and alanine:glyoxalate aminotransferase (AGT) (Noguchi and Takada, 1978; Noguchi and Takada, 1979). Further oxidation of glyoxylate to oxalic acid is prevented by its conversion to glycine in a reaction catalyzed by AGT, which allows it to enter the gluconeogenic pathway (Rowsell, et al., 1972). Deficiency in peroxisomal AGT activity results in primary hyperoxaluria, type I (Danpure and Jennings, 1986; Danpure, et al., 1987), demonstrating the importance of this reaction.

Peroxisomal catabolic pathways degrade a number of important metabolites including D- and L-amino acids, L-α-hydroxy acids, urate (De Duve and Baudhuin, 1966), glutaryl-CoA (Vamecq and van Hoof, 1984), oxalate (Beard, et al., 1985), CoA derivatives (Casteels, et al., 1990), and polyamines (Holtta, 1977). However, the most thoroughly understood catabolic pathway is that for the β-oxidation of fatty acids, especially very long chain fatty acids (VLCFA) and long chain unsaturated fatty acids.

β-oxidation can be divided into three steps: the activation of a fatty acid by the attachment of CoA; the β-oxidation reactions *per se*; and the

removal of the end products from the compartment. Fatty acids are converted to their corresponding acyl-CoA compounds in the presence of CoA, ATP, and acyl-CoA synthase (Krisans, et al., 1980; Mannaerts, et al., 1982; Shindo and Hashimoto, 1978). The peroxisome has its own acyl-CoA synthases which appear to be associated with the organelle membrane (Mannaerts, et al., 1982). The peroxisomal β-oxidation enzymes that oxidize each fatty acyl-CoA chain are located in the organelle matrix (Osumi and Hashimoto, 1978). These enzymes are acyl-CoA oxidase, a multifunctional enzyme that exhibits enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase activities (Kunau, et al., 1987), and 3-oxoacyl-CoA thiolase (Miyazawa, et al., 1981). The peroxisomal β-oxidation system is unique in mammals in that it oxidizes specific types of fatty acids that are not oxidized in the mitochondria, such as VLCFA (Kawamura, et al., 1981; Singh, et al., 1984), long-chain unsaturated fatty acids (Bremer and Osmundsen, 1984), long-chain dicarboxylic acyl-CoAs (Mortensen, et al., 1982), prostaglandin (Pace-Asciak and Granstrom, 1983), and certain xenobiotic compounds (Yamada, et al., 1984). Another unusual feature of peroxisomal β-oxidation in mammals is that fatty acid chain shortening does not proceed all the way to acetyl-CoA but stops at medium-chain acyl-CoA esters which are exported via carnitine acyltransferases to the mitochondria for further oxidation (Farrell and Bieber, 1983). Unlike mitochondrial transport, carnitine assists in the export of β-oxidation products from the peroxisomes, but not the import of substrates (Markwell, et al., 1973; Markwell, et al., 1976; Mayazawa, et al., 1983).

### 1.1.2 Peroxisomes in plants

Plant microbodies can be divided into three groups: peroxisomes of the leaf, glyoxysomes of fatty seeds, and other non-specialized microbodies (Tolbert, 1981). Together with the chloroplast and mitochondrion, the leaf peroxisome participates in a process called photorespiration, in which illuminated plants consume oxygen, ATP and NADPH, and evolve carbon dioxide, and which competes with photosynthetic carbon dioxide fixation (Larson and Kershaw, 1976). In photorespiration, oxygen replaces carbon

dioxide as the substrate for ribulose bisphosphate carboxylase (RuBP carboxylase-oxygenase or Rubisco) to generate phosphoglycolate, which is then dephosphorylated to glycolate. Glycolate leaves the chloroplast and enters the peroxisome, where it is oxidized to glyoxylate by glycolate oxidase and converted to glycine with the help of serine:glyoxylate transaminase. Glycine enters the mitochondrion, and two molecules of glycine are converted to one molecule of carbon dioxide, a product of the photorespiration, and one molecule of serine, which reenters the peroxisome. A serine transaminase converts serine to hydroxypyruvate which is then reduced to glycerate using one molecule of NADPH. In the cytosol, glycerate is phosphorylated using one molecule of ATP to 3-phosphoglycerate, which reenters the chloroplast and is converted to ribulose 1,5-bisphosphate. Overall, photorespiration dissipates the carbon dioxide fixation, consumes energy, and as a consequence, is a major limitation in plant growth (Ogren, 1984).

A specialized peroxisome in fatty seeds, the glyoxysome, is responsible for the initial reactions that convert fatty acids to sucrose and provide energy during seed germination (Beevers, 1975). In addition to β-oxidation enzymes, glyoxysomes also contain isocitrate lyase and malate synthase, key enzymes of the glyoxylate cycle (Hogg, 1969). The remaining glyoxylate cycle enzymes are components of the tricarboxylic acid cycle and are present in the mitochondrion. In some plant seeds, the glyoxysomes contain additional glyoxylate cycle enzymes, namely citrate synthase and aconitase (Breidenbach and Beevers, 1967; Cooper and Beevers, 1969). Peroxisomal structures containing catalase, uricase, and glycolate oxidase are found in other plant tissues as well (Breidenbach and Beevers, 1967; Cooper and Beevers, 1969).

### 1.1.3 Peroxisomes in protozoans

In protozoans, the content and function of microbodies vary depending upon the species (Müller, 1975; Opperdoes, 1987). Aerobic species, such as *Tetrahymena pyriformis*, have microbodies that perform typical

peroxisomal oxidative reactions such as β-oxidation (Lloyd, et al., 1971; Müller, et al., 1968). In *Trypanosoma brucei*, peroxisomes contain enzymes involved in β-oxidation and plasmalogen synthesis. *T. brucei* peroxisomes are unique in that they also harbor a complete glycolytic pathway, and thus they are referred to as glycosomes (Opperdoes, 1987). The compartmentalization of the glycolytic pathway is thought to serve as a means of concentrating pathway enzymes and intermediates, thereby increasing the effective rate of glycolysis. Hydrogenosomes, another class of microbodies that are observed in many anaerobic flagellates, are involved in pyruvate metabolism, and for this purpose contain pyruvate synthase, acetate thiokinase, and a hydrogenase (Müller, 1975). The organelles also contain superoxide dismutase, adenylate kinase, and sometimes, circular DNA (Lindmark and Müller, 1973; Lindmark and Müller, 1974; Müller, 1975). Hydrogenosomes are unique among microbodies in that they contain nucleic acids but lack matrix proteins containing the carboxy-terminal peroxisomal targeting signal, Ser-Lys-Leu-COOH. In addition, they lack hydrogen peroxide-generating oxidases. Instead of producing $H_2O_2$ as reducing product, hydrogenosomes generate hydrogen (Lindmark and Müller, 1973). Because of these differences, there is doubt as to whether hydrogenosomes are true peroxisomes.

### 1.1.4 Peroxisomes in yeasts

Peroxisomes play an essential role in the metabolism of a variety of carbon and nitrogen sources in yeasts (van den Bosch, et al., 1992; Veenhuis and Harder, 1987). However, the presence of many of the required pathways and therefore, the ability to utilize these nutrients, is species specific. Virtually all yeast species contain the glyoxylate pathway enzymes malate synthase and isocitrate lyase in their peroxisomes (glyoxysomes), thus enabling them to grow on $C_2$ compounds such as ethanol or acetate.

Many yeast species also harbor a complete β-oxidation system in their peroxisomes, which together with the glyoxylate pathway, allows them to metabolize fatty acids such as oleate. Examples of these species include

*Candida tropicalis, Candida albicans, Candida utilis, Candida maltosa, Yarrowia lipolitica, Pichia pastoris* and *Saccharomyces cerevisiae*. The pathway for fatty acid metabolism is diagrammed in Figure 1.1. Fatty acids are first converted to fatty acyl-CoA with the help of peroxisomal acyl-CoA synthetase and transported into the peroxisome. Upon entering the peroxisome, acyl-CoA is oxidized to 2-enoyl-CoA, converted to 3-hydroxyacyl-CoA, oxidized again to 3-ketoacyl-CoA, and then cleaved to generate acetyl-CoA and an acyl-CoA product that is shorter by two carbons (Veenhuis and Harder, 1987).

A few yeast species such as *Candida boidinii, Hansenula polymorpha* and *P. pastoris* are capable of utilizing methanol as their sole carbon and energy source, an ability that requires a unique pathway involving three peroxisomal enzymes (Anthony, 1982; Douma, et al., 1985; Goodman, 1985; Veenhuis, et al., 1983). As diagrammed in Figure 1.2, the first step of the pathway involves alcohol oxidase (AOX), a peroxisomal enzyme that oxidizes methanol to formaldehyde. This reaction generates $H_2O_2$ which is broken down to water and oxygen by the second peroxisomal enzyme, catalase. Formaldehyde is further metabolized by one of two paths. In one path, it exits the peroxisome to the cytosol and is further oxidized to formate and then carbon dioxide by two dehydrogenases that generate reducing power for the cell. In the other path, formaldehyde condenses with xylulose-5'-monophosphate ($Xu_5P$) in a reaction catalyzed by the third peroxisomal enzyme, dihydroxyacetone synthase, to generate two $C_3$ compounds, glyceraldehyde-3-phosphate and dihydroxyacetone. These molecules then enter a cyclic pathway that regenerates $Xu_5P$ plus glyceraldehyde-3-phosphate for the construction of cellular constituents.

Certain yeast species are known to metabolize specific organic nitrogen sources via peroxisomal pathways. These compounds (and their requisite peroxisomal enzymes) include: alkylated amines such as methylamine or choline (amine oxidase) (van Dijken and Bos, 1981; Zwart, 1983), uric acid (urate oxidase) (Veenhuis, et al., 1985) and D-alanine (D-amino acid oxidase) (Zwart, et al., 1983).

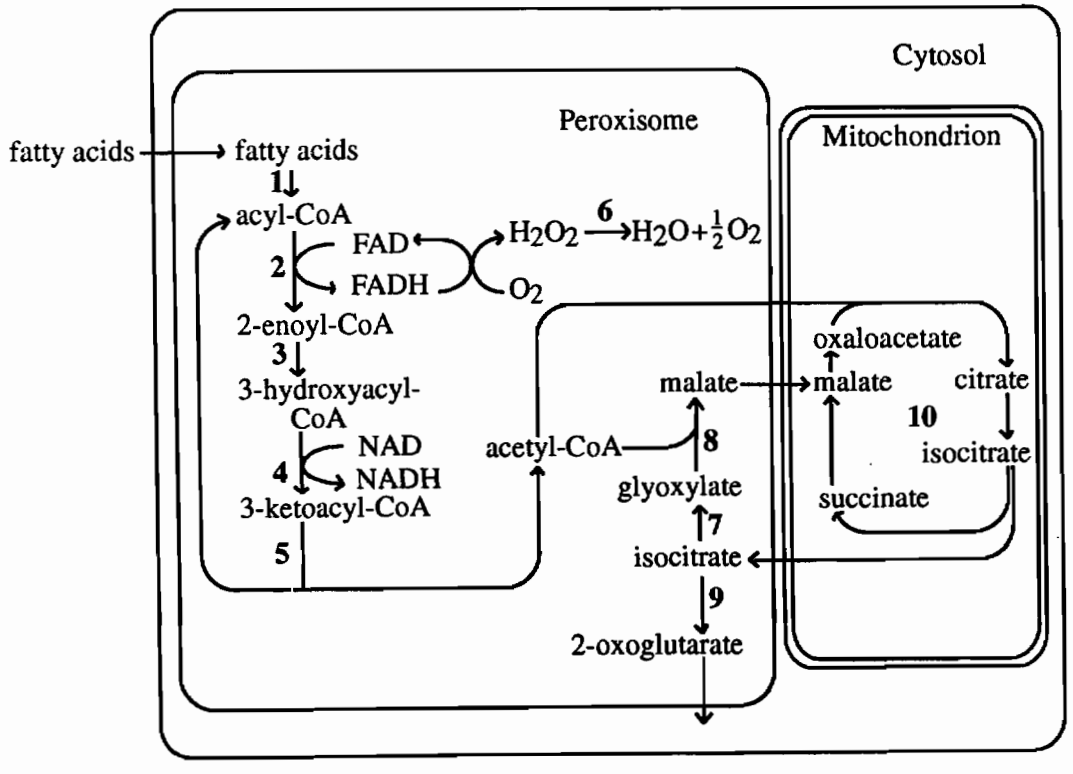


Figure 1.1 The fatty acid β-oxidation pathway in yeasts. The pathway was defined in species of *Candida* and assumed to be the same in *P. pastoris*. 1. acyl-CoA synthase; 2. acyl-CoA oxidase; 3. 2-enoyl-CoA hydratase; 4. 3-hydroxyacyl-CoA dehydrogenase; 5. 3-ketoacyl-CoA thiolase; 6. catalase; 7. isocitrate lyase; 8. malate synthase; 9. NADP-linked isocitrate dehydrogenase; 10. TCA cycle enzymes.

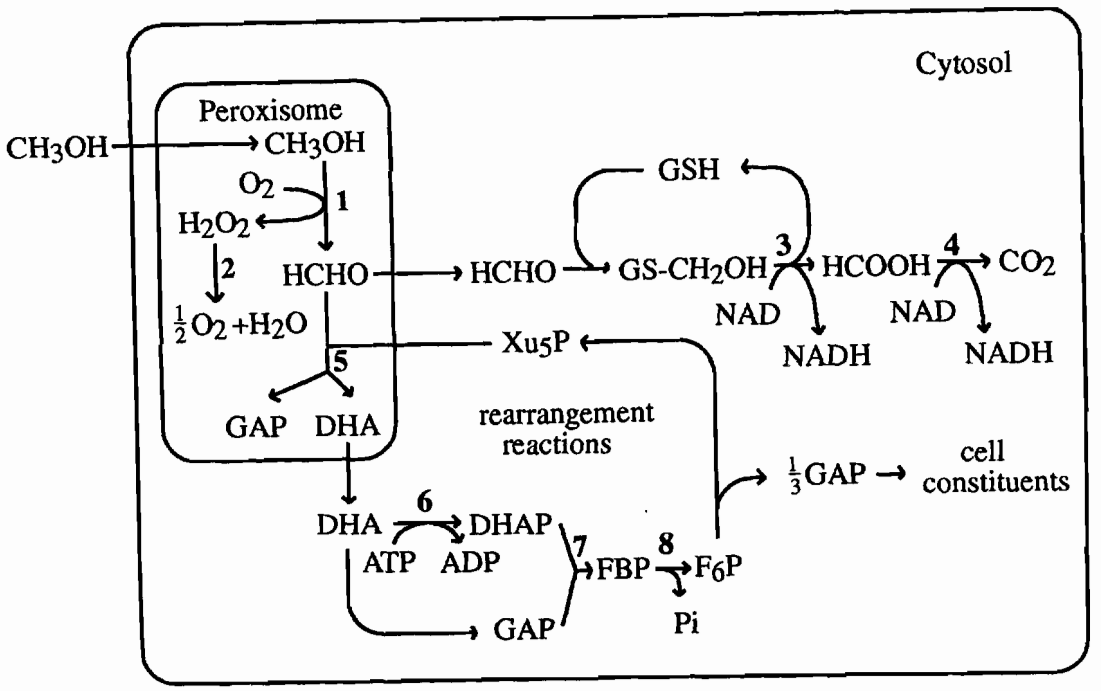


Figure 1.2 The methanol pathway in yeasts. 1. alcohol oxidase; 2. catalase; 3. formaldehyde dehydrogenase; 4. formate dehydrogenase; 5. dihydroxyacetone synthase; 6. dihydroxacetone kinase; 7. fructose 1,6-bisphosphate aldolase; 8. fructose 1,6-bisphosphatase.

## 1.2 Peroxisome biogenesis

### 1.2.1 General features

Peroxisomes are single-membrane-enclosed organelles that are believed to proliferate through growth and division from pre-existing peroxisomes (Borst, 1989; Lazarow and Fujiki, 1985; Tolbert, 1981). Based on this, peroxisome biogenesis mechanisms must include the integration of lipids and specific integral membrane proteins into the peroxisomal membranes, the import of specific sets of proteins into the peroxisomal matrix, the growth and fission of the organelles and their segregation into daughter cells upon cell division. A further biogenesis mechanism unique to peroxisomes is the peroxisome induction phenomenon, i.e., the process by which the number, size and enzymatic content of peroxisomes dramatically change in response to specific environmental stimuli. With the exception of the targeting signals for peroxisomal matrix proteins and the peroxisome induction mechanism (discussed below), little is known about these mechanisms at the molecular level.

Peroxisomes do not contain nucleic acids (Kamiryo, et al., 1982) and therefore, peroxisomal proteins are encoded by nuclear genes. Peroxisomal proteins are synthesized on cytosolic or free ribosomes and post-translationally imported (Lazarow and Fujiki, 1985). Hydrolysis of ATP appears to be required for import but not the proton gradient that exists across the peroxisomal membrane (Imanaka, et al., 1987; Wendland and Subramani, 1993). If, as believed, peroxisomes bud from pre-existing peroxisomes, at least one peroxisome must somehow be faithfully directed to each daughter cell during division to serve as source for new peroxisomes (Lazarow, 1993; Veenhuis, et al., 1987).

### 1.2.2 Peroxisome targeting sequences

During growth and proliferation of peroxisomes, cytosol-synthesized peroxisomal proteins must be accurately delivered to their proper locations within the peroxisome. Although the details of the molecular mechanisms

have not been elucidated, the targeting signals used by peroxisomal matrix proteins have been identified (Keller, et al., 1991; Swinkels, et al., 1991). To date, two peroxisomal targeting signals (PTSs), PTS-1 and PTS-2, have been characterized. PTS-1 is a carboxyl-terminal tripeptide sequence (SKL and several conservative variations) (Keller, et al., 1991; Swinkels, et al., 1991). The sequence is necessary and sufficient to direct non-peroxisomal proteins to the peroxisome (Fung and Clayton, 1991; Gould, et al., 1989) and is present at the carboxy-terminus of a large number of peroxisomal matrix enzymes from a wide spectrum of organisms including mammals, insects, plants, protozoans and fungi (Keller, et al., 1991). In addition, antibodies specific to SKL react with matrix proteins from peroxisomes, glyoxysomes, and glycosomes, a result that supports the idea that these microbody types are related (Keller, et al., 1991; Swinkels, et al., 1992).

PTS-2 is a targeting signal found at the amino terminus of two rat 3-ketoacyl-CoA thiolases (Swinkels, et al., 1991). Although most peroxisomal matrix proteins are synthesized at their mature sizes, the PTS-2 of rat thiolases exist as presequences of 36 and 26 amino acids, respectively, that are cleaved after import into peroxisomes. As shown for PTS-1, PTS-2 is sufficient to direct cytosolic reporter proteins into peroxisomes (Bodnar and Rachubinski, 1990; Hijikata, et al., 1990; Swinkels, et al., 1991). The *S. cerevisiae* thiolase sequence is similar to rat PTS-2 at its amino terminus, but the yeast protein is not cleaved after import (Swinkels, et al., 1991). Based on sequence similarity, yeast thiolase is most likely also imported via a PTS-2-based mechanism, although this has not been demonstrated directly. Other than thiolase, few peroxisomal matrix proteins appear to have PTS-2 and therefore, this matrix targeting mechanism appears minor relative to the PTS-1-based mechanism.

Many peroxisomal matrix proteins appear not to have either PTS-1 or PTS-2. Therefore, additional PTSs (and peroxisomal targeting mechanisms) are likely to exist (Borst, 1989; Gould, et al., 1989; Kragler, et al., 1993; Small and Lewin, 1989; Small, et al., 1988). In addition, none of the peroxisomal integral membrane proteins (PMPs) described to date have sequence

similarity to PTS-1 or PTS-2. In peroxisome-deficient mutants (see below), PMPs remain associated with matrix protein import-defective organelles (Höhfeld, et al., 1991; Santos, et al., 1988). This observation suggests that PMPs may be directed to the peroxisome membrane by mechanism(s) independent of those for matrix proteins. The *PAS3* gene product (Pas3p) from *S. cerevisiae* is believed to be a PMP (Höhfeld, et al., 1991). When a fusion protein composed of the amino-terminal half of Pas3p fused to β-galactosidase was expressed in *S. cerevisiae,* β-galactosidase activity was found associated with the peroxisomal membranes. Thus, information for targeting Pas3p appears to exist within this segment of Pas3p. Further fusions of this sort may result in the identification of critical sequences required for the targeting of this putative PMP (Höhfeld, et al., 1992).

### 1.2.3 Peroxisome induction

A unique feature of peroxisomes is their ability to undergo rapid and dramatic changes in size, number and enzymatic content (Lock, et al., 1989; Veenhuis, et al., 1981; Veenhuis, et al., 1979). The most extreme example of this is observed in methylotrophic yeasts where the one or two small peroxisomes that occupy less than 1% of cell volume in glucose-grown cells increase enormously in size and number, and eventually occupies 70% of the cell volume after induction by methanol (Veenhuis, et al., 1983). Upon induction, peroxisomal membranes are observed to extend into looped structures in apparent preparation for import of the peroxisomal matrix enzymes. Growth of the organelle is followed by fission, generating more peroxisomes (Lazarow, et al., 1982; Veenhuis, et al., 1978). Along with organelle proliferation, massive induction occurs in the synthesis of each of the three peroxisomal methanol-pathway enzymes, AOX, catalase and dihydroxyacetone synthase. AOX, which is not present in glucose-grown cells, has been observed to accumulate to approximately 30% of total soluble protein in cells shifted to methanol medium (Couderc and Baratti, 1980).

The peroxisomal content of rat liver cells is greatly increased in animals fed with hypocholesterolemic drugs such as clofibrate or certain

other xenobiotic compounds (Lazarow and Fujiki, 1985; Reddy and Lalwani, 1983). Along with peroxisome proliferation, specific peroxisomal enzymes are also greatly induced, such as carnitine acetyltransferase, carnitine octanoyltransferase, acyl-CoA:DHAP acyltransferase, but not catalase (Hajra and Bishop, 1982; Hashimoto, 1982; Reddy and Lalwani, 1983; Tolbert, 1981). The frequency of liver carcinomas also increases significantly in response to peroxisome proliferating drugs (Reddy and Qureshi, 1979). Since the drugs do not interact and damage DNA directly, it is thought that the hepatocarcinogenic nature of these compounds may be due to the greatly increased levels of peroxisomal oxidative reactions that occur upon proliferation and the DNA-damaging activated-oxygen species they generate. Thus, peroxisomes may be a key component in a second pathway by which certain chemicals induce cancers (Conway, et al., 1989; Kasai, et al., 1989; Reddy and Lalwani, 1983; Warren, et al., 1980).

Key features of the molecular mechanism controlling the peroxisome proliferative response in mammals have been elucidated. Overall, the mechanism is strikingly similar to that described for steroid hormone response (Issemann and Green, 1990). Peroxisome proliferator-responsive elements (PPREs) have been identified upstream of several genes encoding peroxisomal enzymes including rat acyl-CoA oxidase (Dreyer, et al., 1992; Osumi, et al., 1991; Tugwood, et al., 1992), rat hydratase-dehydrogenase (Zhang, et al., 1993), and rat peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (Zhang, et al., 1992). The PPRE in the rat acyl-CoA oxidase promoter region has been defined as two repetitive motifs of the sequence AGG(A/T)CA separated by a single nucleotide and is sufficient to activate transcription of reporter genes in response to known peroxisome proliferators (Dreyer, et al., 1992; Osumi, et al., 1991; Tugwood, et al., 1992). The promoter regions of both rat hydratase-dehydrogenase and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase contain PPREs (Zhang, et al., 1993; Zhang, et al., 1992). Genes encoding transcription factors that bind to PPREs and appear to be key elements responsible for proliferation have been cloned and named peroxisome proliferator-activated receptors (PPARs). The

predicted sequence of the gene products indicates they are members of the soluble receptor superfamily of transcriptional activators. Like other members of the family, the PPARs each exhibit a putative ligand binding domain, a transactivating domain, and potential zinc finger DNA binding domain. The cloned gene products include the 9-*cis* retinoid acid-responding retinoid X receptor-α (RXRα) (Mangelsdorf, et al., 1990), and the clofibric acid responding PPAR from mouse (Issemann and Green, 1990) and *Xenopus* (Dreyer, et al., 1992). These receptors exhibit DNA binding affinity and synergistic interaction with respect to both DNA binding and transcription activation.

## 1.3 Mutations affecting peroxisome biogenesis

### 1.3.1 Peroxisome-deficient mutants in mammals

The importance of peroxisomes in humans is demonstrated by a family of lethal genetic disorders affecting peroxisomes referred to collectively as Zellweger syndrome (Lazarow and Moser, 1989). The syndrome was first described in 1964 based on the abnormal morphology of affected individuals, usually infants (Bowen, et al., 1964). Zellweger patients typically have severe hypotonia, a high forehead, a large anterior fontanelle, midface hypoplasia, and other deformities. Currently there is no effective therapy for treatment of these patients, who often die shortly after birth. Cytologically, patient cells are grossly deficient in morphologically recognizable peroxisomes and this organelle deficiency appears to be the root cause of the disease (Goldfischer, et al., 1973). Zellweger syndrome is the only known human genetic disease believed to be caused by a general malfunction of an organelle.

Biochemically, most peroxisomal enzymes are either missing (e.g., acyl-CoA oxidase, multifunctional enzymes of the β-oxidation pathway, DHAP acyl-transferase, and alkyl-DHAP synthase) or mislocalized in the cytosol (e.g., catalase, D-amino acid oxidase, and L-α-hydroxyacid oxidase) in Zellweger cells (Lazarow and Moser, 1989). For one of the missing enzymes, acyl-CoA oxidase, evidence suggests that the enzyme is synthesized but is not

imported into the peroxisome and, as a consequence, is rapidly degraded in the cytosol (Schram, et al., 1986). Metabolically, most pathways that involve a peroxisomal enzyme are impaired. These include very long chain fatty acid, pipecolic acid and phytanic acid degradation, and cholesterol and plasmalogen synthesis.

Recent studies suggest that peroxisome deficiency in Zellweger syndrome is due to a defect in the molecular machinery for peroxisomal protein import, specifically import of PTS-1-containing proteins (McCollum, et al., 1993; Walton, et al., 1992a). Although normal peroxisomes are not present in Zellweger cells, abnormal vesicles containing PMPs are observed (Santos, et al., 1988; Wiemer, et al., 1989). These vesicles, referred to as "peroxisomal ghosts," contain thiolase, an enzyme harboring PTS-2, but do not contain enzymes harboring PTS-1. Furthermore, PTS-1-containing proteins such as luciferase, or albumin conjugated with a peptide that contains the PTS-1 tripeptide sequence, are observed to localize to peroxisomes when microinjected into normal cells, but not when injected into Zellweger cells (Walton, et al., 1992a).

Peroxisome-deficient Chinese hamster ovary (CHO) cell lines have also been isolated and characterized (Tsukamoto, et al., 1990). These CHO mutant cell lines are devoid of normal peroxisome structures. Catalase is mislocalized to the cytosol, and acyl-CoA oxidase and thiolase are not correctly processed into their mature sizes in these mutants.

### 1.3.2 Peroxisome-deficient mutants in yeasts

The yeast species *S. cerevisiae*, *P. pastoris*, and *H. polymorpha* are each being used to study peroxisome biogenesis. Peroxisome-deficient mutants have been isolated in all three species. Aside from the general advantages of yeasts as model systems, these yeasts have specific advantages for peroxisome studies. *S. cerevisiae* is well characterized with regard to biochemical, molecular genetic and cell biological aspects of the organism. With regard to peroxisome studies, *S. cerevisiae* is known to induce a peroxisomal β-oxidation pathway when cultured on oleic acid (Erdmann,

et al., 1989). *P. pastoris* and *H. polymorpha* are methanol-utilizing yeast species and, primarily due to their methylotrophy, have certain advantages over the *S. cerevisiae* for peroxisome studies. First, in response to methanol, peroxisome proliferation is enormous relative to oleate-induced *S. cerevisiae* (Veenhuis, et al., 1987; Veenhuis, et al., 1983). *H. polymorpha* and *P. pastoris* peroxisomes take up 10-20% of cell volume in methanol shake-flask cultures and approximately 70% of cell volume in cells growing in methanol-limited chemostat cultures (Veenhuis, et al., 1983). In *S. cerevisiae*, oleate also induces peroxisome proliferation, but not nearly to this extent. Thus, it is easier to observe peroxisomes in methylotrophic yeasts and to manipulate them for biochemical studies. Importantly, proteins involved in peroxisome biogenesis appear to be present at significantly higher levels in the methanol-induced yeasts and therefore, it should be easier to isolate and to determine their subcellular locations. Furthermore, *H. polymorpha* and *P. pastoris* grow well on methanol as their sole carbon source. In contrast, *S. cerevisiae* grows poorly on oleate. Therefore, experiments such as mutant isolation, genetic analysis and gene cloning, where relative growth rate on the peroxisome-dependent substrate (oleate or methanol) must be assessed, are more easily performed with methylotrophs.

The first *S. cerevisiae* peroxisome-deficient mutants (*pas* mutants) were reported by Erdmann *et al.* (Erdmann, et al., 1989). The strains were isolated by growing mutagenized cell populations on acetate medium and subsequently replica plating the resulting colonies onto oleate medium. Mutant strains that grew on acetate but not on oleate were collected and oleate-induced cells of these mutants were subjected to electron microscopic examination. Two strains, *pas1-1* and *pas2*, lacked detectable peroxisome structures. Cell fractionation studies of these mutants indicated that both catalase and 3-oxoacyl-CoA thiolase, two peroxisomal β-oxidation-pathway enzymes, were mislocalized to the cytosol. Subsequently, additional peroxisome-deficient mutants have been isolated and, to date, *pas* mutants in 13 complementation groups have been described (Kunau and Hartig, 1992). Based on electron microscopy (EM) studies, three types of *pas* mutants have

been distinguished. Type I mutants (*pas1*, *pas2*, *pas3*, and *pas5*) have no detectable peroxisomes, and peroxisomal matrix enzymes such as catalase and epimerase are present at normal levels but are mislocalized to the cytosol. It is speculated that these mutants may be defective in peroxisome formation. Type II mutants (*pas4* and *pas6*) have a few small peroxisomes that do not proliferate in response to oleate induction. As with Type I mutants, peroxisomal enzymes are induced to normal levels and are mostly cytosolic, although a small amount of enzyme appears to be properly imported. These mutants are thought to be defective in peroxisome proliferation. The Type III group contains only one mutant, *pas7*, and has morphologically normal appearing peroxisomes. In addition, catalase and most other peroxisomal enzymes are properly targeted to the organelle. However, thiolase is mislocalized to the cytosol. Since thiolase is believed to be targeted via PTS-2, this mutant may be specifically defective in a component of the PTS-2 import machinery.

*H. polymorpha* peroxisome-deficient mutants (*per* mutants) were first described by Cregg *et al.* (Cregg, et al., 1990). To isolate these mutants, mutagenized cultures of the yeast were screened for mutants that were methanol-utilization-defective ($Mut^-$) and also ethanol-utilization-defective ($Eut^-$) but still able to grow on glucose. The resulting mutants were examined by EM and two *per* mutants were identified. Backcrossing the mutants revealed that peroxisome deficiency was linked to the $Mut^-$ but not the $Eut^-$ phenotype. Biochemical and immunocytochemical studies demonstrated that all peroxisomal enzymes examined were present but mislocalized to the cytosol in each *per* mutant. Interestingly, the *per* mutants grew well on ethanol even though at least two peroxisomal enzymes that are required for ethanol growth, isocitrate lyase and malate synthase, appeared to be in the cytosol.

Subsequently, additional *H. polymorpha per* mutants were isolated with defects in a total of 14 genes (*PER1* through *PER14*) (Didion and Rogenkamp, 1990; Tan, et al., 1993; Titorenko, et al., 1993; Waterham, et al., 1992). Three distinct *per* mutant phenotypes are distinguishable based on EM

examination of methanol-induced cells. Per$^-$ mutants, like the Type I mutants of *S. cerevisiae*, were completely devoid of peroxisomes. In addition, methanol-induced Per$^-$ mutant cells contained a large cytosolic crystalloid structure composed primarily of active AOX (van der Klei, et al., 1991). In all Per$^-$ mutants, AOX, catalase, and dihydroxyacetone synthase were present but mislocalized to the cytosol. Peroxisomal membrane proteins (PMPs) in each Per$^-$ cell appeared in an unusual punctate structure (Sulter, et al., 1993). A second group of mutants, called Pim$^-$, were similar to the Type II mutants of *S. cerevisiae* in that they each contain only a few small peroxisomes and most peroxisomal enzymes are mislocalized to the cytosol. Like Per$^-$ mutants, each Pim$^-$ mutant also harbored a large cytosolic AOX crystalloid and other peroxisomal enzymes were mislocalized to the cytosol as well (Waterham, et al., 1992). The third group, referred to as Pss$^-$ mutants, induced peroxisomes that are normal in size and number but harbored an aberrant crystalline matrix substructure (Titorenko, et al., 1993). In contrast to the *S. cerevisiae pas* mutant groups, different mutant alleles of the same gene sometimes were of different peroxisome phenotype. In fact, one gene (*PER3*) had Per$^-$, Pim$^-$ and Pss$^-$ alleles (Titorenko, et al., 1993; Titorenko, et al., 1992). Finally, dominant-negative mutant alleles were identified for two genes, *PER5* and *PER7* (Tan, et al., 1993).

Peroxisome-deficient mutants (*per* mutants) in 10 complementation groups have been reported for *P. pastoris* (Gould, et al., 1992; Liu, et al., 1992). These mutants were obtained from mutagenized cultures of *P. pastoris* by isolating strains that were Mut$^-$ and oleate-utilization-defective (Out$^-$), but still able to grow on other carbon sources including ethanol, glycerol and glucose. With the possible exception of one isolate, every *P. pastoris* mutant that fit this phenotypic profile was a *per* mutant upon EM examination. In methanol- or oleate-induced *per* mutant cells, catalase was present and active but appeared to be mislocalized to the cytosol. In contrast to catalase and to AOX in *H. polymorpha per* mutants, little or no AOX was found in *P. pastoris per* mutants. It was suggested that, similar to certain peroxisomal enzymes in peroxisome-deficient mammalian cells, AOX may be synthesized but is

rapidly degraded, as a consequence of its inability to be imported (Liu, et al., 1992).

## 1.4 Isolation and analysis of genes required for peroxisome biogenesis

### 1.4.1 Mammalian peroxisomal genes

Two mammalian genes whose products are required for peroxisome biogenesis have been isolated to date. *PAF-1* was cloned by functional complementation of a peroxisome-deficient CHO cell line using a rat liver cDNA library (Tsukamoto, et al., 1991). *PAF-1* encodes a 35-kD protein that restores missing peroxisomal biochemical functions to the mutant CHO cell line. These functions include: catalase import, acyl-CoA oxidase dimerization, the maturation of 3-ketoacyl-CoA thiolase, and DHAP acyltransferase activity. The *PAF-1* product appears to be a peroxisomal membrane protein, a result that is consistent with the predicted presence of two potential transmembrane domains in its sequence. The human homologue of *PAF-1* was cloned by hybridization with the rat gene and the two gene products are predicted to be 80% identical. Both genes corrected peroxisome deficiency in a fibroblast cell line derived from a Japanese Zellweger patient, making this gene particularly interesting from the standpoint of this genetic disease (Shimozawa, et al., 1992).

A second mammalian peroxisomal gene, PMP70, is an abundant peroxisomal membrane protein and was cloned first from a rat liver cDNA expression library using anti-PMP70 antibodies as a probe and then from human cDNA library using rat liver PMP70 cDNA as a probe (Kamijo, et al., 1992; Kamijo, et al., 1990). The hPMP70 and rPMP70 genes share 90.6% identity in nucleotide sequences, and 95% identity and 99.1% similarity in predicted amino acid sequences (Kamijo, et al., 1992). rPMP70 mRNA and protein levels are induced by hypolipidemic drugs. The carboxy-terminus of the protein appears to be exposed to the cytosol, and shares homology with ATP binding domains of several known ATPases that are involved in active transport across membranes (Kamijo, et al., 1990). PMP70 complemented

defects in two Zellweger cell lines, a result that suggests these lines were defective in PMP70 and demonstrating that the PMP70 product is essential for peroxisome biogenesis (Gärtner, et al., 1992).

### 1.4.2 Yeast peroxisomal genes

Peroxisome-deficient mutants of *S. cerevisiae* and *P. pastoris* have been used to clone wild-type peroxisomal genes by functional complementation. In *S. cerevisiae*, genes for *PAS1*, *PAS2*, and *PAS3* were isolated from a genomic DNA library by their ability to restore oleate growth to each respective *pas* mutant host. The *PAS1* sequence predicts a 117 kD protein (Pas1p) containing two novel ATP binding motifs that share homology with Sec18p and NSF, proteins that are known to be essential for the fusion of vesicles to Golgi compartments (Erdmann, et al., 1991) The *PAS2* product (Pas2p) is predicted to be a 21-kD protein related to ubiquitin-conjugating (UBC) enzymes (Wiebel and Kunau, 1992). Pas1p and Pas2p were not detectable in oleic acid-induced cells, presumably due to their low abundance; therefore, their location within the cell could not be determined. The predicted *PAS3* product (Pas3p) was found to be a 51-kD peroxisomal membrane protein (Höhfeld, et al., 1991). The location of Pas3p was consistent with the presence of two potential membrane-spanning domains in its sequence (Höhfeld, et al., 1991).

In *P. pastoris*, the cloning of one peroxisomal gene, *PAS8*, has been reported (McCollum, et al., 1993). *pas8* mutants appear to be specifically defective in the import of PTS-1-containing peroxisomal proteins, since several of these proteins including AOX, catalase, dihydroxyacetone synthase and luciferase were not imported, whereas the presumed PTS-2-directed enzyme thiolase was. This observation is similar to that with Zellweger patient cells, making this mutant of particular interest. The *PAS8* sequence predicted a product (Pas8p) of 65 kD with a region of sequence homology to the tetratricopeptide family of sequences that are believed to be important in certain protein-protein interactions (Goebl and Yanagida, 1991). Pas8p appears to be peroxisomal-membrane-associated, and *in vitro* translated Pas8p

specifically bound to a peptide containing the PTS-1 sequence SKL. This result suggests that Pas8p may be the PTS-1 receptor or a factor that specifically interacts with the PTS-1 receptor (McCollum, et al., 1993).

### 1.5 Importance of peroxisomes in humans

Three current lines of investigation dramatize the importance of peroxisomes in human health. The first line involves the study of genetic disorders affecting the peroxisome. These diseases can be divided into two groups. The first includes diseases affecting single peroxisomal enzymes such as acyl-CoA oxidase deficiency, enoyl-CoA hydratase deficiency, 3-oxoacyl-CoA thiolase deficiency, and acatalasemia (Goldfischer, 1988). Recently, it was discovered that amyotrophic lateral sclerosis, also known as Lou Gehrig's disease, is caused by a deficiency in Cu/Mn superoxide dismutase, a known peroxisomal enzyme (McNamara and Fridovich, 1993; Rosen, et al., 1993). The second group includes disorders of peroxisome biogenesis, such as Zellweger syndrome, infantile Refsum disease, neonatal adrenoleukodystrophy (ALD), and chondrodysplasia punctata, Rhizomelic type, which are all due to a general deficiency of peroxisomes in patient cells (see section 1.3.1 for discussion) (Lazarow and Moser, 1989). Both groups of diseases are usually fatal, demonstrating the crucial role of peroxisomes and peroxisomal enzymes in human survival.

The second line of research involves the role of peroxisome-proliferating drugs, such as clofibrate, in reducing cholesterol levels and in inducing cancer. Clofibrate was investigated as a potential therapeutic drug to reduce cholesterol levels in patients with ischemic heart disease (Havel and Kane, 1973). Unfortunately, the drug was found to induce hepatocarcinomas in rodents (Reddy and Qureshi, 1979). Tumor induction appears to be a consequence of the peroxisome proliferative effect of the drug which also induces peroxisomal fatty acid β-oxidation enzymes (but not catalase) which then generate excess $H_2O_2$ and activated oxygen species. The low levels of catalase allow these toxic compounds to escape the peroxisome and damage DNA (Conway, et al., 1989; Kasai, et al., 1989; Reddy and Lalwani, 1983;

Warren, et al., 1980). As a group, these peroxisome proliferators are referred to as non-genotoxic carcinogens, due to the indirect mechanism by which they damage genetic material, and are employed as models for the study of this phenomenon. It is clear from this work that regulating peroxisomal enzyme content is important to the well-being of eukaryotic cells.

The third line of work involves the potential role of peroxisomes in regulating the level of cholesterol, an important contributor to heart disease in humans. Peroxisomes contain a number of enzymes required for cholesterol synthesis and are the sole location of all enzymes required for its degradation to bile acids (see section 1.1.1 for details). In fact, cholesterol has been produced *in vitro* from mevalonic acid in reactions containing purified peroxisomes and cytosolic extracts (Thompson, et al., 1987). Each enzyme required for cholesterol synthesis also exists in at least one other compartment of the cell. Therefore, the contribution of the peroxisome to this process has been unclear. Recently, it was shown that cholesterol levels are abnormally low in fibroblasts from Zellweger patients (Hodge, et al., 1991) as well as the blood of these patients (Barth, et al., 1987; Scotto, et al., 1982). Thus, the peroxisome appears to be critical in determining human cholesterol levels.

## 1.6 Issues in peroxisome biogenesis and function

### 1.6.1 Current perceptions

Our present understanding of the mechanisms that operate in peroxisome biogenesis is crude at best. Peroxisomes are single-membrane-enclosed acidic compartments that are thought to be generated by growth and fission of preexisting peroxisomes. Peroxisomal proteins are encoded by nuclear genes, synthesized on cytosolic ribosomes and imported post-translationally by a process that requires ATP hydrolysis. Virtually nothing is known about the molecular mechanisms involved in these processes. There are two partial exceptions to this statement. The first exception involves the transcriptional machinery that responds to specific signals to induce

peroxisome proliferation in mammals. The signal is transmitted to the nucleus via soluble nuclear receptors which activate transcription of specific peroxisomal genes (see section 1.2.3 for discussion). The second exception is the specific amino acid signals that direct proteins to the peroxisomal matrix (PTSs) (see section 1.2.2 for discussion). Two such PTSs have been defined and it is speculated that others are yet to be discovered. On the other hand, nothing is known concerning the targeting of peroxisomal integral membrane proteins. Furthermore, no information is yet available on the machinery that recognizes a PTS and translocates the protein into the organelle. It is speculated that since there is more than one PTS, there may also be independent PTS-specific pathways for each. Studies of peroxisome-deficient mutants support this conjecture. Zellweger and CHO cell lines, and *P. pastoris pas8* mutants appear to be specifically defective in PTS-1-containing protein import (McCollum, et al., 1993). Conversely, the Type III mutant of *S. cerevisiae* properly imports PTS-1-containing proteins, but not thiolase, which is believed to be imported via a PTS-2 sequence.

### 1.6.2 New approaches

Until recently, the application of modern molecular genetic and biochemical methods to the peroxisome field had been relatively unsuccessful. The first major breakthrough came from the laboratory of Subramani in the late 1980s. They applied recombinant DNA techniques and transient transfections of tissue culture cells to define peroxisomal targeting signals (Gould, et al., 1989; Gould, et al., 1990a; Gould, et al., 1987; Gould, et al., 1988; Gould, et al., 1990b; Gould and Subramani, 1988; Keller, et al., 1987). Three recent advances offer hope that additional insights into the mysteries surrounding the organelle will be forthcoming. These include: the isolation of peroxisome-deficient mutants of yeasts and their use for cloning of peroxisomal genes; the application of microinjection techniques to study peroxisomal protein import; and the use of permeabilized cell systems for investigation of peroxisomes.

The application of the formidable strengths of yeast genetics and biochemistry has resulted in rapid advances in our understanding of diverse problems including cell cycle control and protein secretion (Deshaies, et al., 1989; Hartwell and Weinert, 1989; Pringle and Hartwell, 1981). The key element in applying a yeast system is the ability to efficiently isolate mutants defective in the process of interest. For peroxisome studies, this breakthrough was described in 1989 for *S. cerevisiae* and in 1990 for *H. polymorpha* (Cregg, et al., 1990; Erdmann, et al., 1989). With these publications came the recognition that, in yeasts, peroxisomes are required only for growth on certain substrates such as oleate and methanol and therefore, that yeast peroxisome defects are conditional mutations which only affect growth on these substrates. The isolation of yeast peroxisome-deficient mutants provided the means of cloning peroxisomal genes by functional complementation, several of which have now been reported. It was hoped that the predicted amino acid sequences of these genes would provide useful insights into the function of their products. However, in most instances, the sequences have revealed novel proteins with little similarity to others. Thus, researchers must proceed with the difficult task of determining the function of these proteins with few clues from their primary structures to guide them. It is worth noting that the identification of the *PAS8* gene product of *P. pastoris* as a candidate for the PTS-1 receptor is the first example where biochemical evidence for a specific function has been provided for a cloned peroxisomal gene (McCollum, et al., 1993).

The recent application of microinjection and permeabilized cell techniques to study peroxisome function should allow peroxisome researchers to compensate for the lack of a reliable and efficient *in vitro* system for the peroxisome. *In vitro* systems have been crucial in unraveling the details of complex biological processes such as peptide transit across membranes of the endoplasmic reticulum and mitochondrion, and vesicle movement between compartments of the secretory pathway. Although much effort has gone into developing *in vitro* import with purified peroxisomes (Imanaka, et al., 1987; Lazarow, et al., 1982; Lazarow, et al., 1991),

a reliable system has not emerged. Recently, it was reported that microinjected peroxisomal matrix enzymes such as luciferase are properly imported into peroxisomes of mammalian cells (Walton, et al., 1992a; Walton, et al., 1992b). When the PTS-1-directed enzyme luciferase was coinjected with an excess of a Ser-Lys-Leu-COOH containing competitor polypeptide, luciferase import was blocked, indicating the transport process involves a saturable receptor (Walton, et al., 1992a) Furthermore, when injected into both a human 3T3 cell line and a Zellweger cell line, luciferase was imported into peroxisomes only in normal cells. In contrast, *P. pastoris* AOX, which is thought to be imported through another PTS, was imported in both cell lines. These results support the notion that Zellweger cells are specifically defective in PTS-1 protein import. Variations on this approach should make it possible to distinguish and define various import routes utilized by different peroxisomal proteins. It may even be possible to inject the products of cloned peroxisomal genes into the different mutant Zellweger and CHO cell lines and thereby identify the missing functions in each cell line and possibly elucidate the role of specific gene products.

Permeabilized cells have recently been applied to the study of peroxisomal protein import. Using Streptolysin-O permeabilized CHO cells, import of luciferase and albumin conjugated with PTS-1 peptides was observed and shown to be dependent on ATP and cytosolic factors (Wendland and Subramani, 1993). An advantage of permeabilization relative to microinjection is that the former technique should facilitate the identification of individual cytoplasmic components required for peroxisomal biogenesis.

### 1.7 Thesis overview

This thesis describes the cloning and analysis of *PER6*, a gene required for peroxisome biogenesis in *P. pastoris*. The gene was cloned by functional complementation of a *per6* mutant strain that was one of a number of peroxisome-deficient mutants isolated in this laboratory (Liu, et al., 1992). I describe the localization of the gene to a small fragment by subcloning,

Northern filter hybridization analysis, and the DNA sequence of the gene. *PER6* appears to contain a 1296-bp open reading frame which potentially encodes a 432-amino acid protein of 49 kD. I describe the construction of a mutant strain in which a large portion of the open reading frame was deleted, and the use of this construct to genetically demonstrate that the cloned gene was the same as that which is defective in the originally isolated *per6* mutant. The predicted amino acid sequence of the *PER6* product revealed several interesting features which are discussed. Finally, I describe the synthesis of the *PER6* product in *E. coli*, and its purification for use as antigen to raise antibodies for future biochemical and immunocytological experiments.

# CHAPTER 2
# MATERIALS AND METHODS

## 2.1 Media and strains

*Pichia pastoris* and *Escherichia coli* strains used are listed in Table 2.1. *P. pastoris* strains were cultured at 30°C in YPD medium (1% yeast extract, 2% peptone, and 2% glucose) or YNB medium (0.67% yeast nitrogen base without amino acids) supplemented with one of the following carbon sources: 0.4% glucose, 0.5% methanol, 0.5% ethanol, or 0.1% oleic acid (with 0.5% Tween 40 to solubilize oleate). Amino acids were added to 50 μg/ml as required. Mating and sporulation were induced by nitrogen starvation on medium composed of 0.5% sodium acetate, 1% potassium chloride, and 1% glucose. *E. coli* strains were cultured at 37°C in LB medium (0.5% yeast extract, 1% glucose, and 1% sodium chloride). For selection of plasmids, ampicillin was added to a concentration of 0.1 mg/ml.

## 2.2 *P. pastoris* genomic DNA extractions

*P. pastoris* strains were grown in small YPD cultures overnight, and aliquots of these cultures were used to inoculate 200 ml YNB 0.5% glucose liquid cultures to a starting $OD_{600}$ of approximately 0.1. Cultures were harvested at an $OD_{600}$ of 1.0 to 2.0 by centrifugation and successively washed by centrifugation at 1,500 x g with 5 ml of water, SED buffer (1 M sorbitol, 25 mM EDTA, 50 mM DTT) and 1 M sorbitol. Washed cell pellets were resuspended in 5 ml of ST buffer (1 M sorbitol and 0.1 M Tris-HCl, pH 7.0), and incubated with 50 μg/ml Zymolyase T100 (ICN, Irvine, CA) for 30 minutes at 30°C to digest cell walls. The resulting spheroplast preparations were centrifuged at 1,000 x g and resuspended in 5 ml of lysis buffer (0.2% SDS, 5 mM EDTA, 50 mM NaCl, 10 mM Tris-HCl, pH 7.4). The viscous cell

Table 2.1 *P. pastoris* and *E. coli* strains used

| ***P. pastoris* Strains** | **Genotype** | **Source or reference** |
|---|---|---|
| JC100 | Wild type | NRRL Y-11430,[a] Cregg, et al., 1985 |
| JC114 | *per6* | Liu, et al., 1992 |
| JC116 | *per6 his4* | This study |
| JC117 | *per5 his4* | This study |
| JC214 | *per6Δ::SHIS4 his4 arg4* | This study |
| GS200 | *his4 arg4* | This study |

| ***E. coli* Strains** | **Genotype** | **Source or reference** |
|---|---|---|
| MC1061 | *hsdR mcrB araD*139 Δ(*araABC-leu*)7679 Δ*lacX*74 *galU galK rpsL thi* | Meissner, et al., 1987 |
| XL-1 Blue | *supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46 *thi relA*1 *lac*⁻ F' [*proAB*⁺ *lacI*$^{q}$ *lacZ*ΔM15 Tn*10*(*tet*$^{r}$)] | Bullock, et al., 1987 |
| TB1 | *ara*Δ(*lac proAB*) *rpsL* (ø80 *lacZ*ΔM15) *hsdR* | Johnston, et al., 1986 |

[a] Northern Regional Research Laboratories, Peoria, Ill.

lysates were treated with 50 µg/ml each of proteinase K and RNase A for 30 minutes at 30°C. An equal volume of chloroform:isoamylalcohol (24:1) was added to each preparation and gently mixed. Aqueous and organic phases were then separated by centrifugation at 12,000 x g for 20 minutes. The aqueous (top) phase of each preparation was collected and gently mixed with an equal volume of PCA. Phases were again separated by centrifugation as described above. The top aqueous phases (approximately 3 to 4 ml each) were then transferred to tubes containing 10 ml ice-cold 100% ethanol, and the chromosomal DNA preparations were mixed and precipitated by gently inverting the tubes. Each precipitated DNA sample was spooled onto a plastic rod, redissolved immediately in 1 ml of TE buffer, and dialyzed overnight at 4°C against 100 volumes of TE buffer.

**2.3 Gel electrophoresis, blotting, and hybridization**

DNA samples were separated by size on 0.8% agarose gels in TBE buffer (0.045 M Tris-borate, pH 8.0, 1.0 mM EDTA) following standard procedures (Sambrook, et al., 1989). RNA samples were separated by size on 1.5% agarose gels prepared in E buffer (0.02 M 3-[N-morpholino]propane-sulfonic acid [MOPS], 5 mM NaOAc, 5 mM EDTA, pH 7.0) that contained 18% formaldehyde deionized with AG501-X8 resin (BioRad, Richmond, CA) by a standard procedure (Sambrook, et al., 1989). Capillary transfer of nucleic acids onto nylon membranes was performed as described by Sambrook et al. (Sambrook, et al., 1989). The prehybridization mix for DNA-DNA (Southern) filter hybridizations contained 50% formamide, 6 x SSPE (0.9 M NaCl, 0.06 M $NaH_2PO_4$, pH 7.4, 6 mM $Na_2EDTA$), 1 x P (0.02% bovine serum albumin [BSA], 0.02% polyvinylpyrollidone, 0.02% Ficoll 400,000, 0.1% sodium pyrophosphate, 1% sodium dodecyl sulfate [SDS], 0.1% Tris-HCl, pH 7.5), and 0.1 mg/ml of sheared and denatured salmon sperm DNA. The prehybridization mix for RNA-DNA (Northern) filter hybridizations contained 50% formamide, 1 x P, 10% dextran sulfate, 1 M sodium chloride, and 0.1 mg/ml denatured, sheared salmon sperm DNA. Southern and Northern filters were preincubated in heat-sealed bags with the

prehybridization mix for at least five hours at 42°C before addition of radioactive probes.

DNA fragments were labeled with $\alpha^{32}$P-dCTP for use as hybridization probes by the random hexamer priming method (Sambrook, et al., 1989). Approximately 50 to 100 ng of template DNA in 3 µl of TE buffer was denatured by boiling for 3 minutes and cooling on ice for 1 minute. The denatured DNA was then added to 7.5 µl of LS buffer (0.4 M N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] [HEPES] [pH 6.6], 0.8 mg/ml BSA, 0.1 M Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 20 mM β-mercaptoethanol, 0.11 mM EDTA, 10 OD unit/ml hexamers, 40.3 mM dATP, dTTP, dGTP respectively), 20 µCi $^{32}$P-dCTP and 0.5 µl Klenow fragment of *E. coli* DNA polymerase I. The reaction was incubated at room temperature for at least three hours, and the reaction mix was then adjusted to a volume of 50 µl with TE buffer. To separate the labeled probe DNA from unincorporated $^{32}$P-dCTP, the mix was centrifuged through a Boehringer G-25 column (Boehringer Mannheim Corporation, Indianapolis, IN) at 3,000 x g for 5 minutes. The eluate containing the labeled probe was brought to 200 µl, and radioactivity was determined by scintillation counting.

Probes were denatured by boiling for 3 minutes and rapidly cooling on ice. Approximately $10^6$ to $10^7$ counts per minute (cpm) of labeled probe was added to filters in prehybridization solution and incubation was continued at 42°C for 12 to 24 hours. After hybridization, the filter was washed twice for 10 minutes each at room temperature in buffer containing 0.1 x SSPE and 1% SDS, and then a third time in the same solution for 20 minutes at 67 °C. Finally, the filter was wrapped in plastic and exposed to X-ray film for 1 to 5 days with an enhancer screen (DuPont, Ontario, CA) at 70°C.

### 2.4 Transformation of DNA into *P. pastoris* by the spheroplast method

A fresh single yeast colony was inoculated into 10 ml of YPD medium and cultured for approximately 24 hours at 30°C. Care was taken to maintain the culture in mid-logarithmic growth phase ($OD_{600} < 0.5$) by dilution into

fresh YPD medium. This culture was then used to inoculate a second YPD culture of 100 ml at a starting density of approximately 0.0001 $OD_{600}$ units. This second culture was grown for approximately 15 hours at 30 °C with shaking to a density of 0.2 $OD_{600}$ units.

The culture was harvested by centrifugation and the cells were washed successively by centrifugation at 1,500 x g in 10 ml water, SED, and 1 M sorbitol. The resulting washed cell pellet was resuspended in 5 ml SCE buffer (1 M sorbitol, 10 mM EDTA, 0.1 M sodium citrate, pH 5.8), and treated with 12 µg Zymolyase T100 at 30°C for 15 to 30 minutes to remove cell walls. The generation of spheroplasts during Zymolyase treatment was monitored by removing 100-µl aliquots of the mixture and adding them to 1 ml of 1 M sorbitol and 1 ml of 0.2% SDS. Spheroplast formation was indicated by lysis of cells in SDS, observed visually by a reduction in turbidity, and by an increase in viscosity of the sample, an indication that high molecular weight DNA had been released. Care was taken from this point on to handle spheroplasts gently in all subsequent manipulations. The spheroplasts were collected and washed by centrifugation at 1000 x g in 10 ml of 1 M sorbitol and 10 ml CaS buffer (10 mM $CaCl_2$, 1 M sorbitol) and were then gently resuspended in 0.6 ml CaS buffer.

Spheroplast samples of 100 µl were added to 12 x 75 mm polypropylene tubes containing DNA samples to be transformed. The DNA spheroplast mixtures were incubated for 20 minutes at room temperature before the addition of 1 ml of 20% polyethylene glycol (PEG)-1000 dissolved in 10 mM Tris-HCl, pH 7.4, 10 mM $CaCl_2$. Incubation at room temperature in PEG was continued for 15 minutes and the spheroplasts were harvested by centrifugation, resuspended in 150 µl of SOS medium (1 M sorbitol, 30% YPD, 10 mM $CaCl_2$), and allowed to recover at room temperature for 30 minutes. Spheroplast samples were brought to 1 ml with 1 M sorbitol and gently mixed. Samples of 0.1 and 0.9 ml were added to 10 ml of regeneration agar containing 2% agar, 0.6 M KCl, 0.67% YNB, and 2% glucose held at 55°C, mixed gently, and poured over the surface of a Petri plate containing a 10-ml

solidified layer of the same regeneration agar medium. Plates were incubated at 30°C and transformants formed colonies after 3 to 5 days.

**2.5 Construction of subcloning plasmids**

Five subfragments from pYT6 were cloned into individual *P. pastoris-E. coli* shuttle vectors that contained either the *Saccharomyces cerevisiae HIS4* (*SHIS4*) gene or the *P. pastoris HIS4* gene and transformed into JC116 (*per6 his4*) to test for their ability to complement the mutation in *PER6*, as diagrammed in Figure 3.2. A *Sac*I fragment was removed from pYT6 by *Sac*I digestion and self ligation. This construct allowed us to test the remaining P. pastoris DNA in pYT6, a *Sac*I to *Bam*HI fragment (coordinates 12.0 to 14.5 in Figure 3.1). A *Bam*HI fragment (coordinates 10.5 to 14.5 in Figure 3.1) and a *Bgl*II fragment (coordinates 12.4 to 9.7 in Figure 3.1) were each tested by insertion into the unique *Bam*HI site in pYM8. Two *Cla*I fragments (coordinates 11.1 to 14.1 and 8.2 to 11.1 in Figure 3.1, respectively), were inserted into the unique *Cla*I site in pYM4 (Cregg, et al., 1985). A *Sca*I fragment (coordinates 6.9 to 10.2 in Figure 3.1) was inserted into the *Eco*RV site in pYM8.

**2.6 Construction of DNA sequencing template plasmids**

For DNA sequencing, two sets of plasmids were constructed that each contained a nested set of subfragments of the region of interest. Each plasmid contained a region complementary to an oligonucleotide primer just 3' to different portions of the region to be sequenced. To create these template plasmids, a 1.5 kb *Cla*I-*Dra*I (coordinates 11.1 to 12.6 on pYT6 in Figure 3.1) fragment was inserted into pBS SK+ in both orientations. For one orientation, the fragment was ligated into *Cla*I and *Hin*cII digested pBS SK(+) to create pCH4. For the other orientation, the fragment was inserted into the same vector digested with *Cla*I and *Eco*RV to create pCR1. pCH4 and pCR1 were digested with *Kpn*I and *Xho*I and treated with Exonuclease III for six minutes at 37°C. Aliquots of these reactions were removed every 30 seconds during this period, a time interval predicted to remove approximately 200 bp

from each molecule. The resulting deletion products were further incubated with S1 nuclease to remove the remaining single strand and treated with the Klenow fragment of DNA polymerase I in the presence of dNTPs to make the DNA termini flush ended. The resulting molecules were recircularized by ligation and transformed into *E. coli* strain XL-1 Blue. Plasmids in transformants were extracted and examined for deletions of the desired sizes and a series of pCH4- and pCR1-derived plasmids was collected that contained deletion start points every 200 to 300 bp. These nested sets of plasmids were used as templates for double-stranded dideoxynucleotide sequencing using -40 primer. A single open reading frame that started beyond the DraI site was revealed. To determine the sequence of the 5' terminus of *PER6*, additional sequence was obtained in this direction by sequencing using synthetic oligonucleotides as primers and pYT6 as the template.

**2.7 DNA sequence determination**

The sequence of cloned DNA fragments was determined by the dideoxy-chain termination method (Sanger and Coulson, 1975) using a commercially available kit (USB, Cleveland OH). The template plasmids were prepared using the rapid boiling method (Sambrook, et al., 1989), followed by extraction with two volumes of buffered phenol, and then two further extractions with an equal volume of PCA. The quality and quantity of plasmid DNA was determined by examination of samples after electrophoresis through agarose gels. For sequencing reactions, 3-5 μg of each template DNA in 5 μl TE buffer was mixed with 1 μl of primer (10 ng/μl) and denatured by addition of 1 μl of 1 N NaOH and incubation for 10 minutes at 37°C. The mixture was then neutralized with 1 μl of 1 N HCl followed by addition of 2 μl of Sequenase reaction buffer (Hsiao, 1991). The standard procedure for Sequenase polymerization reactions using $^{35}$S-labeled dATP was followed as described by the supplier (USB, Cleveland OH).

DNA fragments resulting from sequencing reactions were separated by electrophoresis through 0.5% acrylamide gels of 385 mm x 310 mm x

0.4 mm at a constant wattage of 55 watts. Immediately prior to electrophoresis, samples were denatured by boiling and rapid cooling on ice. After electrophoresis, gels were transferred to filter paper, dried by vacuum at 1 cm Hg using Gel Dryer Sr., Slab gel dryer SE1160 (Hoefer Scientific Instruments, San Francisco, CA) and exposed to X-ray film. DNA sequences were determined from X-ray films using a Kodak Gel Reader (IBI, New Haven, CT), and sequences was analyzed using the MacVector 3.5 software program (IBI, New Haven, CT).

**2.8 Construction of the disruption plasmid pUZ12**

A restriction map of pUZ12 is shown in Figure 2.1. The first step in its construction was to insert a 4.0 kb *Bam*HI *P. pastoris* fragment from pYT6 (coordinates 10.5 to 14.5 in Figure 3.1) into the unique *Bam*HI site of pBR322. The resulting plasmid was named pRW6. This *Bam*HI fragment appeared to contain the entire coding sequence of *PER6* along with 5' and 3' flanking sequences. In the second step, pYM8 was digested with *Bam*HI and *Nru*I, to release a 4.0 kb fragment that contains the *S. cerevisiae HIS4* (*SHIS4*) gene. The *SHIS4* fragment was purified and ligated into the *Bam*HI and *Sma*I digested pBW5 (a derivative of pUC19 in which an *Mlu*I site had been converted into a *Sac*I site by insertion of an oligonucleotide adaptor). The *SHIS4* fragment was then released from pBW5 by digestion with *Bam*HI and *Mlu*I, and inserted into *Bgl*II and *Mlu*I digested pRW6. This step also deleted 612 bp of *PER6*, and the resulting plasmid was named pUZ12. Prior to transformation into *P. pastoris* strain GS200, pUZ12 was digested with *Bam*HI to yield a 7.9 kb fragment composed of the *SHIS4* gene flanked one side by 5' *PER6* sequences and on the other with 3' *PER6* sequences as shown in Figure 3.7.

**2.9 Expression of Per6p in *E. coli***

A DNA fragment from *PER6* that encodes the carboxyl-terminal 298 amino acids of Per6p was expressed in *E. coli* as a fusion protein with *E. coli* maltose binding protein (MBP). Materials required for the construction and

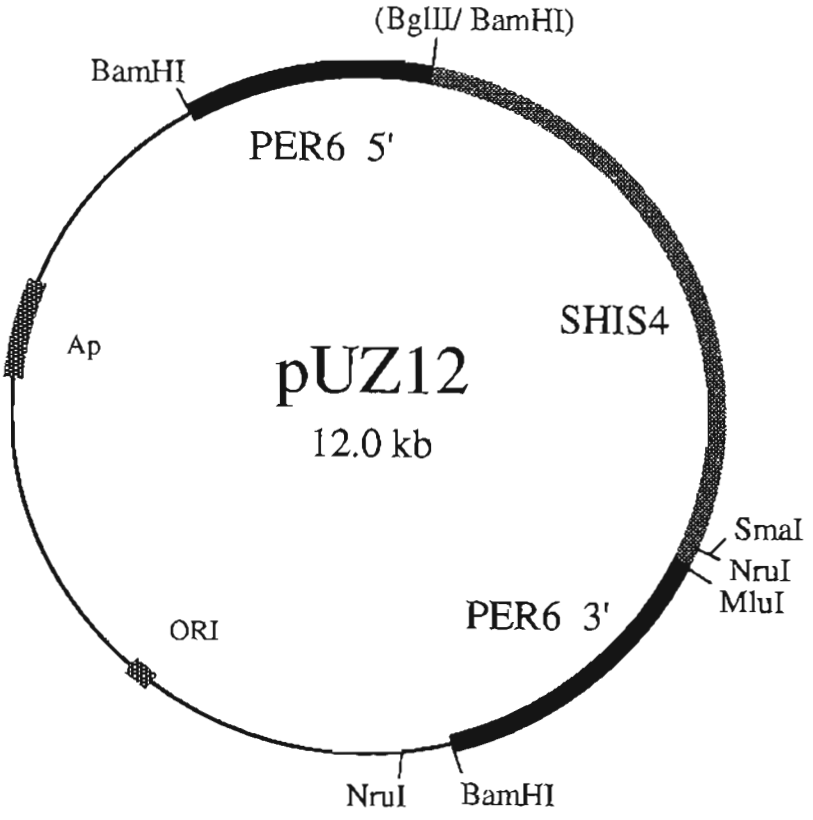


Figure 2.1 Restriction enzyme map of pUZ12, the plasmid used to delete *PER6* in *P. pastoris*. pUZ12 is composed of an *E coli* origin of replication (ORI); an ampicillin resistence gene (Ap); a *S. cerevisiae HIS4* gene (SHIS4); and 5' and 3' fragments from *PER6* as shown.

expression of the MBP-Per6p fusion vector were purchased as a kit from New England Biolabs (Beverly, MA) and used as recommended by the supplier. A 908 kb *Bgl*II-*Hin*cII DNA fragment from pYT6 that encodes most of Per6p was inserted into expression vector pMAL supplied in this kit. Prior to this insertion, restriction sites within the pMAL polylinker cloning sites were modified so that the *PER6* fragment would be fused in the proper translational reading frame with MBP. This was achieved through the insertion of two adaptor oligonucleotides into pMAL. In the first insertion, two oligonucleotides of the following sequences were synthesized:

SR1 5' AATTCCGGATCCCTGCAGGATATCA 3'

SR2 5' AGCTTGATATCCTGCAGGGATCCGG 3'

These oligomers were inserted into *Eco*RI and *Hin*dIII digested pMAL. For this, 200 pmoles of each oligonucleotide was mixed in 10 µl of buffer A (500 mM Tris-HCl, pH 8.0, 100 mM $MgCl_2$) and brought to a total volume of 100 µl with water. The mixture was overlaid with 50 µl of mineral oil, heated to 90°C for 3 minutes and cooled slowly to room temperature over a period of at least 2.5 hours to allow the oligonucleotides to anneal. The oligomers were then ligated into *Eco*RI and *Hin*dIII cut pMAL, the ligation products transformed into *E. coli* strain XL-1 Blue and transformed cells screened for ones that harbored pMAL with an SR oligonucleotide insert by restriction digest patterns. One correctly constructed plasmid was named pBW1 and subjected to further modification.

For the second adaptor insertion step, the following oligonucleotide was synthesized:

TG 5' GAGGCCTCTGCA 3'

The oligomer was allowed to self-anneal by heating and slow cooling as described above and then ligated with *Pst* I digested pBW1. The resulting plasmid was named pBW2 and contained a unique *Stu*I site at the polylinker. Finally, pBW2 was digested with *Bam*HI and *Stu*I and the *Bgl*II-*Hin*cII fragment from *PER6* was inserted to produce the MBP-Per6p expression plasmid pMW6 (Figure 2.2).

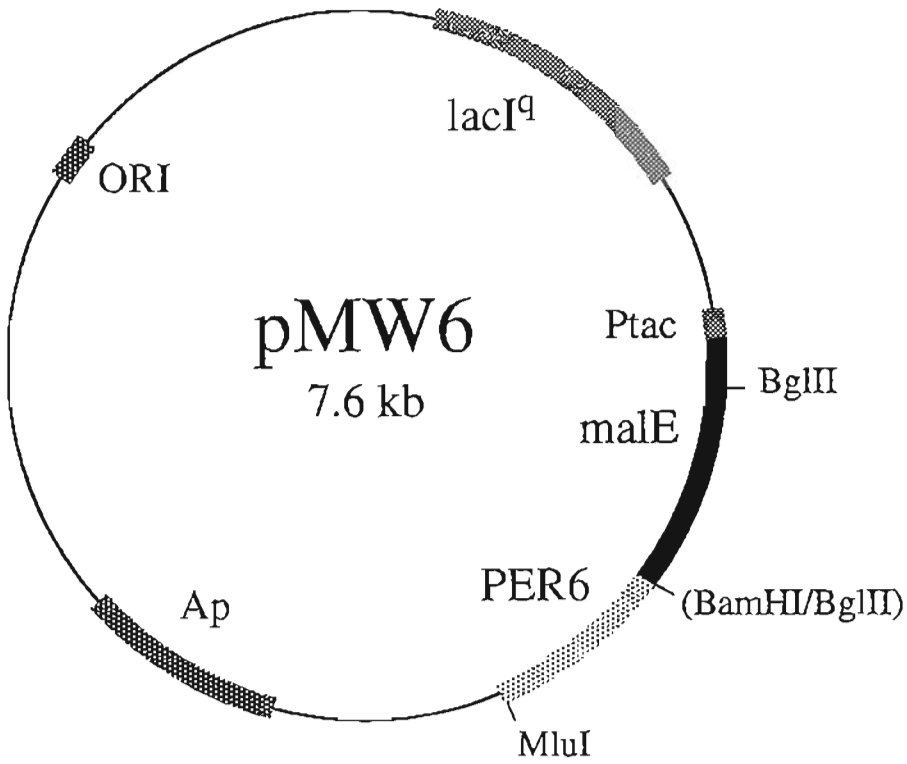


Figure 2.2 Restriction enzyme map of pMW6, the plasmid used to express MP6 in *E. coli*. pMW6 contains an *E. coli* origin of replication (ORI); an ampicillin resistence gene (Ap); a *lacI$^q$* gene that encodes a Lac repressor; and a *malE-PER6* fusion that is under an inducible $P_{tac}$ promoter

To express the MBP-Per6p fusion protein (MP6), pMW6 was transformed into *E. coli* strain TB-1. A transformant, named TBM6 was cultured in 1 liter of LB-ampicillin medium to an $OD_{600}$ of 0.5, and the production of the fusion protein was induced by addition of isopropylthioglactoside (IPTG) to a concentration of 0.3 mM. After two hours, the culture was harvested by centrifugation and stored frozen at -20°C overnight in 50 ml of amylose column buffer (10 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA) . This suspension was thawed in cold water and cells were disrupted by 10 cycles of sonication in 15 second pulses using an ultrasonic processor (Heat Systems-Ultrasonic, Inc., Plainview, NY) set at output 4. The preparation was kept in an ice-water bath during the sonication to prevent the heating of the sample, and held on ice for a minimum of 30 seconds between each sonication cycle. The supernatant was collected after centrifugation for 30 minutes at 9,000 x g and diluted 2.5 times with amylose column buffer, and applied to a chromatography column containing 15 ml of amylose resin (NEB, Beverly, MA). The supernatant was loaded on the column at a flow rate of 1 ml/minute and the column was then washed at the same rate with 120 ml of amylase column buffer. The fusion protein was eluted with 50 ml of column buffer containing 0.01 M maltose, and MP6 containing fractions were identified on SDS-Page gels. MP6 fractions were pooled and dialyzed overnight against 3 changes of 100 x volume of 20 mM Tris-HCl, pH 8.0, 25 mM NaCl. The dialyzed protein preparation was then loaded onto a column containing 5 ml of DEAE sepharose CL-6B resin (Pharmacia, Uppsala, Sweden) equilibrated with low salt buffer (10 mM Tris-HCl, pH 8.0, 25 mM NaCl). The column was washed with 15 ml of the same buffer, eluted with 50 ml NaCl gradient from 25 mM to 500 mM in 20 mM Tris-HCl, pH 8.0, and 1 ml fractions were collected. The flow-through, wash, and eluted fractions were examined for protein concentration by absorbance at 260 nm, and fractions containing significant amounts of protein were examined on SDS-PAGE gels stained with Coomassie blue. Fractions that appeared to contain mostly full-sized MP6 were pooled and concentrated to

approximately 1 mg/ml using a Centriprep 10 column (Amicon, Beverly, MA).

Rabbit polyclonal antibodies against MBP-Per6p were prepared by Josman Laboratories (San Jose, CA).

**2.10 Miscellaneous procedures**

Other recombinant DNA procedures were performed as described in Sambrook et al. (Sambrook, et al., 1989). Most restriction enzymes and other DNA modifying enzymes were purchased from New England Biolabs (Beverly, MA) and used as directed by the supplier. RNAs from *P. pastoris* were extracted as described by Köhrer and Domdey (Kohrer and Domdey, 1991). SDS-Polyacrylamide gels were composed of 4% stacking gels on top of 10% separation gels. SDS-Page and Western blotting were performed as described in Sambrook et al. (Sambrook, et al., 1989) using a Mini-PROTEAN® II cell from BioRad (Richmond, CA). Antigens were detected on membranes using the alkaline phosphatase-coupled antibody procedure (Sambrook, et al., 1989) with reagents purchased from BioRad (Richmond, CA).

# CHAPTER 3
# RESULT

## 3.1 Cloning the *Pichia pastoris PER6* gene

### 3.1.1 Summary of the isolation and characterization of the *per6* mutant

The *PER6* gene was cloned by functional complementation of a *P. pastoris per6* mutant. This mutant was one of a collection of peroxisome deficient mutants (*per* mutants) that was affected in eight different genes isolated in this lab (Liu, et al., 1992). The *per* mutants were isolated by screening a collection of methanol-utilization-defective ($Mut^-$) mutants for ones that were also oleic acid-utilization-defective ($Out^-$) but still able to grow on other carbon sources such as glucose, glycerol and ethanol. Methanol-induced cells of these mutants were examined by electron microscopy and observed to be without morphologically recognizable peroxisomes. Biochemical studies provided supporting evidence for defective peroxisome biogenesis in each mutant. In methanol- or oleate-induced cultures of these strains, the peroxisomal matrix enzyme catalase was present at normal levels. However, subcellular fractionation studies indicated that the enzyme was mislocalized to the cytosol (Liu, et al., 1992).

### 3.1.2 Cloning of the *PER6* gene

The *PER6* gene was isolated from a *P. pastoris* genomic DNA library constructed in the *E. coli-P. pastoris* shuttle vector pYM8 (Cregg, et al., 1985). The vector is composed of a *P. pastoris* autonomous replication sequence (PARS1), the *S. cerevisiae* histidinol dehydrogenase gene (*HIS4*) which provides a selectable marker when the plasmid is transformed into *his4* *P. pastoris* strains, and sequences from *E. coli* plasmid pBR322 including an *E. coli* origin of replication and an ampicillin resistance gene ($Amp^r$) for

selection in *E. coli* strains. The *P. pastoris* genomic DNA library was constructed by insertion of genomic DNA fragments into the unique *Bam*HI site in pYM8 (Tan and Cregg, unpublished data). pYT6 was isolated from this library by its ability of transforming JC116 (*per6 his4*) into Mut$^+$ phenotype (Tan and Cregg, unpublished data), which suggested that this plasmid most likely contained the *PER6* gene. However, it was conceivable that the gene was not *PER6* but a gene with suppressor activity. In addition, it was possible that the *P. pastoris* DNA fragment in pYT6 did not contain the whole *PER6* gene but only that portion of the gene that included the region that was mutated in the chromosomal *per6* gene. Section 3.3 describes genetic experiments that strongly indicate that the DNA fragment in pYT6 does contain *PER6*. For discussion purposes, it is assumed that the cloned gene is *PER6*.

## 3.2 Localization of *PER6* coding sequences

### 3.2.1 Subcloning *PER6*

As a first step in identifying sequences encoding *PER6*, a restriction endonuclease cleavage site map of pYT6 was constructed (Figure 3.1). The plasmid harbored a *P. pastoris* DNA fragment of approximately 6.5 kb inserted at the *Bam*HI site of pYM8. Five *P. pastoris* DNA subfragments from pYT6 were examined for the presence of *PER6* by their ability to functionally complement the *per6* mutation in strain JC116. This was accomplished by subcloning each fragment into a *HIS4* gene containing plasmid. The resulting vectors were then transformed into strain JC116 by selection for His$^+$ prototrophy and the resulting colonies were replica plated on to methanol medium plates. As shown in Figure 3.2, a 3-kb *Cla*I fragment (coordinates of 11.1 to 14.1 on the map shown in Figure 3.1) was the smallest fragment able to transform the *per6* strain JC116 to Mut$^+$, a result that suggested that this fragment contained at least a portion of *PER6*.

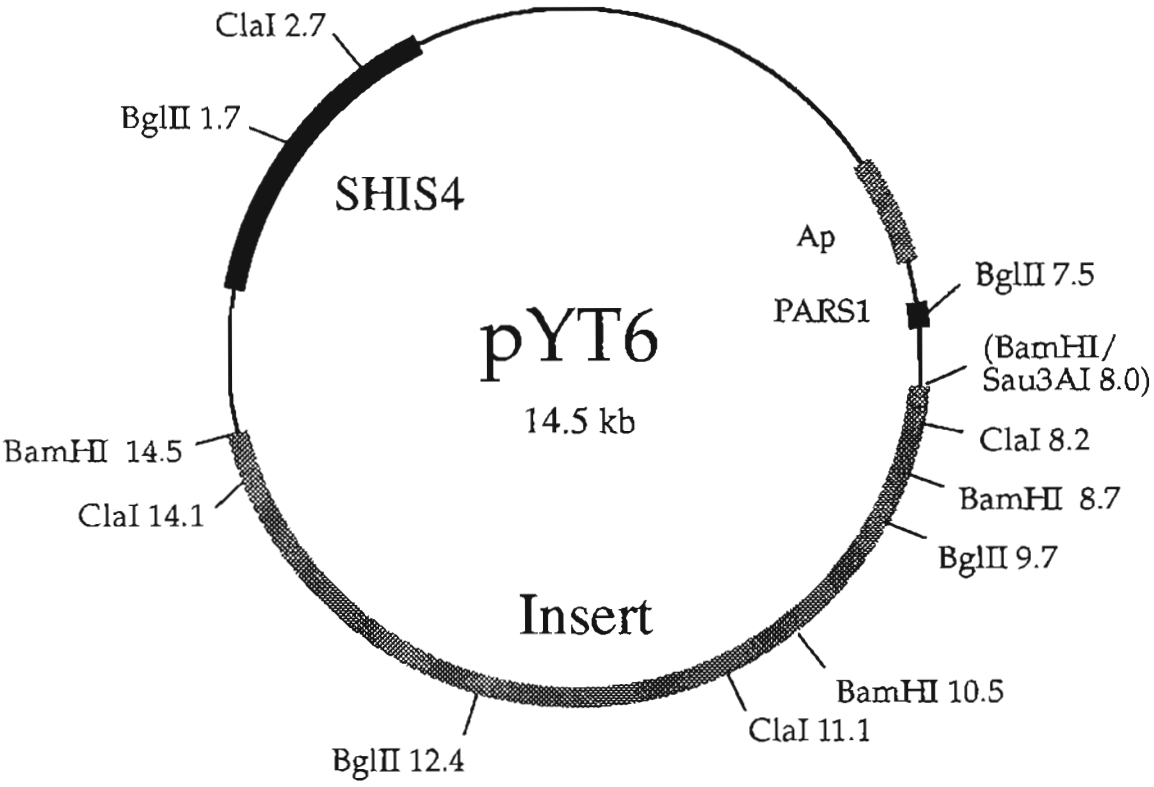


Figure 3.1 Restriction enzyme map of pYT6, a plasmid that complements the *P. pastoris per6* mutant. pYT6 contains a PARS1 sequence that allows it to replicate autonomously in the *P. pastoris* genome; an ampicillin resistent gene (Ap); a *S. cerevisiae HIS4* gene (SHIS4); and a 6.5 kb fragment of *P. pastoris* genomic DNA.

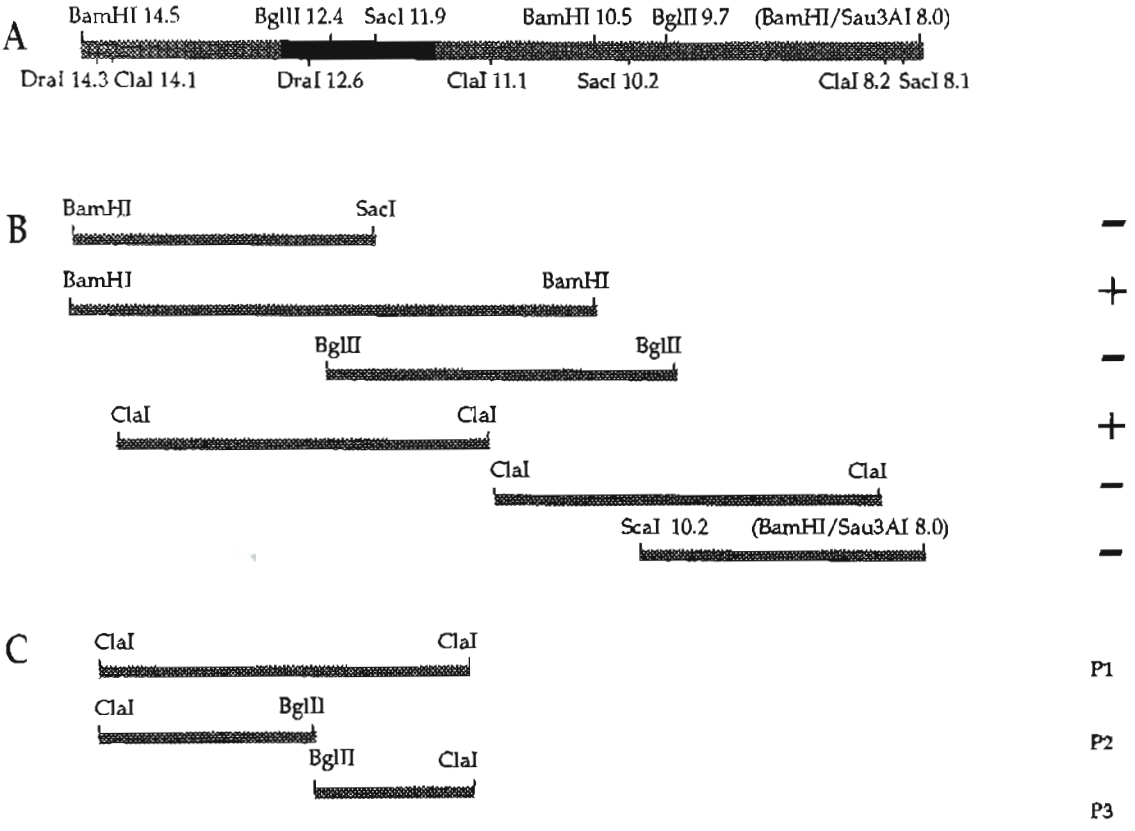


Figure 3.2 Restriction enzyme map of subfragments of the pYT6 insert: (A), the 6.5 kb *P. pastoris* genomic DNA insert in pYT6 (solid box indicates the location of an open reading frame); (B), DNA subfragments tested for complementation of the *per6* mutant (+ indicates complementation to Mut$^+$; - indicates no complementation); (C), Subfragments used as probes in Northern blot of Figure 3.3.

### 3.2.2 Northern blot analysis

Northern filter hybridization experiments were performed to further narrow the approximate location of the *PER6* sequences and to estimate the size of the putative *PER6* transcript. For these studies, RNA was prepared from each of two *P. pastoris* wild-type cultures, one grown in YNB methanol medium and the other in YNB glucose medium. Total RNA was extracted from the cells, separated by electrophoresis through formaldehyde agarose gels, and blotted onto nylon membranes. As hybridization probes, three DNA fragments were labeled with $^{32}P$ (Figure 3.2, C). Probe 1 contained the *Cla*I fragment of 3.0-kb which was shown to be sufficient to correct the *per6* defect in the subcloning experiments (coordinates 11.1 to 14.1 in Figure 3.1); probes 2 and 3 were subfragments of the *Cla*I fragment after digestion with *Bgl*II (probe 2 coordinates, 14.1 to 12.4 ; probe 3 coordinates, 12.4 to 11.1 in Figure 3.1). A single message of 1.4 kb was detected with all three probes (Figure 3.3). This message was a good candidate for the transcription product of *PER6* since each probe included at least a portion of the 3-kb *Cla*I fragment that functionally complemented a *per 6* strain and since the steady state level of the transcript was significantly higher in methanol-grown cells than in glucose grown cells. Probe 3 composed of the *Cla*I-*Bgl*II fragment (coordinates 11.1 to 12.4) showed a significantly more intense band than that from probe 2 (coordinates 12.4 to 14.1), suggesting that most of the *PER6* message is transcribed from within the former fragment.

## 3.3 Determination and analysis of the nucleotide sequence of the *PER6* locus

### 3.3.1 DNA sequence determination

A 1.5-kb *Cla*I-*Dra*I fragment (coordinates 11.1 to 12.6 in Figure 3.1) that was predicted from the subcloning and Northern blot results to contain most of *PER6* was selected for DNA sequencing by the dideoxy-chain termination method (Sambrook, et al., 1989). In order to sequence both strands, this fragment was inserted in both orientations into plasmid pBS SK+

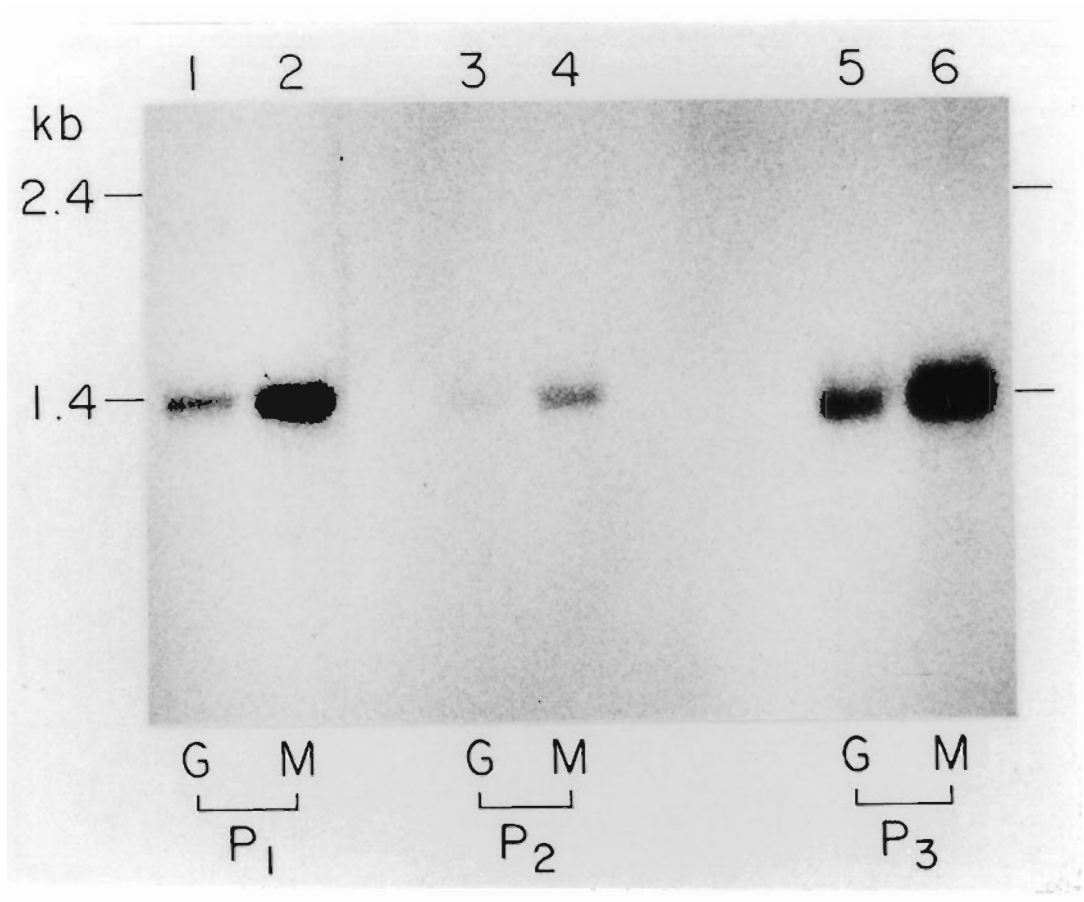


Figure 3.3 Northern blot of *PER6* transcript. Total RNA was extracted from *P. pastoris* wild-type cells cultured in glucose (G) and methanol (M). The three labeled probes used are shown in Figure 3.2 (C).

to create pCR1 and pCH4. Each of these vectors was then utilized to create a set of subvectors, which together provided a set of unidirectional nested deletions in the *PER6* DNA fragment each starting adjacent to a fixed pBS SK+ sequence (see Materials and Methods for details). Each subvector was then subjected to double-stranded DNA sequencing reactions using a single oligonucleotide primer that was complementary to a fixed sequence located adjacent to a specific plasmid-*P. pastoris* DNA junction. As described below the DNA sequence revealed a single large open reading frame that appeared to extend beyond the *Dra*I site (coordinate 12.6 in Figure 3.1). Using pYT6 as template and oligonucleotide primers that were complementary to previously sequenced regions, an additional 350 bp of sequence was obtained that overlapped with the previous sequence and contained 250 bp of additional sequence beyond the *Dra*I site.

### 3.3.2 Analysis of *PER6* sequence

The DNA sequence results revealed a single open reading frame of 1,296 bp with the potential of encoding a protein of 432 amino acids (Figure 3.4). The first ATG of this open reading frame was preceeded by an in frame translation stop codon (TAA) 27-bp upstream, and was followed by a second in frame ATG 174-bp downstream. Based on our knowledge of other *P. pastoris* genes and correlation with transcript size, it appeared like that the first in frame ATG in the sequence is the translation start site for the *PER6* protein (Per6p). For purposes of discussion, we assume that the full open reading frame encodes Per6p.

The Per6p sequence revealed one hydrophobic region of 23 amino acids at the amino portion (amino acids 198 through 215) in Figure 3.4, having potential to form an α-helical membrane spanning domain (Figure 3.5). The carboxy-terminal quarter of Per6p (120 amino acids) is rich in acidic amino acids (-24 net charge) with the last nine amino acid residues being hydrophobic. When Per6p was compared with other protein sequences in GenBank, it showed significant similarity to other proteins. One of these proteins, PAF-1p, is encoded by the *PAF-1* gene from rat and is believed

```
                                30                                60
                                 *                                 *
GGTCCTTCAACTCGATGCTAAGTTGCTAGA CAACGAAATATCGATATGCTCTACCGGCAG
                                                     M  L  Y  R  Q>

                                90                               120
                                 *                                 *
CTATCTGGAGCTTTTAACAGCAACAGACTT CCGAGTTGGCTTGGGAGAATCCATTCCAAC
 L  S  G  A  F  N  S  N  R  L   P  S  W  L  G  R  I  H  S  N>

                               150                               180
                                 *                                 *
TATGCCTCTGAGTTAAAGCTCTTACTGGAA CTACTTATCTTTAAAGTAACCGTATGGAAC
 Y  A  S  E  L  K  L  L  L  E   L  L  I  F  K  V  T  V  W  N>

                               210                               240
                                 *                                 *
AAGCACTCAAGCTATGGCCTCACTCTTCAG AATCTGGTAATGTACGATGGTGGTGTTCAT
 K  H  S  S  Y  G  L  T  L  Q   N  L  V  M  Y  D  G  G  V  H>

                               270                               300
                                 *                                 *
AATAAAAAATTCAGGTCGAAACAACAGTCC GAACTCAGGGTTACAAAGAAAATACTTTTA
 N  K  K  F  R  S  K  Q  Q  S   E  L  R  V  T  K  K  I  L  L>

                               330                               360
                                 *                                 *
CTGTCATCCGTGTTGCTTGGGTATTTTGTC AAAAAGATTCAATCGTATGTGTACTCTTTC
 L  S  S  V  L  L  G  Y  F  V   K  K  I  Q  S  Y  V  Y  S  F>

                               390                               420
                                 *                                 *
GAAGATTATGATCTAGAGACTGATGGAGAA GACTTGAGCACCTTAGAGAGAATTAGATTA
 E  D  Y  D  L  E  T  D  G  E   D  L  S  T  L  E  R  I  R  L>

                               450                               480
                                 *                                 *
AAGACTATCAAACTGTTAAAATCCCAGATC TCCACACTGGAGAAAGCACATTCCGTTCTC
 K  T  I  K  L  L  K  S  Q  I   S  T  L  E  K  A  H  S  V  L>

                               510                               540
                                 *                                 *
TCATTGGTAAATTTTGTTACATTTCTGGTA TCTGGAAGTTTTCCTGACCTAACTACTCGA
 S  L  V  N  F  V  T  F  L  V   S  G  S  F  P  D  L  T  T  R>

                               570                               600
                                 *                                 *
ATCCTTAACATTAGATTCAAACCATTGGTT ACTACGCAAGTGGCCTTCGCTTCAAACCCA
 I  L  N  I  R  F  K  P  L  V   T  T  Q  V  A  F  A  S  N  P>

                               630                               660
                                 *                                 *
GAAACGATATCCTATGAATTTCAAAATAGA CAACTAGTGTGGAACACATTGACAGAATTT
 E  T  I  S  Y  E  F  Q  N  R   Q  L  V  W  N  T  L  T  E  F>
```

```
                                690                                 720
                                 *                                   *
ATTGTGTTTATTTTGCCAGCATTATCAGTA CCTAAGTTTACCAAGTCACTGGTAAGCTCG
 I  V  F  I  L  P  A  L  S  V   P  K  F  T  K  S  L  V  S  S>

                                750                                 780
                                 *                                   *
ATAACAGGAACTTCACCCAAGTCTAGCCAA GTGACTGACGAGGACCTAAAAGTTTTTTCT
 I  T  G  T  S  P  K  S  S  Q   V  T  D  E  D  L  K  V  F  S>

                                810                                 840
                                 *                                   *
TCTCTTCCGGAAAGAGTATGTGCTATATGT TTCCAGAATTCACAAAATTCTGACTCGGGA
 S  L  P  E  R  V  C  A  I  C   F  Q  N  S  Q  N  S  D  S  G>

                                870                                 900
                                 *                                   *
GCTCAAAATGATATTTCCCTCAACGATACT TTAGTCACCAATCCATACGAAACTACCTGT
 A  Q  N  D  I  S  L  N  D  T   L  V  T  N  P  Y  E  T  T  C>

                                930                                 960
                                 *                                   *
GGACATATTTACTGCTATGTGTGCATTCTT TCAAAATTGCAAATCTTCAAGGAGGAAGGC
 G  H  I  Y  C  Y  V  C  I  L   S  K  L  Q  I  F  K  E  E  G>

                                990                                1020
                                 *                                   *
AAGAATCTCCCAAAGTCAGATCCGAACAAA TACTGGCATTGCTTGAGATGTAATGAGCCA
 K  N  L  P  K  S  D  P  N  K   Y  W  H  C  L  R  C  N  E  P>

                               1050                                1080
                                 *                                   *
GCTTCTTGGTGTCGAGTCTATACTGGAGAT GTGGAAGACGCGTTGAGACAGAAGGCTGTT
 A  S  W  C  R  V  Y  T  G  D   V  E  D  A  L  R  Q  K  A  V>

                               1110                                1140
                                 *                                   *
GAAGAAGTCACAGAGGATGAAGATGCTTCA AGTGAAGACGAGGAAAAAAGGGATCAAGAT
 E  E  V  T  E  D  E  D  A  S   S  E  D  E  E  K  R  D  Q  D>

                               1170                                1200
                                 *                                   *
TCAGAAGGTGCTAAAACTGTTTCCCAAAGT TTCCATCACGTTAATGGATCAGACTATCAA
 S  E  G  A  K  T  V  S  Q  S   F  H  H  V  N  G  S  D  Y  Q>

                               1230                                1260
                                 *                                   *
ACAGCATCATTCATAGAGCAGGCTGAGCTG AATGAAAATGAATACACAGACGGTTCGGAA
 T  A  S  F  I  E  Q  A  E  L   N  E  N  E  Y  T  D  G  S  E>

                               1290                                1320
                                 *                                   *
GTGGAAATATATGATGCTGAAGATGAATAC ACTGATGAAGAAGTAGATGACGACTCTCCA
 V  E  I  Y  D  A  E  D  E  Y   T  D  E  E  V  D  D  D  S  P>
```

```
                                1350                                1380
                                   *                                   *
GGTTTTTTCGTTGGTGCGTTATAGAGTTAC GGTTAACAAGATGGTATTAGAATTTGTTAA
 G  F  F  V  G  A  L  *>

                                1410                                1440
                                   *                                   *
GTAATCATAATCATGTAATGTGTCACGTGA CTGTTTGTATCATTCCTTACCTGGATAACA

                                1470                                1500
                                   *                                   *
ATCTATCTATCTCATCGCCATTCCTCTAGC GTAAATGGGCGCAGAACTATCACTGTTGGC

                                1530                                1560
                                   *                                   *
TCCTACGGCTCAACCAATTGCTCTATCAGC CTATGTGGATTTCCTCAGCAACATTCAATA

                                1590                                1620
                                   *                                   *
CAATAAACCACTGGGGACCTCTCGTTTCCT AAAAACAGTGAAGGGCCTCAACGATCAAGG

                                1650                                1680
                                   *                                   *
GAGCATTGTTGTCAAAGTCCTCGTTAAACC TAATAGCGGCTTGGATCTTAGTGAATGGGT

                                1710                                1740
                                   *                                   *
AGAAAAGTTGGAATTCTTACGACTGAAGCT GCTTGATGTACCAAACGTGATACCATATAA

CTTGGTC
```

Figure 3.4 DNA and predicted amino acid sequence of the *PER6* open reading frame.

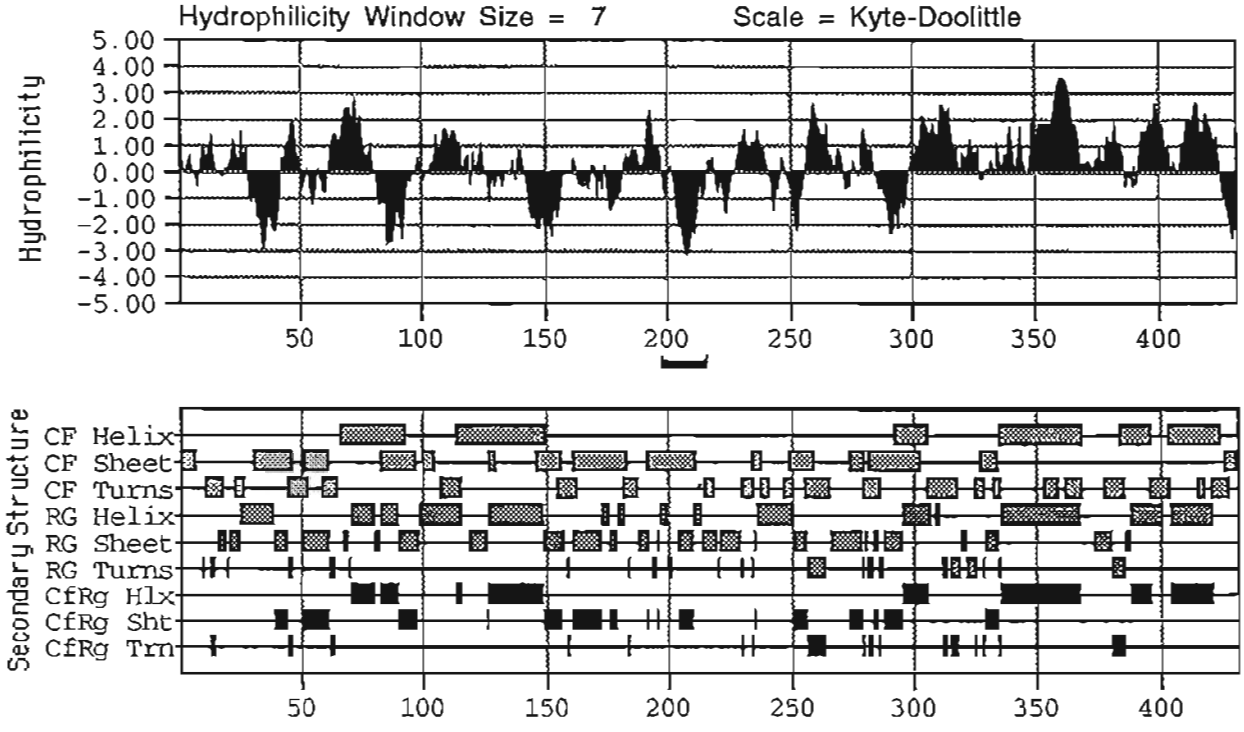


Figure 3.5 Hydrophilicity and predicted secondary structure of Per6p. The potential transmembrane domain is indicated by the solid bar.

to be a peroxisomal integral membrane protein of 35 kD (Tsukamoto, et al., 1991). The region of homology was localized in three closely spaced segments of 37, 24, and 17 amino acids (Figure 3.6). When optimally aligned, the co-linear sets of segments of PAF-1p and Per6p were 37, 50 and 41% identical, and 72, 70 and 58% similar, respectively. The middle segment of similarity overlaps the predicted Per6p transmembrane domain and one of the two predicted for PAF-1p. Seven cysteine residues were also present in this region of both Per6p and PAF-1p, with a CxxxxCxxC stretch located at the same position within the third segments of similarity, one CxxC located at different positions between the second and third segments, and one CxxC located at different positions just to the COOH-side of the third segments.

The acid rich carboxyl portion of Per6p showed homology to several "acid blob" activation domains of a number of transcription factors, including the immediate early protein RSp40 in the Ka strain of suid herpes virus 1 (Zhang and Leader, 1990). However, on closer inspection, the homology was limited to aspartate and glutamate residues in these regions. Interestingly, PAF-1p does not contain an acidic region and when Per6p and PAF-1p regions of similarity are aligned PAF-1p sequence ends at approximately the point at which the acid rich portion of the Per6p sequence begins.

### 3.4 Construction of a *per6* "null" mutant of *P. pastoris*

A *P. pastoris* strain in which a large segment of *PER6* is deleted was constructed by the gene replacement method of Rothstein (Rothstein, 1991). This *per6* "null" (*per6Δ*) mutant strain is useful for at least two types of experiments. The first are genetic experiments to formally demonstrate that the cloned DNA fragment actually contains *PER6*. The second are biochemical and cytological experiments aimed at determining the metabolism and subcellular structure of a *P. pastoris* strain in which *PER6* is completely defective.

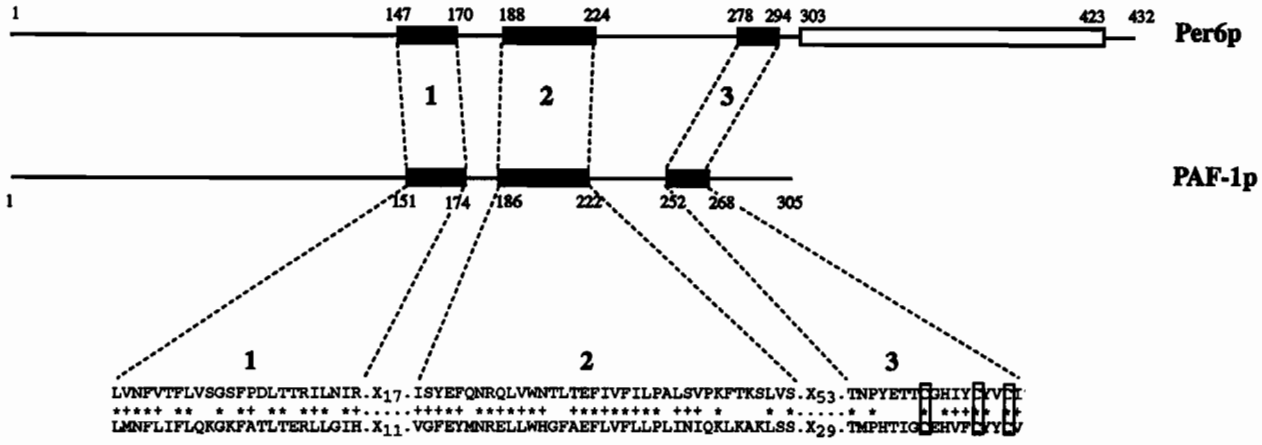


Figure 3.6 Allignment of Per6p and PAF-1p sequences. The solid boxes indicate the regions of similarity, the open box indicates the acidic region in Per6p, and the verticle lines indicate the cysteine residues. * indicates identity and + indicates similarity of amino acids between the two proteins.

### 3.4.1 Construction of the *per6* null strain

Plasmid pUZ12 was constructed to delete approximately 50% of *PER6* coding sequence (nt 400-1012, Figure 3.7) in the *P. pastoris* genome (Figure 2.1). In addition, the *S. cerevisiae HIS4* gene (*SHIS4*) replaced the deleted sequences. Digestion of the vector with *Bam*HI released a 7.9 kb fragment with 2.1 kb of sequence from the 5' locus of *PER6* at one terminus and 1.3 kb of 3' *PER6* locus sequence at the other. These flanking sequences were arranged to direct the *per6Δ* fragment to the *PER6* locus upon transformation into *P. pastoris*. Three μg of this 7.9 kb linear fragment were transformed into GS200 (*his4 arg4*) using the spheroplast transformation method and $His^+$ transformants were selected. A total of 90 transformants were obtained and further tested for Mut phenotype. Approximately 28% were $Mut^-$ and three were selected for further analysis.

### 3.4.2 Southern blot analysis

A Southern filter hybridization experiment confirmed that the *PER6* gene was disrupted. Chromosomal DNA was prepared from three *per6Δ* transformants, along with wild type *P. pastoris* DNA. Samples of each were digested with *Cla* I and *Xho* I. Three sets of digests were analyzed by Southern's technique, each with a different labeled probe. DNAs from all three transformants produced identical banding pattern and therefore, data from only one transformant, JC214, was shown in Figure 3.8. Probe 1 was generated from a 1.6 kb *Bgl*II-*Xho*I fragment of *SHIS4*. Since this gene is not sufficiently homologous to *P. pastoris HIS4* to hybridize, only DNA from the transformants but not wild-type showed a band (Figure 3.8). Probe 2 was composed of a 0.5 kb *Bgl*II-*Sac*I fragment from the *PER6* gene region that had been deleted in pUZ12. As expected, this probe showed no hybridization with transformants' DNA, but hibridized with wild-type DNA. Thus, *PER6* sequences covered by probe 2 are deleted in JC214. Probe 3 was composed of the 3.0 kb *Cla*I fragment from pYT6 that contains all of *PER6* coding sequences plus 1.3 kb flanking sequence at the 5' terminus and 0.4 kb at 3' terminus

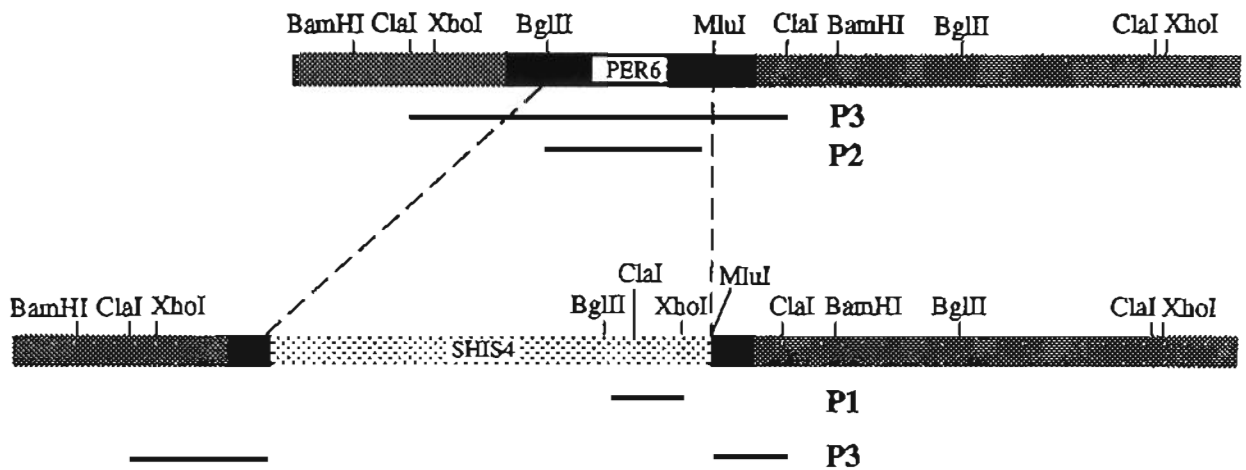


Figure 3.7 Restriction map of *P. pastoris PER6* locus in wild-type (top) and pUZ12 disrupted (bottom) alleles. Location of the three DNA fragments used as labeled probes for Southern blot in Figure 3.8 are shown with solid bars.

(coordinates 11.1-14.1 in Figure 3.1). As expected, probe 3 hybridized with two fragments of 4.5 kb and 1.8 kb in *Cla*I digested JC214 DNA. However, *Xho*I digested JC214 did not produce the predicted banding pattern. We expected two bands of approximately 4.7 kb, which would appear as a doublet. However, in addition to these bands, a 2.6 kb band was present. This extra band could be accounted for by the existence of one or more additional copies of the *PER6* disruption fragment integrated at the *PER6* locus. The presence of multiple copies was also suggested by the high intensity of the 4.7 kb band in JC214 DNA, which was significantly stronger than the corresponding signals in other samples, even though the amount of DNA loaded was approximately the same in all lanes. Two copies of the disruption fragment at *PER6* would be expected to produce three bands of about 4.7 kb, relative to one band in wild-type DNA. Further Southern analysis is needed to verify the presence of multiple copies. Although it would have been ideal to have a *per6Δ* strain with a single copy of the disruption fragment, the potential presence of additional copies at the locus did not interfere with the validity of results obtained with the strain.

### 3.4.3 Genetic evidence that the cloned gene is *PER6*

All three putative *per6Δ* null mutant strains (*per6Δ::SHIS4 his4 arg4*) were utilized in a genetic experiment to determine whether the *P. pastoris* fragment that had been cloned actually contained the same gene that was defective in the original *per6* strain, which was obtained by chemical mutagenesis. The experiment was a complementation test in which each *per6Δ* strain was crossed with JC116 (*per6 his4*). Prototrophic diploids were selected on minimal glucose medium and then tested for ability to grow on methanol. None of these diploids grew on methanol, whereas in a control cross of JC214 with JC117 (*per5 his4*), all diploids grew. The *per6Δ/per6* diploids were further analyzed by sporulation and examination of spore products. Out of approximately 500 spores examined, all were Mut$^-$. As a control, 300 of the spores resulting from *per6Δ/per5* diploids were examined

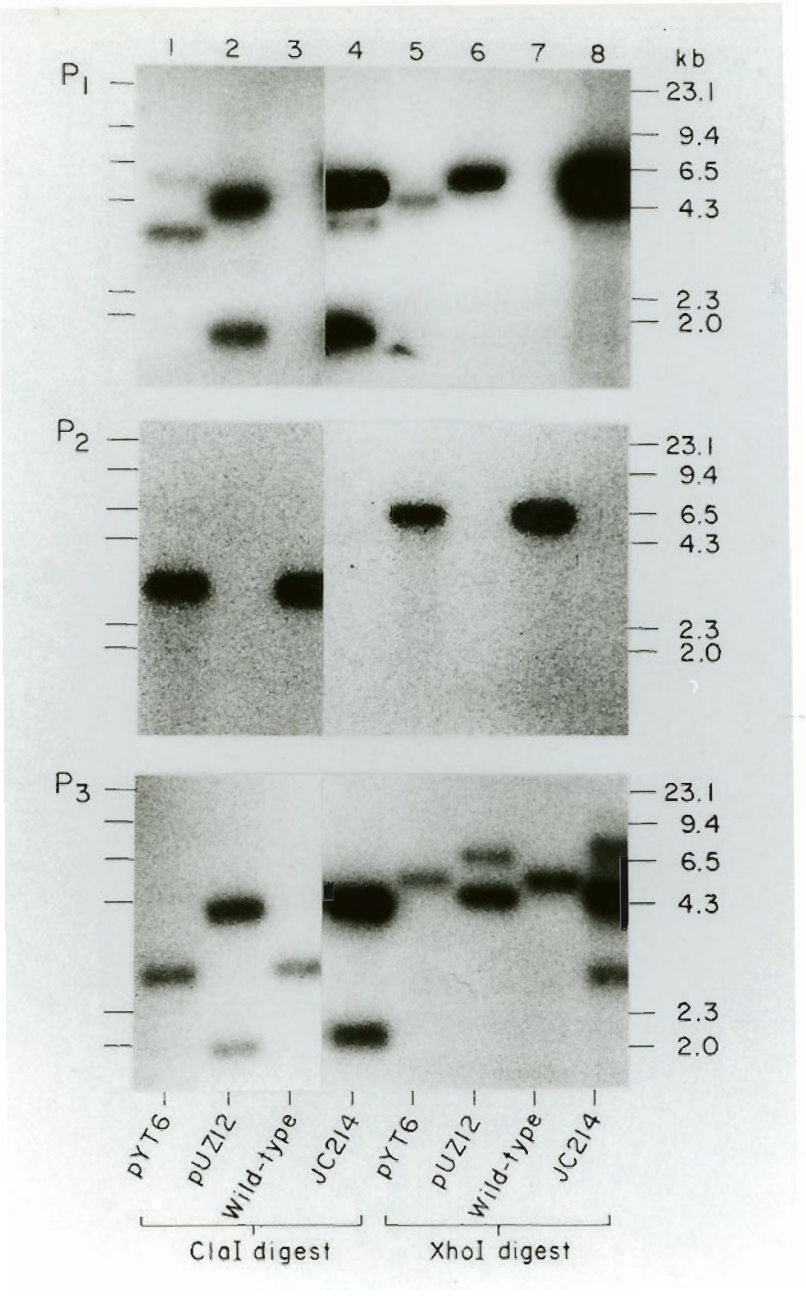


Figure 3.8 Southern blot of *PER6* locus in *P. pastoris* strains. Restriction digests: lanes 1-4, *Cla*I; lanes 5-8, *Xho*I. DNA: lanes 1 and 5, pYT6; lanes 2 and 6, wild-type genomic DNA; lanes 3 and 7, pUZ12; lane 4 and 8, JC214 genomic DNA. Labeled DNA probes: P1, *SHIS4* fragment; P2, *PER6* deleted region; P3, *PER6* plus flanking sequences from the *PER6* locus (see Figure 3.7 for location of the three probes).

and 62% were observed to be Mut$^-$. Thus, the *per6Δ* and original *per6* mutations did not complement and appeared to be defective in the same gene since no Mut$^+$ spore products were recovered. These results strongly suggest that the cloned gene is *PER6*.

### 3.5 Expression of Per6p in the *E. coli*

For biochemical and cytological experiments aimed at examining the subcellular location and function of Per6p, it was desirable to have high quality antibodies against the protein. To rapidly and efficiently produce pure Per6p antigen for antibody generation, the carboxy-terminal 298 amino acids of Per6p were synthesized in *E. coli* cells as a fusion to the bacterial maltose binding protein. Rabbit polyclonal antibodies were then raised against the fusion protein, MP6, and are in the process of being affinity purified.

#### 3.5.1 Construction of the maltose binding protein-Per6p fusion vector

A 964-bp *Bgl*II-*Hin*cII fragment from pYT6 that was predicted to encode amino acids 134 to 432 (the carboxy-terminus) was inserted into a derivative of vector pMAL that had been modified to receive this fragment and express it as an in frame fusion with maltose binding protein (MBP) (see Materials and Methods for details). The final fusion vector, pMW6, expressed the fusion product under control of the regulatable *E. coli tac* promoter.

#### 3.5.2 Expression and purification of MP6

MP6 expression in TBM6 was induced with IPTG for two hours before harvesting. Stained SDS-polyacrylamide gels of cell free extracts of induced cells showed a large amount of a protein of approximately 76 kD, the predicted size of the fusion product (Figure 3.9). This protein band was not present in extracts of uninduced TBM6 cells or of pMAL-containing TB-1 induced cells (data not shown).

To purify MP6, cell-free extracts of induced TBM6 were passed through an amylose resin column under conditions that promote binding of MBP. Protein was then eluted from the column with 0.01 M maltose.

Several species of proteins ranging in size from 76 kD to 43 kD eluted with maltose from the column (Figure 3.9). Most proteins smaller than 76 kD appeared to be MP6 degradation products since the same species were observed in Western blots reacted with anti-MBP antibodies (Figure 3.10).

Proteins eluted from the amylose columns were further purified by DEAE ion exchange column chromatography. Proteins were eluted from this column with a salt gradient and fractions were examined by SDS-PAGE. Fractions that contained mostly 76-kD-sized material were pooled and concentrated (Figure 3.9). The purified MP6 fusion was used for antibody production without the removal of the MBP segment, since Western blot experiments showed that anti-MBP antibodies do not react with proteins from *P. pastoris* cells (Figure 3.10).

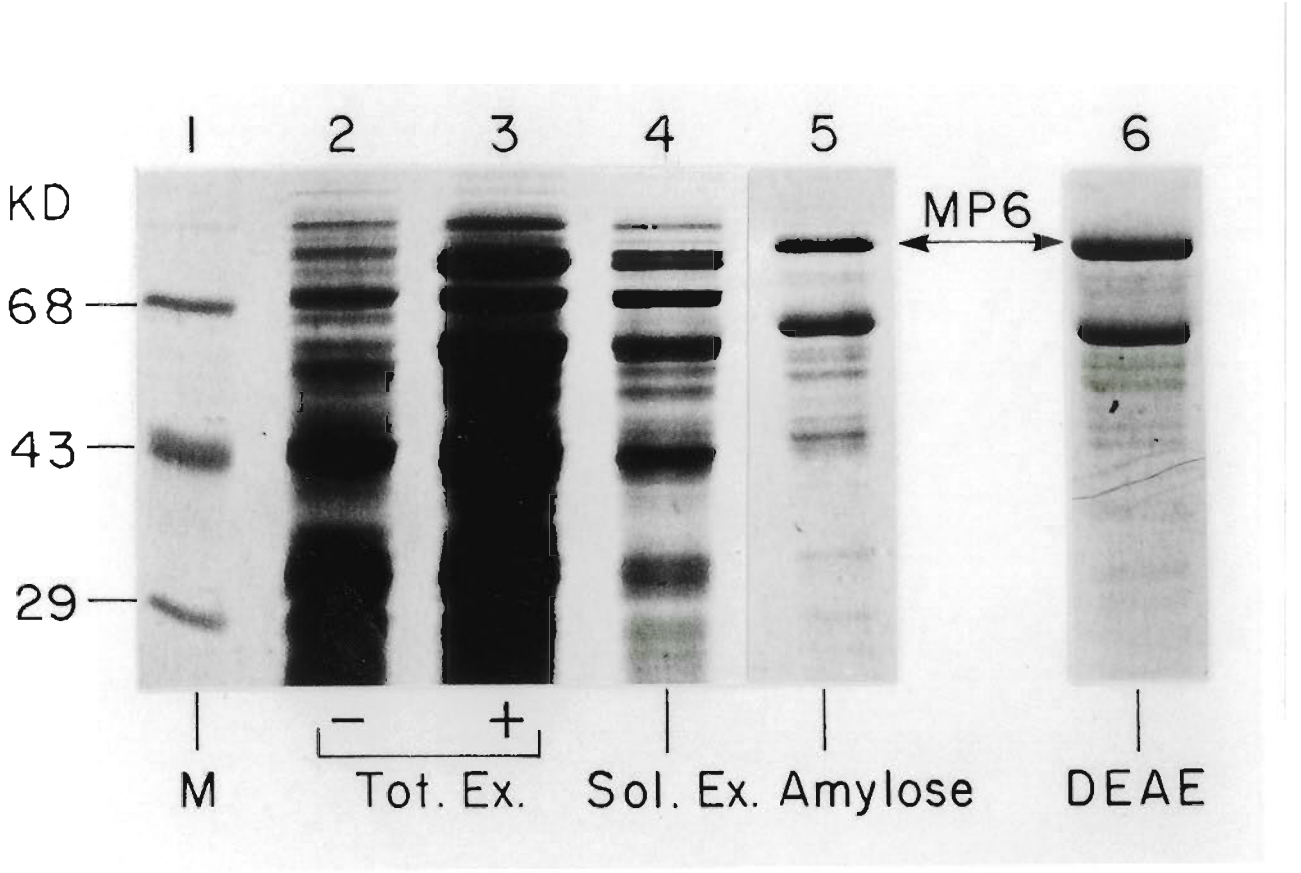


Figure 3.9 SDS-PAGE separation of protein samples from expression and purification of MP6. Lane 1, molecular weight standards; lane 2, crude extract from uninduced TBM6; lane 3, crude extract from IPTG induced TBM6; lane 4, cell free extract of induced TBM6; lane 5, MP6 preparation after elution from amylose resin column; lane 6, MP6 preparation after DEAE resin column; lane 7, molecular weight standards.

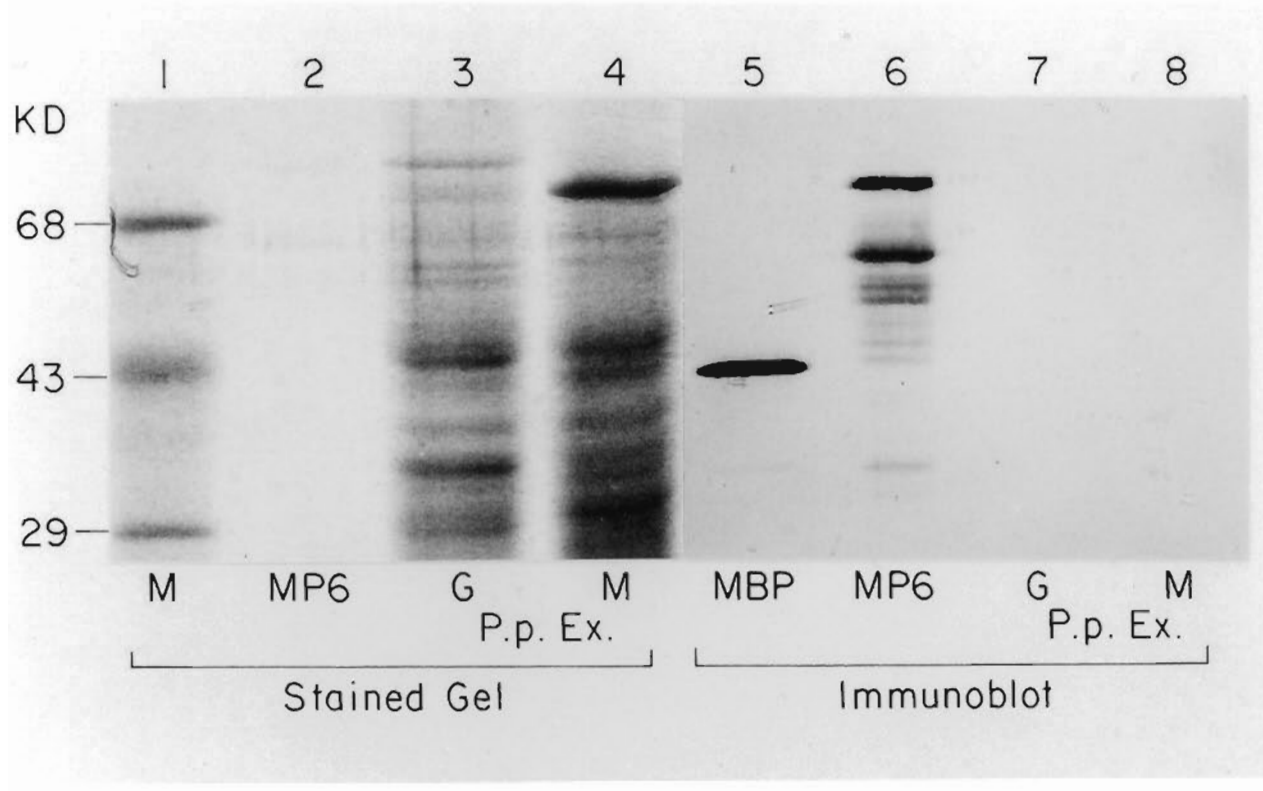


Figure 3.10 Stained SDS-PAGE gel (lanes 1-4) and anti-MBP antibodies immunoblot of MP6 and *P. pastoris* cell free extract (lanes 5-8). Lane 1, molecular weight standards; lanes 2 and 6, 50 ng of purified MP6; lanes 3 and 7, 0.05 mg of glucose grown *P. pastoris* wild-type cell free extract (G); lanes 4 and 8, 0.05 mg of methanol induced *P. pastoris* wild-type cell free extract (M); lane 5, 20 ng of purified MBP.

# CHAPTER 4
# DISCUSSION

## 4.1 A genetic approach toward understanding peroxisome biogenesis

The long-term goal of this laboratory is to elucidate the molecular mechanisms that control the biogenesis and function of peroxisomes. The primary approach toward this end involves the novel application of a molecular genetic strategy that was pioneered by this group. In brief, the strategy entails the isolation of mutants defective in peroxisome biogenesis (*per* mutants), their utilization to clone *PER* genes by functional complementation, and further genetic, biochemical and cytological studies on *PER* gene products. Insights into the role of a specific *PER* protein are gathered from a detailed analysis of the characteristics of the *per* mutants, the predicted amino acid sequence of *PER* products, and other studies made possible as a result of *PER* gene isolation such as the subcellular localization of *PER* products and the identification of proteins that interact with *PER* products.

As a model organism for these studies, the methanol-utilizing yeast *Pichia pastoris* was selected due to its conditional requirement for peroxisomes during growth on methanol or oleic acid and because classical- and molecular-genetic methods are well developed. In previous studies in this laboratory, a significant collection of *per* mutants from *P. pastoris* were isolated and biochemically characterized (Liu, et al., 1992). The mutants lack morphologically recognizable peroxisomes and are affected in eight different *PER* genes. The next logical step in these studies was the utilization of the *per* mutant collection to clone *PER* genes. The subject of this thesis is the isolation and preliminary analysis of one such gene, *PER6*.

### 4.2 Isolation and analysis of the *PER6* gene of *P. pastoris*

The *P. pastoris PER6* gene was isolated by functional complementation of a *per6* mutant of the yeast. For this, a plasmid-based genomic DNA library was introduced into mutant cells and transformants that received a wild-type copy of *PER6* from the library were selected by their restored ability to grow on methanol. Plasmids were recovered from transformed cells that were capable of efficiently retransforming *per6* cells to $Mut^+$. One of these plasmids, pYT6, that contained a 6.5-kb insert of *P. pastoris* DNA was selected for further examination. Subcloning experiments reduced the complementing activity to a DNA fragment of approximately 3 kb, most of which was subsequently sequenced. The DNA sequence revealed a single open reading frame of 1296 bp that was capable of encoding a protein of 432 amino acids and a molecular mass of approximately 49 kD.

We obtained evidence that the cloned DNA fragment actually contained the gene that is defective in *per6* mutants and not suppressor gene. Thus, the open reading frame identified through DNA sequencing likely encodes the *PER6* product (Per6p). First, a *P. pastoris* strain was constructed in which 612 bp of the putative *PER6* open reading frame from amino acids 134 to 338 were removed and replaced with the *S. cerevisiae HIS4* gene. Diploid lines constructed from this *per6Δ* strain and the original *per6* mutant failed to complement, i.e., were $Mut^-$, and all spore products derived from these crosses were also $Mut^-$. These results strongly indicate that the deleted region contains *PER6*. Second, Northern blot experiments using labeled DNA fragments containing all or portions of the *PER6* open reading frame revealed a transcript of approximately 1.4 kb, a reasonable size message for a coding sequence of 1296 bp. Furthermore, the steady state level of *PER6* mRNA was significantly higher in *P. pastoris* cells grown on methanol than on glucose, a predicted observation for a peroxisomal gene given the proliferation of the organelles in methanol. Third, a plasmid construct that expressed maltose binding protein (43 kD) fused to the putative carboxy-terminal 298 amino acids of Per6p (33 kD) expressed a fusion product of approximately 76 kD, the predicted size.

It is conceivable that the amino terminus of Per6p is not initiated from the 5' most inframe ATG of the open reading frame but from a second ATG located 173 bp 3' of the first. Given that most proteins are initiated from the first potential in frame ATG, this possibility appears unlikely. However, additional experiments are necessary to rigorously differentiate between the two potential translation start points.

**4.3 Analysis of the predicted amino acid sequence of Per6p**

Assuming that the full open reading frame described above is Per6p, the protein is 432 amino acids in length with a potential α-helical membrane spanning domain located at amino acids 134 to 158 and an acidic rich stretch of 120 amino acids at its carboxy terminus (Figure 3.5). This acidic region showed significant similarity to "acid blob" transcriptional activation domains of several known transcription factors. However, the similarity was limited only to the acidic amino acid residues. Furthermore, this region is not nearly as acidic as those of most known transcriptional activation domains. Per6p does not contain sequences similar to either peroxisomal targeting signals PTS-1 or PTS-2.

A segment of 137 amino acids located approximately in the middle of Per6p showed significant similarity to a sequence near the center of the predicted product of *PAF-1* (Tsukamoto, et al., 1991). *PAF-1* is a rat liver cDNA clone that was isolated by functional complementation of a peroxisome-deficient Chinese hamster ovary cell line (see sections 1.3.1 and 1.4.1 for details). The *PAF-1* product, PAF-1p, has been shown to be a 35 kD peroxisomal integral membrane protein. The rat gene is of particular interest since it is the homolog of a gene that is defective in some Zellweger patients (Shimozawa, et al., 1992). Low but apparently significant similarity between Per6p and PAF-1p was observed within three colinear segments corresponding to amino acids 147-170, 218-224, and 278-294 in the Per6p sequence. The second segment of similarity includes a shared potential membrane spanning domain of Per6p and PAF-1p. In addition, seven cysteine residues were also located within this region of both proteins. The

center-most three cysteines are conserved with regard to their positions within similarity segment three. Although the remaining four cysteines are not positionally conserved, the overall arrangement of the seven cysteine residues was strikingly similar between the two proteins (CxxC...CxxxxCxxC...CxxC). Such cysteine-rich regions have been identified in several other non-nuclear proteins involved in organelle biogenesis (Dulic and Riezman, 1989; Weisman and Wickner, 1992; Woolford, et al., 1990). However, a specific function for the motif in biogenesis, if any, has not been established. The region of similarity does not extend to any additional locations outside the region and the two proteins do not otherwise appear similar in any other way. Thus, the similarity is not sufficient to suggest that Per6p and PAF-1p perform similar functions in peroxisome biogenesis.

A possible function for the regions of similarity is suggested from the fact that the potential membrane spanning domain of Per6p and one of two that were reported in PAF-1p exist within the regions. These sequences could constitute an evolutionarily conserved PTS for certain peroxisomal integral membrane proteins (PMPs). Such sequence conservation between a mammalian and a yeast protein is not without precedent. Both of the known matrix protein PTSs, PTS-1 and PTS-2, are also highly conserved in mammals and yeasts (Gould, et al., 1989; Keller, et al., 1991; Swinkels, et al., 1991). Although a similar motif has not been observed in any other PMP, very few PMPs have been described to date and, as with matrix proteins, more than one targeting mechanism may exist.

### 4.4 Speculation on the role of Per6p in peroxisome biogenesis

Preliminary results from the laboratory of Dr. Suresh Subramani (University of California at San Diego) suggest a possible role for Per6p in peroxisome biogenesis. His laboratory recently reported the isolation and characterization of another *P. pastoris* peroxisomal gene, *PAS8* (McCollum, et al., 1993). Several interesting features were noted with regard to the *PAS8* product (Pas8p). First, Pas8p is associated with the peroxisomal membrane in *P. pastoris* but does not appear to be an integral membrane protein. Second,

the predicted sequence of Pas8p contains seven sets of the tetratricopeptide repeat (TPR), a sequence motif involved in protein-protein interactions with other TPR-bearing proteins or with proteins harboring a motif complementary to TPR (Goebl and Yanagida, 1991). Since Pas8p does not contain a potential membrane spanning domain, they suggested that the protein may associate with the peroxisomal membrane through interaction with a PMP via its TPR sequences. Third, *in vitro* translated Pas8p specifically binds peptides ending in the PTS-1 sequence Ser-Lys-Leu-COOH. Thus, Pas8p is a candidate for a PTS-1 receptor protein.

With regard to a potential function for Per6p, Pas8p appears to be membrane associated in cells of each of the peroxisome-deficient mutants of *P. pastoris* except one, *per6*. In this mutant, Pas8p appears to be located in the cytosol (S. Subramani, personal communication). This result suggests that Pas8p may associate with the peroxisomal membrane through interaction with Per6p and that, in the *per6* mutant, this interaction is defective. Thus, Per6p may anchor Pas8p to the outer surface of the peroxisomal membrane, either directly as a PMP or indirectly through interaction with yet another protein. In this hypothetical model, if Pas8p is the PTS-1 receptor, then Per6p would be the PTS-1 receptor receptor.

Based on this model and the presence of TPR sequences in Pas8p, we examined Per6p for TPR and other sequence motifs that are thought to interact with TPRs. No such motifs arre apparent in Per6p. Thus, if Pas8p and Per6p do interact, it may be through some other yet unidentified protein-protein interaction domains.

### 4.5 Future experiments

We have succeeded in isolating and characterizing the *PER6* gene from *P. pastoris*. The gene appears to play an essential role in peroxisome biogenesis since *per6* mutants are defective in the process. How Per6p functions remains to be determined.

The most immediate goal is to determine the subcellular location of Per6p. Toward that end, we have synthesized and purified large amounts of

Per6p as a fusion protein in *E. coli* cells and used the fusion protein to generate anti-Per6p rabbit polyclonal antiserum. Once a specific antibody preparation is available Per6p will be localized by subcellular fractionation and immunocytochemical experiments. Based on previous discussion, we hypothesize that Per6p may be a PMP.

A second near-term goal is to further characterize *per6* mutants of *P. pastoris* with regard to the location of peroxisomal matrix proteins and PMPs, and the presence or morphology of any peroxisomal "ghost"-like structures. The *per6*Δ strain we constructed will be particularly useful for these experiments since it will allow us to observe these characteristics in a strain that is most likely completely defective in *PER6* function. We are especially interested in determining whether thiolase is imported in a *per6* strain. If, as shown for Pas8p, Per6p is specifically defective in import of PTS-1 containing matrix enzymes but not in PTS-2-containing thiolase (McCollum, et al., 1993), it would support the notion that Per6p is also part of a PTS-1-based import system.

A third goal is to further investigate the possibility that Pas8p and Per6p interact. Anti-Per6p will be used to immunoprecipitate Per6p and proteins complexed with Per6p. If a complex containing Pas8p and Per6p (and perhaps other proteins) is identified immunologically, we will define the interacting sequences within the proteins. For this, selected deletion mutations of *PAS8* and *PER6* will be expressed in *P. pastoris* and the effect of these deletions on Pas8p-Per6p interaction will be assessed.

We would also like to follow up on the observation that PAF-1p and Per6p share a region of similarity. It would be interesting to test whether Per6p and PAF-1 could functionally substitute for one another by expression of their genes in the respective heterologous mutant hosts. We would also like test our conjecture that the similar regions are part of a PMP targeting signal. The region could be expressed as a fusion with a non-peroxisomal protein and the ability of the Per6p sequence to target the fusion to the peroxisomal membrane assessed. In addition, the importance of specific amino acids within the region, such as the potential Zinc-finger-forming

cysteines, can be determined by site-directed mutagenesis of their coding sequences and the resulting Per6p mutant strains examined for phenotype, peroxisome morphology, and mutant Per6p localization and Pas8p interaction. With these and other experiments, we expect to gain insight into the function of Per6p in peroxisome biogenesis.

## REFERENCES


Anthony, C. (1982). The Biochemistry of Methylotrophs. New York, Academic Press, Inc.

Appelkvist, E.-L., Reinhart, M., Fischer, R., Billheimer, J. and Dallner, G. (1990). Presence of individual enzymes of cholesterol biosynthesis in rat liver peroxisomes. Arch. Biochem. Biophys. *282*(2): 318-325.

Barth, P. G., Schutgens, R. B. H., Wanders, R. J. A., Heymans, H. S. A., Moser, A. E., Moser, H. W., Bleeker-Wagemakers, E. M., Jansonius-Schultheiss, K., Derix, M. and Nelck, G. F. (1987). A sibship with a mild variant of Zellweger syndrom. J. Inher. Metab. Dis. *10*: 253-259.

Baudhuin, P., Beaufay, H. and de Duve, C. (1965). Combined biochemical and morphological study at particulate fractions from rat liver. J. Cell Biol. *26*: 219-243.

Beard, M. E., Baker, R., Conomous, P., Pugatch, D. and Holzman, E. (1985). Oxidation of oxalate and polyamines by rat peroxisomes. J. Histochem. Cytochem. *33*: 460-464.

Beevers, H. (1975). Organelles from caster bean seedlings: Biochemical roles in gluconeogenesis and phospholipid synthesis. Regulation of Developmental Processes in Plants. Jena, Fischer. 159-172

Beevers, H. (1979). Microbodies in higher plants. Annu. Rev. Plant Physiol. *30*: 1-26.

Böck, P., Kramar, R. and Pavelka, M. (1980). Peroxisomes and related particles in animal tissues. Cell Biology Monographs. Wien, New York, Springer-Verlag. 1-239

Bodnar, A. G. and Rachubinski, R. A. (1990). Cloning and sequence determination of cDNA encoding a second rat liver peroxisomal 3-ketoacyl-CoA thiolase. Gene *91*: 193-199.

Borst, P. (1989). Peroxisome biogenesis revisited. Biochim. Biophys. Acta *1008*: 1-13.

Bowen, P., Lee, C. S. N., Zellweger, H. and Lindenberg, R. (1964). A familial syndrom of multiple congenital defects. Bull. Johns Hopkins Hosp. *114*: 402.

Breidenbach, R. W. and Beevers, H. (1967). Association of the glyoxylate cycle enzymes in a novel subcellular particle from caster bean endosperm. Biochem. Biophys. Res. Comm. 27(4): 462-469.

Bremer, J. and Osmundsen, H. (1984). Fatty acid oxidation and its regulation. Fatty Acid Metabolism and Its Regulation. Amsterdam, Elsevier Science Publishers B. V. 113-154

Brown, M. S. and Goldstein, J. L. (1980). Multivalent feedback regulation of HMG CoA reductase, a control mechanism coordinating isoprenoid synthesis and cell growth. J. Lipid Res. *21*: 505-517.

Bullock, W. O., Fernandez, J. M. and Short, J. M. (1987). A high efficiency plasmid transforming recA *Escherichia coli* strain with beta-galactosidase selection. BioTechniques *5*: 376-379.

Casteels, M., Schepers, L., van Eldere, J., Eyssen, H. J. and Hannaerts, G. P. (1990). Inhibition of 3α, 7α, 12α-trihydroxy-5β-cholestanoic acid oxidation and of bile acid secretion in rat liver by fatty acids. J. Biol. Chem. *263*: 4654-4661.

Chance, B. and Oshino, N. (1971). Kenetics and mechanisms of catalase in peroxisomes of the mitochondrial fraction. Biochem. J. *122*: 225-233.

Conway, J. G., Tomaszewski, K. E., Olson, M. J., Cattley, R. C., Marsman, D. S. and Popp, J. A. (1989). Relationship of oxidative damage to the hepatocarcinogenicity of the peroxisome proliferators di(2-ethylhexyl)phthalate and Wy-14,643. Carcinogenesis *10*(3): 513-519.

Cooper, T. G. and Beevers, H. (1969). Mitochondria and glyoxysomes from caster bean endosperm. Enzyme constituents and catalytic capacity. J. Biol. Chem. *244*: 3507-3513.

Couderc, R. and Baratti, J. (1980). Oxidation of methanol by the yeast *P. pastoris*. Purification and properties of alcohol oxidase. Agric. Biol. Chem. *44*: 2279-2289.

Cregg, J. M., Barringer, K. J., Hessler, A. Y. and Madden, K. R. (1985). *Pichia pastoris* as a host system for transformations. Mol. Cell. Biol. *9*: 3376-3385.

Cregg, J. M., van der Klei, I. J., Sulter, G. J., Veenhuis, M. and Harder, W. (1990). Peroxisome-deficient mutants of *Hansenula polymorpha*. Yeast *6*: 87-97.

Danpure, C. J. and Jennings, P. R. (1986). Peroxisomal alanine-glyoxylate aminotransferase deficiency in primary hyperoxaluria type I. FEBS Letters *201*: 20-24.

Danpure, C. J., Jennings, P. R. and Watts, R. W. E. (1987). Enzymological diagnosis of primary hyperoxaluria tupe I by measurement of hepatic alanine:glyoxylate aminotransferase activity. Lancet *1*: 289-291.

De Duve, C. and Baudhuin, P. (1966). Peroxisomes (microbodies and related particles). Physiol. Rev. *46*: 323-357.

Deshaies, R. J., Kepes, F. and Bohni, P. C. (1989). Genetic dissection of the early stages of protein secretion in yeast. Trends Genet. *5*: 87-93.

Didion, T. and Rogenkamp, R. (1990). Deficiency of peroxisome assembly in a mutant of the methylotrophic yeast *Hansenula polymorpha*. Curr. Genet. *17*: 113-117.

Douma, A. C., Veenhuis, M., de Koning, W., Evers, M. and Harder, W. (1985). Dihydroxacetone synthase is localized in the peroxisomal matrix of methanol-grown *Hansenula polymorpha*. Arch. Microbiol. *143*: 237-243.

Dreyer, C., Krey, G., Keller, H., Givel, F., Helftenbein, G. and Wahli, W. (1992). Control of the peroxisomal β-oxidation pathway by a novel family of nuclear hormone receptors. Cell *68*: 879-887.

Dulic, V. and Riezman, H. (1989). Characterization of the *END1* gene required for vacuole biogenesis and gluconeogenic growth of budding yeast. EMBO J. *8*(5): 1349-1359.

Erdmann, R., Wiebel, F. F., Flessau, A., Rytka, J., Beyer, A., Frohlich, K.-U. and Kunau, W.-H. (1991). *PAS1*, a yeast gene required for peroxisome biogenesis, encodes a member of a novel family of putative ATPases. Cell *64*: 499-510.

Erdmann, R., Veenhuis, M., Mertens, D. and Kunau, W.-H. (1989). Isolation of peroxisome-deficient mutants of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci., USA *86*: 5419-5423.

Farrell, S. O. and Bieber, L. L. (1983). Carnitine octanoyltransferase of mouse liver peroxisomes: Properties and effect of hypolipidemic drugs. Arch. Biochem. Biophys. *222*: 123-132.

Fung, K. and Clayton, C. (1991). Recognition of a peroxisomal tripeptide entry signal by the glycosomes of *Trypanosoma brucei*. Mol. Biochem. Parasitol. *45*: 261-264.

Gärtner, J., Moser, H. and Valle, D. (1992). Mutations in the 70K peroxisomal membrane protein gene in Zellweger syndrome. Nature Genetics *1*: 16-23.

Goebl, M. and Yanagida, M. (1991). The TPR snap helix: A novel protein repeat motif from mitosis to transcription. Trends in Biochem. Sci. *16*: 173-177.

Goldfischer, S., Moore, C. L., Johnson, A. B., Spiro, A. J., Valsamis, M. P., Wisniewski, H. K., Ritch, R. H., Norton, W. T., Rapin, I. and Gartner, L. M. (1973). Peroxisomal and mitochondrial defects in the cerebro-hepato-renal syndrome. Science *182*: 62-64.

Goldfischer, S. L. (1988). Peroxisomal diseases. The Liver: Biology and Pathology. New York, Raven Press, Ltd. 2, ed. 255-267

Goodman, J. M. (1985). Dihydroxacetone is an abundant constituent of the methanol-induced peroxisome of *Candida boidinii*. J. Biol. Chem. *260*(11): 7108-7113.

Gould, S. J., Keller, G.-A., Hosken, N., Wilkinson, J. and Subramani, S. (1989). A conserved tripeptide sorts proteins to peroxisomes. J. Cell Biol. *108*: 1657-1664.

Gould, S. J., Keller, G.-A., Schneider, M., Howell, S. H., Garrard, L. J., Goodman, J. M., Distel, B., Tabak, H. and Subramani, S. (1990a). Peroxisomal protein import is conserved between yeast, plants, insects and mammals. EMBO J. *9*(1): 85-90.

Gould, S. J., Keller, G.-A. and Subramani, S. (1987). Identification of a peroxisomal targeting signal at the carboxy terminus of firefly luciferase. J. Cell Biol. *105*: 2923-2931.

Gould, S. J., Keller, G.-A. and Subramani, S. (1988). Identification of peroxisomal targeting signals located at the carboxy terminus of four peroxisomal proteins. J. Cell Biol. *107*: 897-905.

Gould, S. J., Krisans, S., Keller, G.-A. and Subramani, S. (1990b). Antibodies directed against the peroxisomal targeting signal of firefly luciferase recognize multiple mammalian peroxisomal proteins. J. Cell Biol. *110*: 27-34.

Gould, S. J., McCollum, D., Spong, A. P., Heyman, J. A. and Subramani, S. (1992). Development of the yeast *P. pastoris* as a model organism for a genetic and molecular analysis of peroxisome assembly. Yeast *8*: 613-628.

Gould, S. J. and Subramani, S. (1988). Firefly luciferase as a tool in molecular and cell biology. Analytical Biochem. *175*: 5-13.

Hajra, A. K. and Bishop, J. E. (1982). Glycerolipid biosynthesis in peroxisomes via the acyl-dihydroxyacetone phosphate pathway. Annu. N. Y. Acad. Sci. *386*: 170-182.

Hajra, A. K., Burke, C. L. and Jones, C. L. (1979). Subcellular localization of acyl-coenzyme A: Dihydroxyacetone phosphate acyltransferase in rat liver peroxisomes (microbodies). J. Biol. Chem. *254*(21): 10896-10900.

Hanson, R. F., Szczepanick-van Leeuwen, P., Williams, G. C., Grabowski, G. and Sharp, H. L. (1979). Defects of bile acid synthesis in Zellweger's syndrome. Science *203*: 1107-1108.

Hartwell, L. H. and Weinert, T. A. (1989). Check points: Controls that ensure the order of cell cycle events. Science *246*: 629-634.

Hashimoto, T. (1982). Individual peroxisomal β-oxidation enzymes. Annu. N. Y. Acad. Sci. *386*: 5-12.

Havel, R. J. and Kane, J. P. (1973). Drugs and lipid metabolism. Annu. Rev. Pharmac. *161*: 287-308.

Hijikata, M., Wen, J.-K., Osumi, T. and Hashimoto, T. (1990). Rat peroxisomal 3-ketoacyl-CoA thiolase gene. J. Biol. Chem. *265*(8): 4600-4606.

Hodge, V. J., Gould, S. J., Subramani, S., Moser, H. W. and Krisans, S. K. (1991). Normal cholesterol synthesis in human cells requires functional peroxisomes. Biochem. Biophys. Res. Comm. *181*(2): 537-541.

Hogg, F. (1969). Peroxisomes in *Tetrahymena* and their relation to gluconeogenesis. Annu. N. Y. Acad. Sci. *168*: 281-291.

Höhfeld, J., Mertens, D., Wiebel, F. F. and Kunau, W.-H. (1992). Defining components required for peroxisome assembly in *Saccharomyces cerevisiae*. Membrane Biogenesis and Protein Targeting. Amsterdam, Elsevier Science Publishers B. V. 185-237

Höhfeld, J., Veenhuis, M. and Kunau, W.-H. (1991). PAS3, a *Saccharomyces ceverisiae* gene encoding a peroxisomal integral membrane protein essential for peroxisome biogenesis. J. Cell Biol. *114*(6): 1167-1178.

Holtta, E. (1977). Oxidation of spermidine and spermine in rat liver: Purification and properties of polyamine oxidases. Biochemistry *16*(1): 91-100.

Hsiao, K.-C. (1991). A fast and simple procedure for sequencing double stranded DNA with Sequenase. Nucl. Acids Res. *19*(10): 2787.

Hsieh, B. and Tolbert, N. E. (1976). Glyoxylate aminotransferase in peroxoisomes from rat liver and kidney. J. Biol. Chem. *251*: 4408-4415.

Imanaka, T., Small, G. M. and Lazarow, P. B. (1987). Translocation of acyl-CoA oxidase into peroxisomes requires ATP hydrolysis but not a membrane potential. J. Cell Biol. *105*: 2915-2922.

Issemann, I. and Green, S. (1990). Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. Nature (London) *347*: 645-650.

Johnston, T. C., Thompson, R. B. and Baldwin, T. O. (1986). Nucleotide sequence of the *luxB* gene of *Vibrio harveyi* and the complete amino acid sequence of the beta subunit of bacterial luciferase. J. Biol. Chem. *261*: 4805-4811.

Kamijo, K., Kamijo, T., Ueno, I., Osumi, T. and Hashimoto, T. (1992). Nucleotide sequence of the human 70kDa peroxisomal membrane protein: a member of ATP-binding cassette transporters. Biochim. Biophys. Acta *1129*: 323-327.

Kamijo, K., taketani, S., Yokota, S., Osumi, T. and Hashimoto, T. (1990). The 70-kDa peroxisomal membrane protein is a member of the Mdr (P-glycoprotein)-related ATP-binding protein superfamily. J. Biol. Chem. *265*(8): 4534-4540.

Kamiryo, T., Abe, M., Okazaki, K., Kato, S. and Shimamoto, N. (1982). Absence of DNA in peroxisomes of *Candida tropicalis*. J. Bacteriol. *152*: 269-274.

Kasai, H., Okadi, Y., Nishimura, S., Rao, M. S. and Reedy, J. K. (1989). Formation of 8-hydroxydeoxyguanosine in liver DNA of rats following long-term exposure to a peroxisome proliferator. Cancer Res. *49*(10): 2603-2605.

Kawamura, N., Moser, H. W. and Kishimoto, Y. (1981). Very long chain fatty acid oxidation in rat liver. Biochem. Biophys. Res. Comm. *99*(4): 1216-1225.

Keller, G.-A., Gould, S., Deluca, M. and Subramani, S. (1987). Firefly luciferase is targeted to peroxiso
mes in mammalian cells. Proc. Natl. Acad. Sci., USA *84*: 3264-3268.

Keller, G.-A., Krisans, S., Gould, S. J., Sommer, J. M., Wang, C. C., Schliebs, W., Kunau, W., Brody, S. and Subramani, S. (1991). Evolutionary conservation of a microbody targeting signal that targets proteins to peroxisomes, glyoxysomes, and glycosomes. J. Cell Biol. *114*(5): 893-904.

Keller, G. A., Barton, M. C., Shapiro, D. J. and Singer, S. J. (1985). 3-hydroxy-3-methylglutaryl-coenzyme A reductase is present in peroxisomes in normal rat liver cells. Proc. Natl. Acad. Sci., USA *82*: 770-774.

Keller, G. A., Pazirandeh, M. and Krisans, S. (1986). 3-hydroxy-3-methylglutaryl-coenzyme A reductase localization in rat liver peroxisomes and microsomes of control and cholestyramine-treated animals: Quantitative biochemical and immunoelectron microscopical analyses. J. Cell Biol. *103*: 875-886.

Kohrer, K. and Domdey, H., Ed. (1991). Preparation of high molecular weight RNA. Methods in Enzymology. Guide to Yeast Genetics and Molecular Biology. San Diego, Academic Press, Inc. 398-404

Kragler, F., Langeder, A., Raupachova, J., Binder, M. and Hartig, A. (1993). Two independent peroxisomal targeting signals in catalase A of *Saccharomyces cerevisiae*. J. Cell Biol. *120*(3): 665-673.

Krisans, S., Mortensen, R. M. and Lazarow, P. B. (1980). Acyl-CoA synthetase in rat liver peroxisomes. Computer assisted analysis of cell fractionation experiemnts. J. Biol. Chem. *255*: 9599.

Krisans, S. K., Thompson, S. L., Pena, L. A., Kok, E. and Javitt, N. B. (1985). Bile acid synthesis in rat liver peroxisomes: Metabolism of 26-hydroxycholesterol to 3-beta-hydroxy-5-cholenoic acid. J. Lipid Res. *26*: 1324-1332.

Kunau, W.-H. and Hartig, A. (1992). Peroxisome biogenesis in *Saccharomyces cerevisiae*. Antonie van Leeuwenhoek *62*: 63-78.

Kunau, W. H., Kionka, C., Ledebur, A., Mateblowski, M., Moreno de la Garza, M., Schultz-Borchard, U., Thieringer, R. and Veenhuis, M. (1987). β–oxidation systems in eukaryotic microorganisms. Peroxisomes in Biology and Medicine. Berlin, Springer-Verlag. 128-140

Larson, D. W. and Kershaw, K. A. (1976). Studies on lichen-dominated systems. XVIII. Morphological control of evaporation in lichens. Can. J. Bot. *54*: 2061-2073.

Lazarow, P. B. (1993). Genetic approaches to studying peroxisome biogenesis. Trends in Cell Biol. *3*: 89-93.

Lazarow, P. B. and Fujiki, Y. (1985). Biogenesis of Peroxisomes. Annu. Rev. Cell Biol. *1*: 489-530.

Lazarow, P. B. and Moser, H. W. (1989). Disorders of peroxisome biogenesis. The Metabolic Basis of Inherited Disease. New York, McGraw-Hill Information Services Company. 1479-1509

Lazarow, P. B., Robbi, M., Fujiki, Y. and Wang, L. (1982). Biogenesis of peroxisomal proteins *in vivo* and *in vitro*. Annu. N. Y. Acad. Sci. *386*: 285-300.

Lazarow, P. B., Thieringer, R., Cohen, G., Imanaka, T. and Small, G. (1991). Protein import into peroxisomes *in vitro*. Methods in Cell Biology. Academic Press, Inc. 303-326

Lindmark, D. G. and Müller, M. (1973). Hydrogenosome, a cytoplasmic organelle of the anaerobic flagellate *Tritrichomonas foetus*, and its role in pyruvate metabolism. J. Biol. Chem. *248*(22): 7724-7728.

Lindmark, D. G. and Müller, M. (1974). Superoxide dismutase in the anaerobic flagellates, *Tritrichomonas foetus* and *Monocercomonas* sp. J. Biol. Chem. *249*(14): 4634-4637.

Liu, H., Tan, X., Veenhuis, M., McCollum, D. and Cregg, J. M. (1992). An efficient screen for peroxisome-deficient mutants of *P. pastoris*. J. Bacteriol. *174*(15): 4943-4951.

Lloyd, D., Brightwell, R., Venables, S. E., Roach, G. I. and Turner, G. (1971). Subcellular fractionation of *Tetrahymena pyriformis* ST by zonal centrifugation: Changes in activities and distribution of enzymes during the growth cycle and on starvation. J. Gen. Microbiol. *65*(2): 209-223.

Lock, E. A., Mitchell, A. M. and Elcombe, C. R. (1989). Biochemical mechanisms of induction of hepatic peroxisome proliferation. Annu. Rev. Pharm. Toxicol. *29*: 145-163.

Mangelsdorf, D. J., Ong, E. S., J. A. Dyck and Evans, R. M. (1990). Nuclear receptor that identifies a novel retinoic acid response pathway. Nature (London) *345*: 224-229.

Mannaerts, G. P., van Veldhoven, P., van Broekhoven, A., Vandebroek, G. and De Beer, L. J. (1982). Evidence that peroxisomal acyl-CoA is located at the cytoplasmic side of the peroxisomal membrane. Biochem. J. *204*: 17-23.

Markwell, M. A. K., McGroarty, E. J., Bieber, L. L. and Tolbert, N. E. (1973). Comparison of carnitine acyltransferases in mammalian liver and kidney. J. Biol. Chem. *248*(10): 3426-3432.

Markwell, M. A. K., Tolbert, N. E. and Beiber, L. L. (1976). Comparision of the carnitine acyltransferase activities from rat liver peroxisomes and microsomes. Arch. Biochem. Biophys. *176*: 479.

Mayazawa, S., Ozasa, H., Osumi, T. and Hashimoto, T. (1983). Purification and properties of carnitine octanoyltransferase and carnitine palmitoyltransferase from rat liver. J. Biochem. *94*: 529-542.

McCollum, D., Monosov, E. and Subramani, S. (1993). The *pas8* mutant of *Pichia pastoris* exhibits the peroxisomal protein import deficiencies of Zellweger syndrome cells-- The PAS8 protein binds to the COOH-terminal tripeptide peroxisomal targeting signal, and is a member of the TPR protein family. J. Cell Biol. *121*(4): 761-774.

McNamara, J. O. and Fridovich, I. (1993). Did radicals strike Lou Gehrig? Nature (London) *362*: 20-21.

Meissener, P. S., Sisk, W. P. and Berman, M. L. (1987). Bacteriophage λ cloning system for the construction of directional cDNA libraries. Prot. Natl. Acad. Sci., USA *84*: 4171-4175.

Miyazawa, S., Furuta, S., Osumi, T., Hashimoto, T. and Ui, N. (1981). Properties of peroxisomal 3-ketoacyl-CoA thiolase from rat liver. J. Biochem. *90*: 511-519.

Monnens, L., Bakkeren, J., Parmentier, G., Janssen, G., van Haelst, U., Trijbels, F. and Eyssen, H. (1980). Disturbance in bile acid metabolism of infants with the Zellweger (cerebro-hepato-renal) syndrom. Eur. J. Pediatr. *133*: 31-35.

Mortensen, P. B., Kolvraa, S., Gregersen, N. and Rasmussen, K. (1982). Cyanide-insensitive and clofibrate enhanced beta-oxidation of dodecanedioic acid in rat liver. Biochim. Biophys. Acta *713*: 393-397.

Müller, M. (1975). Biochemistry of protozoan microbodies: peroxisomes, α-glycerophosphate oxidase bodies, hydrogenosomes. Annu. Rev. Microbiol. *29*: 467-483.

Müller, M., Hogg, J. F. and de Duve, C. (1968). Distribution of tricaboxylic acid cycle enzymes and glyoxylate cycle enzymes between mitochondria and peroxisomes in *Tetrahymena pyriformis*. J. Biol. Chem. *243*(20): 5385-5395.

Noguchi, T. and Takada, Y. (1978). Peroxisomal localization of serine:pyruvate aminotransferase in human liver. J. Biol. Chem. *253*: 7598-7600.

Noguchi, T. and Takada, Y. (1979). Peroxisomal localization of alanine:glyoxylate aminotransferase in human liver. Arch. Biochem. Biophys. *196*: 645-647.

Ogren, W. L. (1984). Photorespiration: Pathways, regulation, and modification. Annu. Rev. Plant Physiol. *35*: 415-442.

Opperdoes, F. R. (1987). Compartmentation of carbohydrate metabolism in *Trypanosomes*. Annu. Rev. Microbiol. *41*: 127-151.

Osumi, T. and Hashimoto, T. (1978). Acyl-CoA oxidase of rat liver: A new enzyme for fatty acid oxidation. Biochem. Biophys. Res. Comm. *83*: 479-485.

Osumi, T., Wen, J.-K. and Hashimoto, T. (1991). Two *cis*-acting regulatory sequences in the peroxisome proliferatior-responsive enhancer region of rat acyl-CoA oxidase gene. Biochem. Biophys. Res. Comm. *175*(3): 866-871.

Pace-Asciak, C. and Granstrom, E. (1983). Postaglandins and Related Substances. Amsterdam, Elsevier Science Publishers B. V.

Pedersen, J. I. and Gustafsson, J. (1980). Conversion of 3alpha, 7alpha, 12alpha-tri-hydroxy-5-beta-cholestanoic acid into cholic acid by rat liver peroxisomes. FEBS Letters *121*: 345-348.

Pringle, J. R. and Hartwell, L. H. (1981). The *Saccharomyces cerevisiae* cell cycle. The Molecular Biology of the Yeast *Saccharomyces*. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press. 97-142

Reddy, J. K. and Lalwani, N. D. (1983). Carcinogenesis by hepatic peroxisome proliferators: Evaluation of the risk of hypolipidemic drugs and industrial plasticizers to humans. Crit. Rev. Toxicol. *12*(1): 1-58.

Reddy, J. K. and Qureshi, S. A. (1979). Tumorigenicity of the hypolipidaemic peroxisome proliferator ethyl-alpha-p-chlorophenoxyisobutyrate (clofibrate) in rats. Br. J. Cancer *40*(3): 476-482.

Rhodin, J. (1954). Correlation of ultrastructural organization and functions in normal and experimentally changed proximal convolated tubule cells of the mouse kidney. Ph. D. thesis. Karolinska Institute, Stockholm, Sweden.

Rosen, D. R., Siddique, T., Patterson, D., Figlewicz, D. A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J. P., Deng, H.-X., Rahmani, Z., Krizus, A., McKenna-Yasek, D., Cayabyab, A., Gaston, S. M., Berger, R., Tanzi, R. E., Halperin, J. J., Herzfeldt, B., van den Bergh, R., Hung, W.-Y., Bird, T., Deng, G., Mulder, D. W., Smyth, C., Laing, N. G., Soriano, E., Pericak-Vance, M. A., Haines, J., Rouleau, G. A., Gusella, J. S., Horvitz, H. R. and Jr, R. H. B. (1993). Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature (London) *362*: 59-62.

Rothstein, R. (1991). Targeting, disruption, replacement, and allele rescue: Integrative DNA transformation in yeast. Methods in Enzymology. Guide to Yeast Genetics and Molecular Biology. San Diego, Academic Press, Inc. 281-301

Rowsell, E. V., Snell, K., Carnie, J. A. and Rowsell, K. V. (1972). The subcellular distribution of of rat liver L-alanine-glyoxylate aminotransferase in relation of a pathway for glucose formation involving glyoxylate. Biochem. J. *127*: 155-165.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular cloning: A Laboratory Handbook. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press.

Sanger, F. and Coulson, A. R. (1975). A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. J. Mol. Biol. *13*: 373

Santos, M. J., Imanaka, T., Shio, H. and Lazarow, P. B. (1988). Peroxisomal integral membrane proteins in control and Zellweger fibroblasts. J. Biol. Chem. *263*: 10502-10509.

Schram, A. W., Strijland, A., Hashimoto, T., Wanders, R. J. A., Schutgens, R. B. H., van den Bosch, H. and Tager, J. M. (1986). Biosynthesis and maturation of peroxisomal beta-oxidation enzymes in fibroblasts in relation to the Zellweger syndrome and infantile Refsum disease. Proc. Natl. Acad. Sci., USA *83*: 6156-6158.

Scotto, J. M., Hadchouel, M. and Odievre, M. (1982). Infantile Phytanic Acid Storage Disease, a possible variant of Refsum's disease: Three cases, including ultrastructural studies of the liver. J. Inher. Metab. Dis. *5*: 83-90.

Shimozawa, N., Tsukamoto, T., Suzuki, Y., Orii, T., Shirayoshi, Y., Mori, T. and Fujiki, Y. (1992). A human gene responsible for Zellweger syndrome that affects peroxisome assembly. Science *255*: 1132-1134.

Shindo, Y. and Hashimoto, T. (1978). Acyl-coenzyme A synthetase and fatty acid oxidation in rat liver peroxisomes. J. Biochem. *84*: 1177-1181.

Singh, I., Moser, A. E., Goldfischer, S. and Moser, H. W. (1984). Lignoceric acid is oxidized in the peroxisome: Implications for the Zellweger cerebro-hepato-renel syndrome and adrenoleukodystrophy. Proc. Natl. Acad. Sci., USA *81*: 4203-4207.

Small, G. M. and Lewin, A. S. (1989). Peroxisome proliferation: Mechanisms and biological consequences. Biochemical Society Transactions *18*: 85-87.

Small, G. M., Szabo, L. J. and Lazarow, P. B. (1988). Acyl-CoA oxidase contains two targeting sequences each of which can mediate protein import into peroxisomes. EMBO J. *7*: 1167-1173.

Sulter, G. J., Waterham, H. R., Vrieling, E. G., Goodman, J. M. and Veenhuis, M. (1993). Expression and targeting of a 47 kD integral peroxisomal membrane protein of *Candida boidinii* in wild-type and peroxisome deficient mutants of *Hansenula polymorpha*. FEBS Letters *315*: 211-216.

Swinkels, B. W., Gould, S. J., Bodnar, A. G., Rachubinski, R. A. and Subramani, S. (1991). A novel, cleavable peroxisomal targeting signal at the amino-terminus of the rat 3-ketoacyl-CoA thiolase. EMBO J. *10*(11): 3255-3262.

Swinkels, B. W., Gould, S. J. and Subramani, S. (1992). Targeting efficiencies of various per mutations of the consensus C-terminal tripeptide peroxisomal targeting signal. FEBS Letters *305*(2): 133-136.

Tan, X., Titorenko, V., van der Klei, I., Sulter, G. J., Haima, P., Waterham, H., Evers, M., Cregg, J. M. and Veenhuis, M. (1993). Identification of peroxisomeless mutants of the yeast *Hansenula polymorpha*. Submitted.

Thompson, S. L., Burrows, R., Laub, R. J. and Krisans, S. K. (1987). Cholesterol synthesis in rat liver peroxisomes. Conversion of mevalonic acid to cholesterol. J. Biol. Chem. *262*: 17420-17425.

Thompson, S. L. and Krisans, S. K. (1990). Rat liver peroxisomes catalyze the initial step in cholesterol synthesis. J. Biol. Chem. *265*(10): 5731-5735.

Titorenko, V. I., Waterham, H. R., Cregg, J. M., Harder, W. and Veenhuis, M. (1993). Peroxisome biogenesis in the yeast *Hansenula polymorpha* is controlled by a complex set of interacting gene products. Proc. Natl. Acad. Sci., USA, in press.

Titorenko, V. I., Waterham, H. R., Haima, P., Harder, W. and Veenhuis, M. (1992). Peroxisome biogenesis in *Hanssenula polymorpha*: Different mutations in genes, essential for peroxisome biogenesis, cause different peroxisomal mutant phenotypes. FEMS Microbiol. Lett. *95*: 143-148.

Tolbert, N. E. (1981). Metabolic pathways in peroxisomes and glyoxysomes. Annu. Rev. Biochem. *50*: 113-57.

Tsukamoto, T., Miura, S. and Fujiki, Y. (1991). Restoration by a 35K membrane protein of peroxisome assembly in a peroxisome-deficient mammalian cell mutant. Nature (London) *350*: 77-81.

Tsukamoto, T., Yokota, S. and Fujiki, Y. (1990). Isolation and characterization of Chinese hamster ovary cell mutants defective in assembly of peroxisomes. J. Cell Biol. *110*: 651-660.

Tugwood, J. D., Issemann, I., Anderson, R. G., Bundell, K. R., McPheat, W. L. and Green, S. (1992). The mouse peroxisome proliferator activated receptor recognizes a response element in the 5' flanking sequence of the rat acyl CoA oxidase gene. EMBO J. *11*: 433-439.

Vamecq, J. and van Hoof, F. (1984). Implication of a peroxisomal enzyme in the catabolism of glutaryl-CoA. Biochem. J. *221*: 203-211.

van den Bosch, H., Schutgens, R. B. H., Wanders, R. J. A. and Tager, J. M. (1992). Biochemistry of peroxisomes. Annu. Rev. Biochem. *61*: 157-197.

van der Klei, I. J., Sulter, G. J., Harder, W. and veenhuis, M. (1991). Expression, assembly and crystallization of alcohol oxidase in a peroxisome-deficient mutant of *Hansenula polymorpha*; properties of the protein and architecture of the crystals. Yeast *7*: 15-24.

van Dijken, J. P. and Bos, P. (1981). Utilization of amines by yeasts. Arch. Microbiol. *128*: 320-324.

Veenhuis, M., Harder, W., van Dijken, J. P. and Mayer, F. (1981). Substructure of crystalline peroxisomes in methanol-grown *Hansenula polymorpha*: Evidence for an *in vivo* crystal of alcohol oxidase. Mol. Cell. Biol. *1*: 949-957.

Veenhuis, M., Hoogkamer-Te Niet, M. C. and Middelhoven, W. J. (1985). Biogenesis and metabolic significance of microbodies in urate-utilizing yeasts. Antonie van Leeuwenhoek *51*(1): 33-43.

Veenhuis, M., Keizer, I. and Harder, W. (1979). Characterization of peroxisomes in glucose-grown *Hansenula polymorpha* and their development after the transfer of cells into methanol-containing media. Arch. Microbiol. *120*: 167-175.

Veenhuis, M., Mateblowski, M., Kunau, W. H. and Harder, W. (1987). Proliferation of microbodies in *Saccharomyces cerevisiae*. Yeast *3*: 77-85.

Veenhuis, M., van Dijken, J. P. and Harder, W. (1983). The significance of peroxisomes in the metabolism of one-carbon compounds in yeast. Adv. Microbiol. Physiol. *24*: 1-82.

Veenhuis, M., van Dijken, J. P., Pilon, S. A. F. and Harder, W. (1978). Development of crystalline peroxisomes in methanol-grown cells of the yeast *Hansenula polymorpha* and its relation to environmental conditions. Arch. Microbiol. *117*: 153-163.

Veenhuis, M. and Harder, W. (1987). Metabolic significance and biogenesis of microbodies in yeast. Peroxisomes in Biology and Medicine. Berlin, Springer-Verlag. 436-458

Walton, P. A., Gould, S. J., Feramisco, J. R. and Subramani, S. (1992a). Transport of microinjected proteins into peroxisomes of mammalian cells: inability of Zellweger cell lines to import proteins with the SKL tripeptide peroxisomal targeting signal. Mol. Cell. Biol. *12*(2): 531-541.

Walton, P. A., Gould, S. J., Rachubinski, R. A., Subramani, S. and Feramisco, J. R. (1992b). Transport of microinjected alcohol oxidase from *P. pastoris* into vesicles in mammalian cells: involvement of the peroxisomal targeting signals. J. Cell Biol. *118*(3): 499-508.

Warren, J. R., Simmon, V. F. and Reddy, J. K. (1980). Properties of hypolipidemic peroxisome proliferators in the lymphocyte [3H]thymidine and *Salmonella* mutagenesis assays. Cancer Res. *40*(1): 36-41.

Waterham, H. R., Titorenko, V. I., van der Klei, I. J., Harder, W. and Veenhuis, M. (1992). Isolation and characterization of peroxisomal protein import ($Pim^-$) mutants of *Hansenula polymorpha*. Yeast *8*: 961-972.

Weisman, L. S. and Wickner, W. (1992). Molecular characterization of *VAC*1, a gene required for vacuole inheritance and vacuole protein sorting. J. Biol. Chem. *267*: 618-623.

Wendland, M. and Subramani, S. (1993). Cytosol-dependent peroxisomal protein import in a permeablized cell system. J. Cell biol. *120*(3): 675-685.

Wiebel, F. F. and Kunau, W.-H. (1992). The Pas2 protein essential for peroxisome biogenesis is related to ubiquitin-conjugating enzymes. Nature (London) *359*: 73-76.

Wiemer, E. A. C., Brul, S., Just, W. W., van Driel, R., Brouwer-Kelder, E., van den Berg, M., Weijers, P. J., Schutgens, R. B. H., van den Bosch, H., Schram, A., Wanders, R. J. A. and Tager, J. M. (1989). Presence of peroxisomal membrane proteins in liver and fibroblasts from patients with the Zellweger syndrome and related disorders: Evidence for the existence fo peroxisomal ghosts. Eur. J. Cell Biol. *50*: 407-417.

Woolford, C. A., Dixon, C. K., Manolson, M. F., Wright, R. and Jones, E. W. (1990). Isolation and characterization of *PEP5*, a gene essential for vacuolar biogenesis in *Saccharomyces cerevisiae*. Genet. *125*: 739-752.

Yamada, J., Horie, S., Watanabe, T. and Suga, T. (1984). Participation of peroxisomal β-oxidation system in the chain-shortening of a xenobiotic acyl compound. Biochem. Biophys. Res. Comm. *125*: 123-128.

Zhang, B., Marcus, S. L., Miyata, K. S., Subramani, S., Capone, J. P. and Rachubinski, R. A. (1993). Characterization of protein-DNA interactions within the peroxisome proliferator-responsive element of the rat hydratase-dehydrogenase gene. J. Biol. Chem. *268*: 12939-12945.

Zhang, B., Marcus, S. L., Sajjadi, F. G., Alvares, K., Reddy, J. K., Subramani, S., Rachubinski, R. A. and Capone, J. P. (1992). Identification of a peroxisome proliferator-responsive element upstream of the gene encoding rat peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase. Proc. Natl. Acad. Sci., USA *89*: 7541-7545.

Zhang, G. and Leader, D. P. (1990). The structure of the pseudorabies virus genome at the end of the inverted repeat sequences proximal to the junction with the short unique region. J. Gen. Virol. *71*: 2433-2441.

Zwart, K. B., Overmars, E. H. and Harder, W. (1983). The role of peroxisomes in the metabolism of D-alanine in the yeast *Candida utilis*. FEMS Microbiol. Lett. *19*: 225-231.

## BIOGRAPHICAL SKETCH

The author was born on November 1, 1970, in Beijing, the People's Republic of China. In 1987, she started her college education at the Department of Biology, University of Science and Technology of China, where she was awarded the Outstanding Student Fellowship in three consecutive years. In 1990, she transferred to the Department of Biology, University of San Francisco, and was selected as a Dean's Honor Roll student in the School of Arts and Sciences. As a senior, she participated in Dr. Paul K. Chien's marine biology lab, studying the acute toxicity of heavy metals on earthworm, and Dr. Patricia Shulz's transmission electron microscopy lab, examining the effect of light on choloroplast development in lemna. The above work was presented in four national and Bay Area undergraduate conferences. In 1991, she started her graduate study at the Department of Chemistry, Biochemistry, and Molecular Biology (formerly the Department of Chemical and Biological Sciences), the Oregon Graduate Institute of Science and Technology, under the supervision and direction of Dr. James M. Cregg. During the two years in Dr. Cregg's lab, she worked on the characterization of a gene required for peroxisome biogenesis in the yeast *Pichia pastoris*.