Investigations into Functional Complementation between Mammalian and Yeast Peroxisomal Biogenesis Genes

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Table of Contents

List of Tables

List of Figures

Abstract

Investigations into Functional Complementation between Mammalian and Yeast Peroxisome Biogenesis Genes.

by Kathaleen Ann Hartrum

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Peroxisomes are a class of ubiquitous subcellular organelles whose variable and multiple functions depend upon the organism and tissue in which they reside as well as the nutrient conditions. All contain at least one hydrogen peroxide-producing oxidase and catalase to reduce that peroxide to water and oxygen. Among peroxisomal functions in methylotrophic yeasts are the ß-oxidation of fatty acids and the metabolism of methanol. Although rare, human peroxisome biogenesis defects result in debilitating and lethal disorders most commonly known as Zellweger syndrome. Nevertheless, few details are known about peroxisome biogenesis in general and only three of the many human peroxisome biogenesis genes have been identified and characterized (*PXR1/PTS1R, PAF-1, PMP70*).

The goal of this study was to clone new human peroxisome biogenesis genes by functional complementation of *Pichia pastoris* peroxisome biogenesis (*per)* mutants. *P. pastoris* is easily manipulated in the laboratory and absolutely requires peroxisomes for metabolism of both methanol and oleate. Hence, complementation of a *P. pastoris per* mutant by a mammalian *PER* cDNA clone could be easily observed. The overall design of the first phase of the study was to clone complementing rat cDNAs and then use those to probe a human cDNA library to identify their human homologues. No complementing rat cDNAs were found.

The second phase of the study investigated whether there is functional homology between the human Paf1p and *P. pastoris* Per6p. The amino acid sequences of these two proteins share significant similarity (32% identity, 49% similarity). In addition, there are a number of structural similarities. A major difference between the two proteins is that Per6p contains a C-terminal, acid rich "tail" of about 100 residues which is not present in the Paf1p.

Plasmids were constructed containing one of the following DNA inserts: *PAF-1*, *PER6*, a hybrid gene in which *PAF-1* was fused with the *PER6* "acid tail," or a truncated *PER6* which lacked the "acid tail." The *PAF-1* constructs did not complement the *P. pastoris per6* mutant. However, the *PAF-1* cDNA clone that was used in the study contained a single nucleotide deletion that created a frame-shift mutation which introduced a premature stop codon. This mutation produced a severely truncated protein of only 80 residues, less than a third of the full-length protein (about 304 residues). Therefore, the results are inconclusive. Transformation with the truncated (tail-less) *PER6* revealed that the "acid tail" is essential for Per6p function in *P. pastoris*. Because Paf1p lacks this structure, it is unlikely that it will be able to complement the yeast *per6* mutant.

Chapter One

Introduction

1.1 Historical Background

In 1954 Rhodin described novel cytoplasmic structures in electron micrographs of mouse kidney proximal convoluted tubule cells, which he called microbodies (Rhodin, 1954). During the next decade, similar structures were reported in liver and kidney cells of various mammals and in the protozoan *Tetrahymena pyriformis*. For a time they were thought to be precursors of mitochondria or other cytoplasmic organelles. However, in 1966 deDuve and Baudhuin summarized the biochemical properties of these structures and speculated on their physiological and biological significance (deDuve and Baudhuin, 1966). They referred to microbodies by the functionally descriptive term, peroxisomes, since all of these structures appeared to contain oxidases which generate hydrogen peroxide. Also present in all samples was catalase that reduces hydrogen peroxide to oxygen and water.

Peroxisomes have since been observed in virtually all types of eukaryotic cells. Relative to other organelles, peroxisomes are unusual in that their enzyme content and function vary depending on the organism, tissue, cell type and nutrient conditions. For example, the peroxisomes in the fatty seeds of certain plants contain enzymes of the β-oxidation pathway and the glyoxylate cycle and function in gluconeogenesis during germination (Tolbert, 1981). These microbodies are therefore called glyoxysomes. On the other hand, peroxisomes in the leaves of most plants function in conjunction with chloroplasts and mitochondria in photorespiration. In mammals, peroxisomes contain the enzymes for β–oxidation of very-long-chain fatty acids (Lazarow and deDuve, 1976); synthesis of plasmalogens, cholesterol, and bile acids; amino acid and purine catabolism; and prostaglandin metabolism (reviewed by Subramani, 1993). In fungi, the β-oxidation of all fatty acids occurs in peroxisomes (see section 1.2.2). In addition, peroxisomes

contain the enzymes necessary for methanol metabolism in methylotrophic yeasts (see section 1.3).

Despite the diversity of functions and resident enzymes, there are certain unifying characteristics that define this ubiquitous class of organelles. They are roughly circular, with a diameter ranging from 0.1 μm to 1.0 μm and are bound by a single relatively thin membrane and contain a matrix that is slightly acidic (about pH 6). Their density is 1.21 to 1.25 gm/cm^3 which is denser than mitochondria (1.18 gm/cm$^{3)}$. Finally, almost without exception, they all contain at least one H_2O_2-producing oxidase and catalase.

1.2 β Oxidation in Peroxisomes

1.2.1 Mammalian cells

Saturated fatty acids with 24 or more carbons (very-long-chain fatty acids) are catabolized in mammalian peroxisomes (Lazarow and Moser, 1989). The first step in this process is activation of the fatty acid by a membrane-bound acyl-CoA synthetase. β–oxidation then proceeds through the usual oxidation, hydration, dehydrogenation and thiolysis steps which are mediated by peroxisomal matrix enzymes. The oxidation step generates hydrogen peroxide which is degraded to O_2 and H_2O by catalase. A bifunctional enzyme catalyzes the hydration and dehydrogenation steps. Finally, thiolase catalyzes thiolysis, producing a shorter acyl-CoA which cycles back through β-oxidation, and acetyl-CoA which may subsequently be used for energy generation or biosynthesis. Peroxisomes also efficiently catabolize unsaturated long-chain fatty acids.

1.2.2 Yeasts

Unlike mammalian cells, yeasts do not contain a mitochondrial β-oxidation system. Instead, β oxidation of all fatty acids takes place in peroxisomes (Veenhuis et al., 1987). Hence, peroxisomes are absolutely required for growth on media containing fatty acids, such as oleate, as the only carbon source. The pathway is essentially the same in yeasts and mammalian peroxisomes (Figure 1.1).

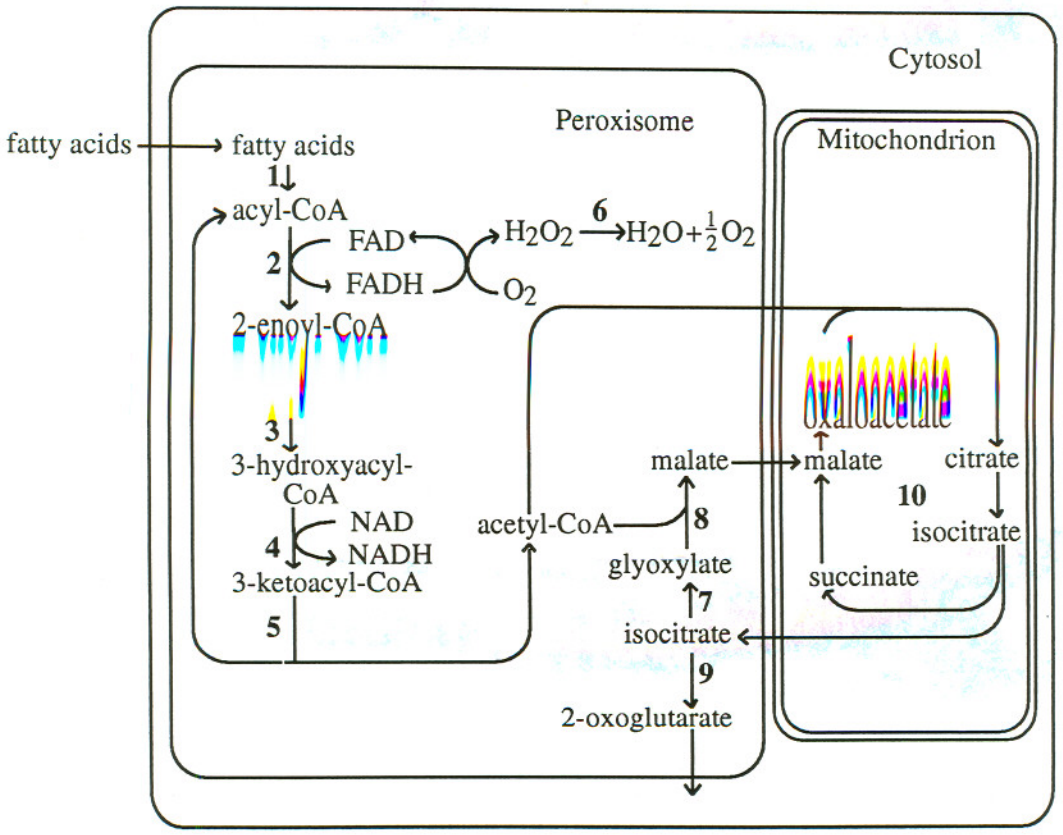

Figure 1.1 The peroxisomal fatty acid β-oxidation pathway in yeasts. The pathway was elucidated in species of *Candida* and is assumed to be the same in *P. pastoris.* (1) acyl-CoA synthase; (2) acyl-CoA oxidase; (3) 2-enol-CoA hydratase; (4) 3-hydroxyacyl-CoA dehydrogenase; (5) 3-ketoacyl-CoA thiolase;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

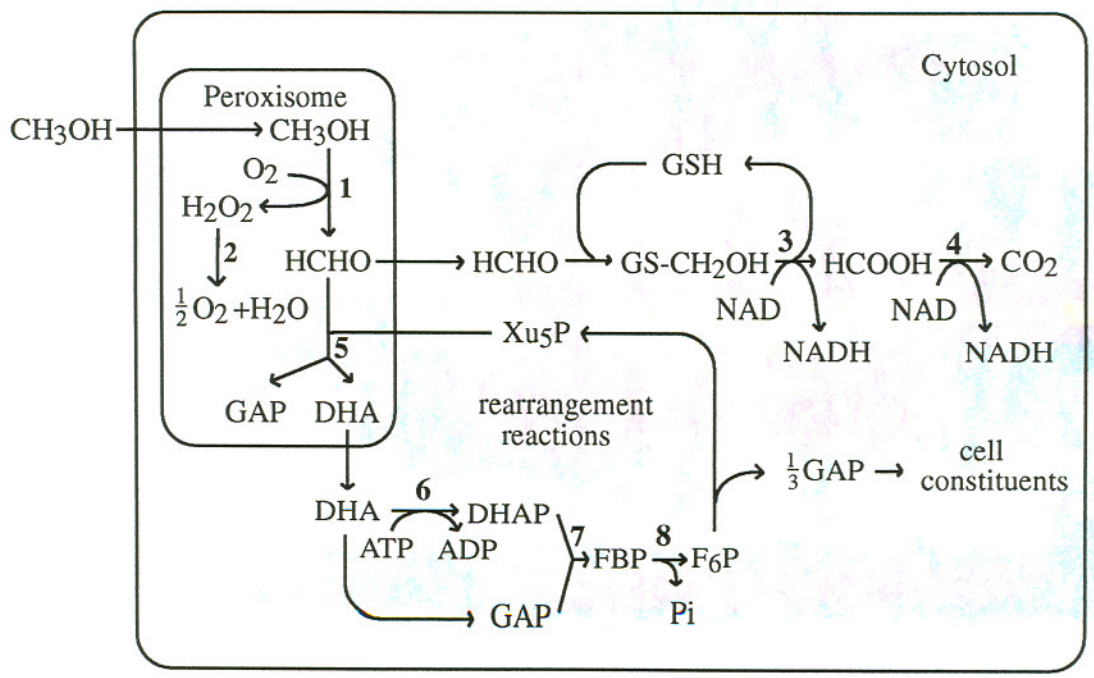

Figure 1.2 The methanol metabolism pathway in methylotrophic yeasts. (1) alcohol oxidase; (2) catalase; (3) formaldehyde dehydrogenase; (4) formate dehydrogenase; (5) dihydroxyacetone synthase; (6) dihydroxyacetone kinase; (7) fructose 1,6-bisphosphate aldolase; (8) fructose 1,6-bisphosphatase.

1.3 Methanol Metabolism in Methylotrophic Yeasts

Methylotrophic yeasts such as *Pichia pastoris* are able to utilize methanol as their sole carbon and energy source (Veenhuis et al., 1983). This methanol utilization pathway requires three peroxisomal enzymes (Figure 1.2). The first is alcohol oxidase which oxidizes methanol to form formaldehyde and hydrogen peroxide. Catalase converts the hydrogen peroxide to water and oxygen. There are two pathways for further formaldehyde metabolism. In one pathway, the formaldehyde exits the peroxisome and is oxidized to formate and then to carbon dioxide by two cytosolic dehydrogenases. This pathway generates reducing power for the cell. The other pathway begins with the third peroxisomal enzyme, dihydroxyacetone synthase, which catalyzes the condensation of formaldehyde and xylulose-5'-monophosphate (Xu5P) to generate glyceraldehyde-3-phosphate and dihydroxyacetone, two C_3 compounds that are subsequently used to construct cellular constituents and to regenerate Xu5P.

1.4 Peroxisome Biogenesis

Peroxisome biogenesis requires three distinct processes: lipid recruitment for the membrane, organellar growth, and fission (proliferation). The mechanisms for these processes remain largely unknown. The currently accepted model of peroxisome propagation is similar to that for mitochondrial and chloroplast proliferation: they grow as a consequence of increased matrix protein import, then undergo fission. It is thought that the organelles generally do not arise *de novo* or from the endoplasmic reticulum.

Peroxisomal membrane phospholipids probably originate in the endoplasmic reticulum as do lipids in other membranous structures (Lazarow, 1989). The lipid composition is similar to that of other cellular membranes except for a low level of sterols (Lazarow, 1984). How these lipids are transported to and incorporated into the peroxisomal membrane is not known.

A unique feature of peroxisomes is that they can be induced to proliferate by certain environmental stimuli. For example, mammalian peroxisome proliferation is induced by hypolipidemic drugs such as clofibrate. Yeasts proliferate peroxisomes in response to certain carbon sources, such as methanol or oleate, whose metabolism requires peroxisomal enzymes. Per8p in *Hansenula polymorpha* and PMP27 in *Saccharomyces cerevisiae* are peroxisomal membrane proteins which, when overexpressed, result in enhanced peroxisome proliferation (Erdmann et al., 1995; Tan et al., 1995; Marshall et al.,

1995). In addition, Per8p is concentrated in the small newly budded peroxisomes relative to the larger, older ones. These observations suggest that Per8p and PMP27 may be actively involved in proliferation. Interestingly, the *per8* deletion mutant is devoid of observable peroxisomes, while the *pmp27* deletion mutant contains a few very large peroxisomes. The specific roles that these proteins play in proliferation is not known.

Recently, evidence was presented that a *de novo* proliferation pathway may exist in which peroxisomes do not derive from pre-existing peroxisomes (Waterham et al., 1993). This evidence involved the isolation of an intriguing temperature-sensitive peroxisome-deficient mutant of *H. polymorpha* which lacks peroxisomes at the restrictive temperature but has wild-type peroxisomes at the permissive temperature. When cells of this mutant were grown at the restrictive temperature and then transferred to the permissive temperature, peroxisomes appeared to regenerate *de novo*.

Peroxisomal growth is primarily the result of the import of newly synthesized proteins into the peroxisomal matrix. Whenever environmental conditions require new peroxisomal enzymes, those proteins are synthesized and imported into the peroxisomal matrix, causing the organelle to expand. Most recent research on peroxisome biogenesis has been directed at understanding this import process. These proteins are synthesized on free cytosolic ribosomes and are imported post-translationally. This import requires both ATP and an HSP70-class protein (Walton et al., 1994). Two distinct peroxisome targeting signals (PTSs) have been identified which direct proteins to the peroxisomal matrix (see section 1.6). Putative receptors which may bind these PTSs have been suggested (see section 1.7).

1.5 Human Peroxisomal Disorders

Several devastating and lethal human diseases result from defects in peroxisome function. Some, such as familial amyotrophic lateral sclerosis (FALS) and X-linked adrenoleukodystrophy (X-ALD), are the result of a single defective peroxisomal protein (Table 1.1). Missense mutations in the peroxisomal Cu/Zn superoxide dismutase are responsible for FALS in certain patients (Rosen et al., 1993). A defective peroxisomal membrane protein required for catabolism of very-long-chain fatty acids is responsible for X-ALD (Mosser et al., 1993; Mosser et al., 1994).

Other human diseases are the result of defects affecting peroxisome biogenesis. These include Zellweger syndrome, neonatal adrenoleukodystrophy (NALD), infantile Refsum's disease (IRD) and classical rhizomelic chondrodysplasia punctata (RCDP).

Complementation studies based on cell fusion experiments indicate that a mutation in anyone of at least ten genes results in these disorders. These defects have been categorized into three types based on the cellular phenotypes of affected individuals (Motley et al.,1994; Slawecki et al., 1995). Type-1 defects result in the inability to import proteins containing the peroxisomal targeting signal-1 (PTS1) sequence and have been identified in two NALD cell lines from complementation group two. A defective PTS1 receptor is thought to be responsible for this type of disorder. Type-2 defects appear to specifically affect the PTS2 import pathway and are responsible for classical RCDP. The affected gene may encode the PTS2 receptor. Finally, type-3 defects diminish or abolish both PTS1 and PTS2 import and are responsible for most of the other peroxisomal biogenesis disorders including Zellweger syndrome and IRD. Three human peroxisomal biogenesis genes have been identified: *PXR1/PTS1R, PAF-1*, and *PMP70*. Mutations in the *PXR1/PTS1R* gene are responsible for type-1 disorders and encode the putative PTS1 receptor (Weimer et al., 1995, Dodt et al., 1995; Fransen et al., 1995). A prematurely truncated PAF-1 product, a peroxisomal integral membrane protein, is responsible for a type-3 Zellweger syndrome cell line disorder (Shimozawa et al., 1992). PMP70 is an integral membrane protein whose function is unknown.

1.6 Peroxisome Targeting Signals

Mitochondria and chloroplasts contain some of their own genes as well as the transcription and translation apparatus necessary for their expression. Thus, some of the proteins specific to these organelles are synthesized endogenously. By contrast, peroxisomes do not contain nucleic acids. Hence, all peroxisomal proteins are most likely encoded by nuclear genes whose products are then translated on free cytosolic ribosomes. These nascent polypeptides are then specifically directed to the peroxisome by targeting signals (PTSs), two of which have been identified and at least two others suggested.

1.6.1 PTS1

The first matrix protein PTS to be described is a carboxy-terminal three-amino-acid sequence. The consensus sequence of this tripeptide is SKL (S = serine, K = lysine, L = leucine), although conservative substitutions are common (Gould et al., 1989). First described in firefly luciferase, this PTS1 sequence has since been demonstrated to be a PTS in mammals, insects, yeasts, and protozoans (de Wet et al., 1987; Keller et al., 1987;

Table 1.1 Human peroxisome-related diseases

CATEGORY	EXAMPLES OR DESCRIPTION	AFFECTED PROTEIN	GENE
SINGLE ENZYME DEFECT	F-ALS (Lou Gehrig's disease) X-ALD (Lorenzo's Oil)	Cu/Zn SOD Acyl-CoA synthetase	
PEROXISOME BIOGENESIS DEFECTS			
Type I	PTS1 import defects NALD (neonatal ALD)	PTS1 receptor	*PXR1/PTS1R*
Type 2	PTS2 import defects RCDP (rhizomelic chondro-dysplasia punctata)	PTS2 receptor?	
Type 3	PTS1 & PTS2 import defects Zellweger syndrome, IRD (Infantile Refsum's disease)		*PAF-1*

Gould et al., 1987; Subramani, 1992). When the sequence is removed from matrix proteins, these proteins remain in the cytosol and when it is added to nonperoxisomal proteins, the proteins are imported into the peroxisome. Unlike targeting signals for most other subcellular compartments, this signal is not cleaved after import but remains a part of the mature protein.

1.6.2 PTS2

PTS2, in contrast to PTS1, is an amino-terminal sequence that is cleaved after import in some organisms (plants and mammals) and not in others (yeasts). It was first described in the two isozymes of rat peroxisomal 3-ketoacyl-CoA thiolase, enzymes that catalyze the last step in fatty acid β oxidation (Swinkels et al., 1991). The consensus sequence of this PTS is RLX5H/QL (de Hoop and AB, 1992; Tsukamoto et al., 1994a). Other matrix proteins containing this presequence include peroxisomal thiolases from humans and yeasts, watermelon glyoxysomal malate dehydrogenase, and amine oxidase from *H. polymorpha.*

1.6.3 Other matrix protein PTSs

Several peroxisomal matrix proteins lack sequences similar to either PTS1 or PTS2. These are thought to contain unidentified PTSs. Evidence for additional PTSs has been presented for two matrix proteins. Acyl-CoA oxidase from *C. tropicalis* contains two non-overlapping internal regions that are required to target this protein to the peroxisome *in vitro* (Small et al., 1988). A region within the first third of *S. cerevisiae* catalase A is sufficient to direct fusion proteins to the peroxisome *in vivo.* This region functions as a PTS even without the C-terminal SKF, a PTS1-like motif which apparently is not a targeting signal in this protein (Kragler et al., 1993). Neither of these putative targeting sequences has been fully characterized.

1.6.4 Implications of the existence of multiple targeting signals

The existence of multiple targeting signals suggests that there are multiple import pathways for peroxisomal matrix proteins. Cells defective in one gene are deficient in their ability to import PTS1 but are competent to import PTS2 proteins. Conversely, cells defective in another gene are deficient in PTS2 import but retain the ability to import PTS1

proteins. It is probable that these pathways overlap significantly. Such a model is suggested by the fact that the defects in most Zellweger genes abolish the import of all matrix proteins into the peroxisomes. These genes may encode common import pathway components. These peroxisome biogenesis mutant phenotypes exist in a diversity of organisms ranging from yeasts to humans, thus implying similar peroxisome assembly processes in all organisms (Zhang et al., 1993; Motley et al., 1994; Slawecki et al., 1995).

1.6.5 Membrane PTSs

Targeting signals for peroxisomal membrane proteins remain a tantalizingly elusive quarry. Most mutants that fail to import matrix proteins possess membranous structures containing peroxisomal membrane proteins. Therefore, these proteins appear to be targeted to and incorporated into the membrane via an apparatus that is separate from that of matrix proteins. An internal region of PMP47 of the yeast *Candida boidinii* is essential for directing this protein to the peroxisomal membrane (McCammon et al., 1994). Two putative membrane spanning α–helical segments and the intervening loop define the region. This region may not actually contain a targeting sequence *per se* but rather contains sequences necessary for anchoring the protein to the membrane during insertion. This is the first and only report of a membrane targeting signal for peroxisomes.

1.7 Targeting Signal Receptors

The elucidation of the two targeting signals described above suggested that the machinery for importing peroxisomal matrix proteins included at least two PTS receptors. In addition, the fact that some mutants are defective in import of one PTS class of protein but competent to import the other suggested that the affected genes may encode receptor proteins.

1.7.1 PTS1 receptor

A *pas8* mutant of *P. pastoris* is defective in PTS1 protein import while retaining the capacity to import PTS2 proteins (McCollum et al., 1993). The affected *PAS8* gene was cloned by functional complementation of a *pas8* mutant and encoded a 68-kDa membrane-associated polypeptide belonging to the tetratricopeptide repeat (TPR) family. The TPR family is characterized by multiple repeats of a 35 amino acid degenerate consensus

sequence. McCollum and coworkers demonstrated that Pas8p specifically binds the SKL tripeptide sequence *in vitro*, thus making it a candidate for the PTS1 receptor (McCollum et al., 1993).

Subsequently, the probable *S. cerevisiae* and *H. polymorpha PAS8* homologues (*PAS10* and *PER 3* , respectively) were reported (van der Leij et al., 1993; van der Klei et al., 1995). Using the two-hybrid screen to search for the PTS1 receptor with an SKL tripeptide as the "bait", DNA fragments containing a portion of the *S. cerevisiae PAS10* gene were isolated (Brocard et al., 1994). This experiment demonstrated that *Pas10p* binds SKL *in vivo,* further supporting the idea that these proteins are PTS1 receptors. Deletions of portions of this gene revealed that a complete and intact TPR domain is essential for interaction with SKL but is not sufficient in itself (Brocard et al., 1994).

Three research groups recently reported cloning the human homologue of the putative PTS1 receptor. Gould's group used the *P. pastoris* Pas8p sequence to search cDNA databases and identified a cDNA (*PXR1*) whose product has 30% identity with Pas8p and a 24% identity with Pas10p. PXR1 binds SKL *in vitro,* and a hybrid protein composed of the N-terminus of Pas8p fused to the TPR domain of PXR1 complements a *pas8* mutant of *P. pastoris* for growth on oleic acid medium. These results lend additional credence to the notion that the yeast and human proteins are functional homologues (Dodt et al., 1995).

The SKL tripeptide sequence was used as the "bait" in a two-hybrid system screen of a human liver cDNA library (Fransen et al., 1995). The recovered gene encoded a protein (PTS1R) that is identical to PXR1 and interacts strongly and specifically with SKL *in vitro* and *in vivo.* This protein was found to be tightly associated with the peroxisomal membrane with its TPR domain exposed on the cytosolic side.

Subramani's was the third group to identify a putative human PTS1 receptor (PTS1R) cDNA clone (Wiemer et al., 1995). In contrast to Fransen, they found that the subcellular localization of this protein is primarily cytosolic with a smaller portion tightly associated with the peroxisomal membrane. The gene was mapped to chromosome band 12p13.3.

All three groups found that the *PXR1/PTS1R* gene complemented peroxisome biogenesis deficiency group 2 mutants which are specifically defective in import of PTS1 proteins. Specific mutations in each defective gene were identified by cloning and DNA sequencing of the mutant gene in each line.

The cloning of the PTS1 receptor from three yeast species and humans represents a significant step forward in elucidating the mechanism of peroxisomal protein import. Even

the apparently divergent data regarding the subcellular localization of the protein may represent a clue to its function. If, for example, the protein shuttles between the peroxisomal membrane and the cytosol, one would expect to find it in both locations. Although TPR proteins have many functions, two TPR proteins are mitochondrial protein import receptors (MOM72 of *Neurospora crassa* and MAS70 of *S. cerevisiae*). These proteins are anchored in the outer mitochondrial membrane by an hydrophobic membrane-spanning region with their TPR domains exposed on the cytosolic surface (van der Leij et al., 1993). Thus, for both mitochondrial and peroxisomal import, TPR motifs may function to recognize and bind the organelle-specific targeting sequence on proteins.

1.7.2 PTS2 receptor

S. cerevisiae pas7 mutants display a phenotype similar to the human peroxisomal biogenesis disorder RCDP (rhizomelic chondrodysplasia punctata) in that both mutant strains have normal-looking peroxisomes that import PTS1 proteins but not the PTS2 protein thiolase. The *PAS7* gene encodes a 375-amino-acid polypeptide that is largely hydrophobic. It contains a WD-40 repeat motif which is thought to mediate protein-protein interaction (Marzioch et al., 1994). The WD-40 motif consists of about 40 amino acid residues with a highly conserved tryptophan-aspartic acid pair and was initially identified within the β subunit of the heterotrimeric G protein transducin (Fong et al., 1986). Lazarow found that Pas7p was primarily located in the peroxisomal matrix (Zhang et al., 1995). These researchers suggested that Pas7p may function as an intra-organellar receptor for thiolase import. According to this model, cytosolic thiolase is recognized by an unidentified extra-peroxisomal receptor and imported through the peroxisomal membrane where Pas7p binds it and pulls it into the matrix. Alternatively, Pas7p may mediate the proper folding of imported thiolase.

1.8 Oligomeric Import of Peroxisomal Matrix Proteins

Recent evidence suggests at that least some oligomeric proteins may be imported into the peroxisomal matrix as fully assembled complexes rather than as unfolded monomers. Studies involving homooligomeric PTS1 and PTS2 proteins indicate that only one subunit of the oligomer needs to contain the PTS in order for the whole oligomer to be imported. *S. cerevisiae* thiolase, a PTS2 protein, is a homodimer in its active form. When a truncated thiolase, lacking the PTS2, is coexpressed with full-length thiolase,

approximately 50% of the truncated thiolase cofractionates with the full-length thiolase in the organellar pellet (Glover et al., 1994). Similar results were obtained in experiments conducted with a PTS1-containing protein (McNew and Goodman, 1994).

Microinjection experiments suggest that proteins can be folded prior to import into the peroxisomal matrix . Using prefolded proteins stabilized by disulfide bonds or by chemical cross-linking, Subramani's group found that such proteins were imported into the peroxisomal matrix of human fibroblast cells (Walton et al., 1995). Furthermore, colloidal gold particles ranging from 4 to 9 nm in diameter and conjugated to proteins bearing the PTS1 sequence were also translocated into the peroxisomal matrix.

These results suggest that the import mechanism for peroxisomal matrix enzymes is different from that of mitochondria and chloroplasts. Two general models have emerged. One suggests that transient large pore-forming complexes assemble within the membrane when new matrix proteins are present or docked on the cytosolic side of the membrane. Import would then proceed through this pore in a manner similar to that of nuclear protein import. However, no such pores have been observed in electron micrographs of peroxisomal membranes. The other model proposes that matrix-destined proteins dock to membrane-bound receptors and are subsequently engulfed by an inwardly directed vesicle. Electron micrographs have not revealed this sort of structure either.

Chapter Two

Functional complementation of *P. pastoris* peroxisome-deficient mutants using a rat cDNA library

2.1 Introduction

In 1973 Goldfischer reported that peroxisomes were apparently absent from the cells of patients afflicted with a family of lethal genetic diseases called Zellweger syndrome (Goldfischer et al., 1973) However, closer examination revealed that aberrant peroxisomes were present in cells of these patients (Santos et al., 1988). At least ten complementation groups of peroxisome-deficient Zellweger cell lines have since been identified indicating that mutations in any one of at least ten genes results in this phenotype (Slawecki et al. 1995). However, with the exception of *PAF-1, PXR1/PTS1R* and *PMP70*, the affected genes remain unidentified. In *P. pastoris*, at least 10 genes are essential for peroxisome biogenesis (Liu et al., 1992; Gould et al., 1992; unpublished results). The *P. pastoris* peroxisome-deficient mutants (*per)* have the same phenotype as human Zellweger cells (i.e., absent or aberrant peroxisomes and peroxisomal enzymes present in the cytosol). In addition, based on amino acid similarity of their products, two of the affected *P. pastoris* genes appear to be homologues of two Zellweger genes. The *P. pastoris PAS8* gene is the putative homologue of the human *PXR1/PTS1R* gene (see section 1.7) and *PER6* is the putative homologue of the human *PAF-1* gene (Waterham et al., 1996; Chapter 3 of this thesis). Thus, many and perhaps all, *P. pastoris per* mutants are defective in genes homologous to those affected in Zellweger syndrome.

Relative to mammalian cells, yeasts are inexpensive and easy to work with. In addition, it is difficult to clone mammalian genes by functional complementation as is routinely done in yeasts. The ability to use yeast mutants to clone mammalian genes would represent a significant contribution to the study of peroxisome biogenesis. This chapter reports efforts to clone rat peroxisomal biogenesis genes by functional complementation of *P. pastoris per* mutants. Toward this end we constructed a rat cDNA library in a *P.*

pastoris expression vector and used it to transform *P. pastoris* mutants. Unfortunately, we were unable to recover a rat cDNA with the ability to complement a *P. pastoris per* mutant.

2.2 Materials and Methods

2.2.1 Media and strains

P. pastoris and *Escherichia coli* strains used are listed in Table 2.1. *P. pastoris* strains were cultured at 30°C in standard YPD medium (1% yeast extract, 2% peptone, and 2% glucose), reduced glucose YPD medium (containing 0.2% glucose), or YNB medium (0.17% yeast nitrogen base without amino acids, and 0.5% ammonium sulfate) supplemented with one of the following carbon sources: 0.4% glucose (YND medium), 0.5% methanol (YNM medium). *E. coli* strains were cultured at 37°C in LB medium (0.5% yeast extract, 1% glucose, and 1% sodium chloride). Ampicillin was added to a concentration of 0.1 mg/ml to select for strains containing plasmids conferring ampicillin resistance (LB / Amp medium).

2.2.2 Construction of the rat liver cDNA library

A rat liver cDNA library was constructed from poly $(A)^+$ RNA prepared from the liver of a male Sprague Dawley rat that had been treated with clofibrate, a hypolipidemic drug known to induce peroxisome proliferation. The rat was fed Teklad rodent diet containing 0.5% (wt/wt) clofibrate for 14 days *ad libitum*.

The library is composed of 3×10^5 independent cDNA clones inserted into the expression vector pHIL-A1 (Invitrogen, San Diego, CA). cDNA was made using a commercially available cDNA synthesis system (BRL, Gaithersberg, MD). Adapters containing the following sequence:

5' AATTCGTTGTCGACTGTCAG 3'
3' CAACAGCTGACAGTC 5'

were ligated to the cDNA. The resulting mixture was resolved in a 1% agarose gel and DNA fragments larger that 400 bp were isolated, phosphorylated and ligated with EcoRI digested, dephosphorylated vector.

The ligation mixture (1µl) was electroporated into DH10B cells and approximately 8.8×10^3 clones resulted. Additional samples of ligation mixture and cells were electroporated until approximately 3×10^5 clones were obtained. Random clones were chosen from each set of ligation reactions and 75% contained inserts. Bacterial colonies were scraped from plates in pools of approximately 10,000 and frozen as glycerol stocks.

Plasmid preparations from saturated 250-ml cultures (inoculated with 1×10^{11} cells) were prepared and used to transform *P. pastoris per* mutant strains.

Table 2.1 *P. pastoris* and *E. coli* strains used for rat/yeast complementation studies

***P. pastoris* strains**	**Genotype**	**Source or reference**
JC100	Wild type	NRRL[a] Y-11430, Cregg et al., 1985
GS115	*his4*	Cregg et al., 1985
589-3	*per4 his4*	Liu et al., 1992
JC121	*per1his4*	This study
JC122	*per2 his4*	This study
JC123	*per3 his4*	This study
JC124	*per4 his4*	This study
JC125	*per5 his4*	This study
JC116	*per 6 his4*	This study
JC127	*per7 his4*	This study
JC128	*per8 his4*	This study
JC129	*pas2 his4*	This study
JC130	*pas6 his4*	This study
***E. coli* strains**	**Genotype1**	**Source or reference**
DH5α	F$^-$ ϕ80d*lac*ZΔM15,*end*A1 *rec*A1 *hsd*R17 (r_K^-, m_K^-) *sup*E44 *thi*-1 *gyr*A9 *rel*A1 Δ(*lac*ZYA-*arg*F) U169 λ$^-$	Promega, Madison, WI.
DH10B	F$^-$ mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80d*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *end* A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ$^-$ *rps*L *nup*G	BRL

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

2.2.3 Transformation of *per* mutants

A typical transformation was performed as follows: a 10-ml overnight culture of cells grown in YPD was used to inoculate a fresh culture in YPD. This culture was maintained in log phase all day and used to inoculate a 300-ml culture to a final OD_{600} of 2.4×10^{-4}. The culture was harvested 17 hours later at an OD_{600} of 0.25. Cells were collected by centrifugation and washed once with 30 ml sterile water, once with 30 ml SED buffer (1 M sorbitol, 25 mM EDTA, 50 mM DTT) and once with 30 ml 1 M sorbitol. The cells were then resuspended in 15 ml of SCE (1 M sorbitol, 0.1 M sodium citrate, 10 mM EDTA, pH 5.8). 7.5 µl of Zymolase T100 (4mg/ml) (ICN, Irvine, CA) was added and the mixture was incubated at 30°C for 10-15 minutes. The spheroplasts were washed once with 30 ml of 1 M sorbitol, once with 30 ml CaS (10 mM $CaCl_2$, 1 M sorbitol) and resuspended in a final volume of 1.8 ml. Liver cDNA library, 20 µg, was mixed with 0.2 ml of cells and incubated at 25°C for 20 minutes. Next, 2.0 ml of a PEG solution (20% polyethylene glycol-3350, 10 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.4) was added and the incubation was continued for another 15 minutes. The cell suspensions were harvested by centrifugation at approximately $1,500 \times g$, resuspended in 0.3 ml of SOS (1 M sorbitol, 0.3x YPD medium, 10 mM $CaCl_2$) and incubated at 25°C for 30 minutes. Finally, 1.7 ml of 1 M sorbitol was added and a 0.5-ml aliquot of the mixture was mixed with 10 ml of regeneration agar (1% glucose, 0.6 M KCl, 0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% agar) and plated (a total of four plates per pool). Typically, 20 µg of DNA gave 5.0×10^4 -1×10^5 yeast His^+ transformants.

In addition to the spheroplast transformations described above, derivatives of *his4 per* mutants from complementation groups *PER1* through *PER8* (Liu et al., 1992) and *PAS2 and PAS6* (Gould et al., 1992) were also transformed by electroporation (Becker et al., 1991). Approximately 2.0×10^4 - 3.0×10^4 His^+ transformants of each per mutant strain were examined.

Finally, as a control to determine that the transformation technique was effective, each mutant was transformed with the cloned wild-type gene and examined on YNM plates to verify complementation.

2.2.4 Transformations and selections

His^+ transformant colonies were screened for those that potentially contained a complementing rat cDNA by replica plating onto YNM and YND plates. YNM plates were incubated at 30°C for approximately 5 days, then left at room temperature for several

weeks. Colonies were fed additional methanol by adding 2 drops of 100% methanol to the lid of each plate every other day.

His^+ transformants that appeared to be Mut^+ (able to utilize methanol as the sole carbon source) on plates were screened for complementation in YNM broth. Cells were precultured in either YPD or YPD with only 0.2% glucose and shifted by centrifugation to YNM medium. Cultures in YNM were sampled daily to determine density at OD_{600}.

To determine whether plasmids were autonomous or had stably integrated, 10-ml YPD broth cultures were inoculated with His^+ Mut^+ colonies from YNM plates and incubated overnight at 30°C. These cultures were diluted to OD_{600} of 0.01 in YPD and grown overnight a second time. The second overnight cultures were used to inoculate three YPD plates to concentrations of approximately 300, 1000, and 3000 cells per plate. The plates were incubated at 30°C for two days until colonies appeared and then replica plated to YNM, YNM + 50 µg/ml histidine, YND, YND + 50 µg/ml histidine and YPD plates.

2.2.5 *P. pastoris* DNA extractions

P. pastoris strains were grown overnight in 10-ml YPD cultures and aliquots of these cultures were inoculated into 200 ml YPD to a starting OD_{600} of approximately 0.0062. Cultures were incubated at 30°C for 16 hours, harvested by centrifugation at an OD_{600} of 1.0 to 2.0 and the pellets stored at -20°C. Pellets were subsequently thawed and successively washed by centrifugation at 1500 × g with 5 ml each of water, SED buffer (1 M sorbitol, 25 mM EDTA, 50 mM DTT) and 1 M sorbitol. Washed pellets were resuspended in 5 ml of ST buffer (1 M sorbitol, and 0.1 M Tris-HCl, pH 7.0), and incubated with 20 µg/ml Zymolyase T100 for 30 minutes at 30°C to digest cell walls. Preparations were then centrifuged at 1000 × g and resuspended in 5 ml of lysis buffer (0.2% SDS, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl). This produced a viscous cell lysate to which was added 100 µg/ml each of proteinase K and RNase A and incubated at 30°C for 30 minutes. An equal volume of PCA (25:24:1 ratio of phenol, chloroform, and isoamyl alcohol) was added and mixed by gently inverting the tube 100 times. This mixture was centrifuged at 12,000 × g for 20 minutes. The top aqueous phase was transferred to a new tube and the PCA extraction was repeated. The resulting aqueous phase was transferred to tubes containing 10 ml of ice cold 100% ethanol and mixed by gently inverting the tubes. The precipitated DNA was then spooled onto a plastic rod, redissolved immediately in 1 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) and dialyzed overnight at 4°C against 100 volumes of TE buffer.

2.2.6 Southern hybridizations

Total DNA was digested with either EcoRI or with SmaI and ApaI. Digested DNA was extracted with PCA, brought to 0.3 M NaOAc (pH 5.2) and precipitated using two volumes of 100% ice-cold ethanol followed by a wash with ice-cold 70% ethanol. The DNA pellets were dried and resuspended in TE buffer. DNA fragments were separated by size on a 0.8% agarose gel in TBE buffer (0.045 M Tris-borate, pH 8.0, 1 mM EDTA). To prepare the gel for capillary transfer of the DNA to a nylon membrane, it was first washed in 0.25 M HCl for 20 minutes, then washed with denaturing solution (0.5 M NaOH, 1.5 M NaCl, 0.002% thymol blue) for one hour and then with a neutralizing solution (0.5 M Tris-HCl, pH 7.0, 1.5 M NaCl). Capillary transfer of the DNA from the agarose gel to the nylon transfer membrane (0.45 micron, MSI, Westboro, MA) was performed as described in Sambrook et al. (1989) using 10 × SSPE (0.01 M Na_2EDTA, 1.5 M NaCl, 0.1M NaH_2PO_4) as the transfer buffer. The prehybridization solution was composed of 50% formamide, 6 × SSPE (0.9 M NaCl, 0.06 M NaH_2PO_4, pH 7.4, 6 mM Na_2EDTA), 1 × P (0.02% bovine serum albumin, 0.02% polyvinylpyrollidone, 0.02% Ficoll 400,000, 0.1% Tris-HCl, pH 7.5) and 0.1 mg/ml of sheared and denatured salmon sperm DNA. Membranes were incubated with the prehybridization mix in heat-sealed bags at 42°C overnight before adding the radioactive probe.

A commercially available kit was used to label DNA with α^{32}P-dCTP for hybridization probes (Amersham International, Amersham, UK) Approximately 25-100 ng of template DNA in 3 µl of H_2O was denatured by boiling for two minutes and immediately chilled on ice for at least one minute. After the labeling reaction the labeled DNA was separated from unincorporated ^{32}P-dCTP, by centrifugation through a Sephadex G-25 column (Boehringer Mannheim, Indianapolis, IN). The eluate containing the labeled probe was boiled for two minutes and immediately chilled on ice. The probe was added to the prehybridization mix in the sealed bag containing the blot and the blot was incubated overnight at 42°C. After hybridization, the filter was washed twice for 10 minutes each at room temperature in buffer containing 0.1 × SSPE + 1% SDS and twice for 15 to 30 minutes each in the same buffer at 65°C. The filter was wrapped in plastic wrap and exposed to X-ray film for 3 to 5 days with an enhancer screen (DuPont, Ontario, CA) at -70°C.

2.2.7 Screening for cDNA inserts by PCR

Two primers, AOX5' and AOX3', were obtained from Invitrogen (San Diego, CA). The AOX5' sequence was GACTGGTTCCAATTGACAAGCT and was

complementary to sequences just 5' of the methionine initiator ATG in the *AOX1* promoter. The AOX3' sequence was GCAAATGGCATTCTGACATCC and was complementary to sequences near the beginning of the *AOX1* terminator region. Two other primers that hybridized near the *AOX1*terminator region (AOXT-1 and AOXT-2) were synthesized by the Center for Gene Research at Oregon State University. The sequence of AOXT-1 was AACTTGAACTGAGGAAC, and AOXT-2 sequence was CATTCTGACATCCTCTT. Vent DNA polymerase was used according to instructions provided by the supplier (NEB, Beverly, MA) For PCR reactions, the total reaction volume of 50 µl was overlaid with 20 µl light mineral oil (Aldrich, Milwaukee, WI) to prevent evaporation. The template for the reaction was 50 ng of genomic DNA or 5 ng of uncut double-stranded plasmid DNA. The concentration of each dNTP was 250 µM and primer concentrations were 1 µM each. PCR reactions were performed in a SingleBlock™ system thermocycler (Ericomp, Inc., San Diego, CA) using the following program: cycle 1, 94°C for 90 seconds; cycles 2 through 46, 94°C for 60 seconds, 60°C for 30 seconds, 72°C for 60 seconds; cycle 47, 72°C for 5 minutes. Amplified DNA was extracted with PCA, precipitated with ethanol and resuspended in TE buffer or water.

A second set of primers (5' pHIL-A1 and 3' pHIL-A1) was also used to amplify cDNA inserts in pHIL-A1 plasmids. These primers were designed to specifically amplify the cDNA insert without *AOX1* promoter or terminator sequences from the plasmid. They contained ten nucleotides from either the promoter or terminator region, followed by the EcoRI site and sequences from the adapter oligonucleotides added to the cDNAs. The sequences of these primers were: 5' pHIL-A1, TTCGAAACGAGGAATTCGTT and 3' pHIL-A1, ATGTCTAAGGGGAATTCGTT.

For PCR reactions with these primers, the concentrations of all components were the same as described above. The reaction conditions were: cycle 1, 94°C for 90 seconds; cycles 2 through 41, 94°C for 60 seconds, 55°C for 30 seconds, 72°C for 120 seconds; and cycle 42, 72°C for 5 minutes.

2.2.8 Recovery of DNA from gels

DNA samples were separated by size on 0.8% agarose gels in TBE buffer following standard procedures. Digested plasmids and PCR products to be inserted into plasmids were purified from gels using a Qiaex gel extraction kit (QIAGEN Inc., Chatsworth, CA).

2.2.9 *E. coli* transformation procedures

$CaCl_2$-competent DH5α *E. coli* cells were prepared using the following procedure. 3.0 ml LB medium was inoculated with cells grown on LB plates, and incubated at 37°C overnight. This culture was added to 300 ml LB and incubated in a 1-liter flask in a 37°C shaking incubator for approximately 3 hours to an OD_{600} of 0.3 to 0.4. 150 ml aliquots of this culture were poured into sterile, ice-cold 250 ml centrifuge bottles and chilled on ice for 10 minutes. The cells were harvested by centrifuging for 10 minutes at 4°C and 5,000 × g and gently resuspended in 100 ml sterile, ice-cold 0.1 M $CaCl_2$. This suspension was held on ice for at least 30 minutes and the cells harvested again by centrifugation. These cells were resuspended in 10 ml of sterile $CaCl_2$/ glycerol solution (90% 0.1 M $CaCl_2$, 10% glycerol), divided into 100-µl aliquots, quick-frozen in liquid nitrogen and stored at -70°C for future use.

To transform ligation reactions into *E. coli*, previously frozen 100-µl glycerol stocks of $CaCl_2$-competent DH5α *E. coli* cells were thawed and kept on ice. The ligation mix containing the linearized (and dephosphorylated when necessary) vector, DNA insert, T4 ligase and ligase buffer was added to the chilled cells and the mixture was incubated on ice for 30 minutes. The cells were then heat shocked for 5 minutes in a 37°C water bath. LB broth (900 µl) was added and the cells were incubated for at least one hour in a 37°C shaker. Cells were harvested by centrifugation, resuspended in 96 µl of LB / Amp broth, spread on LB / Amp agar and incubated overnight at 37°C. If screening colonies for β-galactosidase was required, 40 µl of XGAL (5-bromo-4-chloro-3-indolyl-β-D-galactoside) (20 mg/ml) and 4 µl of IPTG (isopropylthiogalactoside) (200 mg/ml) were added to the plates before the cells were added.

Electroporation transformations of MC1061 *E. coli* cells were performed using total DNA extracted from Mut^+ transformants of *per5* and *per6* . These cells were also spread on LB/Amp plates and incubated overnight at 37°C.

2.2.10 Insertion of PCR products into pBS for sequencing

To clone PCR products, pBS II SK^- plasmid (pBS, Stratagene, San Diego, CA) was cut with EcoRI and dephosphorylated using calf intestine alkaline phosphatase according to the procedure described by the supplier (New England Biolabs, Beverly, MA). PCR products were cut with EcoRI,extracted with PCA, precipitated with isopropanol and resuspended in either water or TE buffer. PCR products were then combined in a 3:1 molar ratio with the cut vector and ligated with T4 ligase following standard procedures (Sambrook et al., 1989). Ligation reactions were transformed into

DH5α *E. coli* by adding 10 µl of the reaction mix to 100 µl of competent cells and following the procedures described in section 2.2.9.

To recover plasmids from transformed cells, single colonies were inoculated into 3 ml LB / AMP broth and incubated in a 37°C shaking incubator for at least six hours. Cells were harvested by centrifugation and the pellet was resuspended in 150 µl TE buffer. To this suspension, 150 µl 0.2 M NaOH, 1% SDS was added, mixed by vortexing and incubated at room temperature for approximately 15 minutes. 150 µl of cold 2 M NaOAc, pH 5, was added and the mixture vortexed and incubated on ice for about 15 minutes. Plasmid DNAs were separated from the other cell components by centrifuging this mixture for 20 minutes at full speed in a mini-centrifuge and removing the supernatant to a fresh tube. Plasmid DNAs were precipitated from the supernatant by addition of 0.7 volumes of 100% isopropanol, vortexing, and centrifuging for 20 minutes. The resulting DNA pellet was washed with 70% ice-cold ethanol, dried and resuspended in TE buffer or water.

2.2.11 DNA sequencing

The Sequenase 7-deaza-dCTP sequencing kit was used according to instructions provided by the supplier (USB, Cleveland, OH). The reaction primers used were the M13 -40 (universal) primer supplied with the kit, and the reverse primer (Stratagene, San Diego, CA) which prime in the pBS vector on opposite sides of the multiple cloning site. Reactions were performed in microtiter plates. Sequencing samples were separated by electrophoresis on acrylamide gels and gels were processed for exposure to X-ray film according to standard methods (Sambrook et al., 1989).

2.3 Results

2.3.1 Initial screen for *per* mutant complementation

A cDNA library was constructed as described in section 2.2.2 from the livers of clofibrate-fed rats. Clofibrate is known to induce high levels of peroxisomal mRNAs in mammalian liver cells, thus enhancing the likelihood of obtaining cDNAs for peroxisomal genes. These cDNAs were inserted into the pHIL-A1 plasmid under the control of the *AOX1* promoter. This plasmid also contains the *HIS4* gene for selection in *his4* strains of *P. pastoris*. The library was then used to transform *his4* derivatives of each of the 10 known *P. pastoris per* mutant groups: *per1* through *per8* (Liu et al., 1992) and *pas2* and *pas6* (Gould et al, 1992). His^+ transformants were selected on YND plates and then screened for methanol utilization on YNM plates.

The basis for this screen was that *per/pas* mutants of *P. pastoris* are unable to utilize methanol as a carbon source while wild-type cells can. Complementation by rat cDNAs encoding homologues of yeast *PER* genes may restore growth of *per* mutants on methanol medium as *P. pastoris PER* genes do. In addition, methanol growth can be monitored very sensitively and even partial complementation by a cDNA would be easily observed.

his4 derivatives of *per3, per4,* and *per6* were transformed with the rat cDNA library using the spheroplast-generation method (Cregg et al., 1985). Of these spheroplast transformants (see section 2.2.3), only one *per3* transformant was able to grow well on YNM plates, *per4* transformants produced many small colonies after 14 days of incubation at room temperature, and nine *per6* transformants grew slowly on YNM plates over a three week period at room temperature. However, we were unable to recover the plasmid from any of these transformants.

The *per6* transformants were screened again on YND and YNM plates grown at room temperature for four weeks. Most of the *per*6 transformants did not show appreciable growth above that of the *per*6 parent mutant on YNM plates. However, two transformants, from pool 10 and pool 13, did show some growth in comparison to the mutant.

All His^+ transformants of *per3, per4* and *per6* that showed any growth on YNM plates were inoculated into YNM broth to determine their growth rates in methanol medium. YNM containing 0.5% and 0.4% methanol was used. However, no growth in liquid media was observed.

In summary, although some His^+ spheroplast transformants of *per3, per4* and *per6* appeared to grow on YNM plates, the rate was barely above that of the parent *per* mutant and significantly less than that of the wild type. In addition, the transformants did not grow in YNM liquid cultures; therefore their growth rates could not be determined.

his4 derivatives of all 10 *per/pas* mutant groups were transformed with the rat cDNA library using electroporation (see section 2.2.3). This yielded 20,000 to 30,000 His^+ transformants of each group on YND plates. After replica plating to YNM plates, the transformants were incubated for 4 days at 30°C, followed by incubation at room temperature for four weeks with methanol feeding. After approximately two weeks, there were two clear Mut^+ transformants: one from complementation group *PER5* and one from *PER6.* After approximately four weeks, nine other Mut^+ transformants appeared on YNM plates: three *PER3* , three *PAS2*, one *PER6*, one *PER2*, and one *PAS6*.

2.3.2 Recovery of plasmids from transformants

Total DNA was extracted from His^+ Mut^+ transformants grown on YND plates and transformed into *E. coli* cells either by $CaCl_2$ or by electroporation methods (see section 2.2.9). Transformed *E. coli* cells were grown overnight at 37°C on LB/Amp plates to select for ampicillin resistance conferred by the pHIL-A1, the plasmid used to construct the rat liver cDNA library. Most yeast DNA samples did not yield ampicillin-resistant *E. coli* colonies. Restriction analysis performed on plasmid DNAs recovered from yeast DNAs that did produce ampicillin-resistant cells revealed that most plasmids did not contain a cDNA insert.

However, restriction analysis of plasmids obtained from a *per5* transformant did contain a 1.2-kb insert. The *per5* mutant was retransformed with this plasmid DNA to determine whether the plasmid actually complemented *per5*. *per5* His^+ transformants with the plasmid appeared to grow significantly on YNM plates but at a slower rate than the original *per5* transformed strain and very slow relative to wild-type *P. pastoris.* Again, no growth was observed in YNM broth. Finally, the 1.2 kb insert was partially sequenced and its putative product had strong sequence similarity to human carboxypeptidase N, a protease. No rat homologue for this protein was present in the databases. The sequence showed no similarity to the *P. pastoris* gene product that complements *per5* (PAS7) (Zhang et al., 1995).

One of the *per2* transformants contained a plasmid with a 2.9-kb insert when characterized by restriction analysis. When sequenced, the only potential open reading frame was found to be in a reverse orientation with the poly $(A)^+$ tail facing the *AOX1* promoter in pHIL-A1. Therefore, it appeared to be incapable of producing a complementing translation product. This conclusion was supported by the inability of this plasmid to restore methanol growth when retransformed into *per2* cells.

Only one of the *per3* transformants contained plasmid with insert (1.2 kb). The sequence of this insert revealed it was homologous to *RAD* 54 from *S. cerevisiae*. Retransformation of *per3* mutants with this plasmid also did not restore the ability to grow on YNM plates.

2.3.3 Plasmid loss studies

The ability to recover only a few plasmids from putative transformants suggested that the plasmids might have either integrated into the *P. pastoris* genome or been lost from cells over successive generations. To determine whether library transformants contained an autonomously replicating plasmid, plasmid loss studies were performed. If plasmid loss

studies showed the plasmid was integrated into the genome, the cDNAs could be recovered using PCR.

Individual colonies were grown in rich (YPD) liquid medium, spread on YPD plates, and then replica plated to selective media (YND). During growth on YPD, most cells will lose an autonomous plasmid. Thus, transformants that contained an autonomous plasmid will produce few His^+ colonies when replica plated to selective medium. If, on the other hand, the plasmid had stably integrated into the genome of transformed *P. pastoris* strains, the number of resulting colonies in this assay that grew on rich and selective media should be identical. The results for *per4* and *per6* transformants unfortunately did not lead to such clear-cut conclusions. Two selective media tests were used. First, growth was compared on YND (without histidine) and YND + histidine. On both of these, growth was equivalent to the growth on YPD. This would indicate that the plasmid had become integrated. Secondly, growth on YNM and YNM + histidine were compared. Growth on YNM was equivalent to growth on YNM + histidine but was markedly less than on YPD and YND plates. YPD plates showed approximately 10^3 colonies, YND plates had approximately 10^2 colonies and YNM plates showed less than 50 very slow-growing colonies even after 2 weeks of incubation at room temperature with methanol feeding. It was assumed that the plasmid had integrated but that growth on methanol represented highly stringent conditions not natural to mammalian gene products, thereby producing decreased growth rates.

One *per3* transformant appeared to contain an autonomous plasmid, but was a Mut^+ revertant (i.e., the plasmid did not contain a complementing cDNA). It produced approximately 50 colonies on YND medium but more than 100 colonies on YND + histidine, indicating that it contained a plasmid with *HIS4*. Similar results were obtained with YNM medium: virtually no colonies on YNM but more than 100 colonies on YNM + histidine. No more work was done with this transformant.

2.3.4 Southern blot analysis

Southern blot analysis was performed on selected *per*6 transformants to determine if these strains contained integrated plasmids or not and, if so, to determine the locus at which the plasmids had integrated. pHIL-A1 contains three regions that are homologous to the *P. pastoris* genome: the *HIS4* gene, the *AOX1* promoter (*AOXp*) and the *AOX1* terminator (*AOXt*). Homologous recombination could have occurred at any of these sites but was predicted to occur most frequently at the *HIS4* locus since this is the longest

sequence of the three. *AOXp* was the next longest and deemed the next most likely point of integration.

For the first blot, total DNA was digested with EcoRI and separated by size on an agarose gel, transferred to a nylon membrane and probed with the labeled and linearized pHIL-A1 plasmid. Most all transformants appearred to contain at least one copy of pHIL-A1. Integration at the *HIS4* locus was predicted to produce three bands: one of 6.0 kb representing the native *AOX1* gene, and two of approximately 5.0 kb representing the fragments containing *AOXp* and *AOXt* in the plasmid. Actual band lengths would be 5.0 kb and 4.7 kb but would appear as one on the gel. Integration at the *AOXp* locus was predicted to produce bands of 1.8 kb, 3.5 kb and 10.2 kb, representing DNA fragments containing AOX_p from the plasmid, the *HIS4*, genomic locus, and a plasmid segment containing both the *HIS4* and the *AOXp*, respectively. Integration at *AOXt* should produce bands of 1.9 kb, 3.5 kb and 10.2 kb. The actual bands observed on blots were estimated to be 1.7 kb, 3.5 kb, and 6.5 kb. The 3.5-kb band represented the native *HIS4* locus if integration occurred at either the *AOXp* or the *AOXt* site. The 1.7-kb band could represent either the fragment containing the *AOXp* (if integration was at *AOXp*) or the *AOXt* (if integration was at *AOXt*).

The EcoRI-digested genomic DNAs were also probed with the *HIS4* gene to confirm that the 3.5-kb band was *HIS4*. Integration at either the *AOXp* locus or the *AOXt* locus was expected to produce two bands at 3.5 kb and 10.2 kb. Surprisingly, three bands were observed of approximately 3.0 kb, 3.5 kb and >6.1 kb. The 3.0-kb band was determined to be the wild-type *HIS4* locus since it was the only one that was present in both the wild-type and untransformed *per6* control DNAs. The identities of the other two bands are unknown.

Additional blots using DNAs digested with other restriction enzymes were also inconclusive with regard to whether the plasmids had integrated at *AOXt* or *AOXp*.

In summary, Southern blot analysis determined that most strains contained vector sequences that were not integrated at *HIS4*. However, the patterns were not consistent with predictions for integration at either *AOXp* or *AOXt*. Although it is possible that the vectors integrated at an unknown site in the *P. pastoris* genome, it is more likely that the vectors are integrated at the *AOX* locus but our knowledge of the restriction site positions was not sufficient to make accurate predictions of the expected Southern blot pattern. Finally, based on the intensity of the hybridization signal, one transformant probably contained multiple copies of the vector.

2.3.5 Recovery of cDNAs from transformed yeast strains by PCR amplification

PCR was used to recover cDNAs from the genomes of *per* mutants transformed with the cDNA library. The amplified fragments were then to be ligated into pBS for sequencing and then into pHIL-A1 and used to retransform *per* mutants. Three sets of *AOX1* primers (AOX5' + AOX3', AOX5' + AOXT-1 and AOX5' + AOXT-2) and one set of pHIL-A1 primers (5'pHIL-A1 + 3'pHIL-A1) were used under selected PCR conditions (section 2.2.7). The AOX5' and AOX3' primer combination did amplify a DNA fragment. When sequenced, this fragment was discovered to be the *AOX1* promoter sequence. However, no insert was amplified from any of the *per* transformants.

2.4 Discussion and Conclusions

Attempts to clone a rat liver cDNA that complements one of the ten known *P. pastoris per* mutants were not successful. Although initial results appeared promising in that His^+ transformants of several *per* mutants appeared to grow on methanol (albeit at a very slow rate), experiments designed to recover a putative complementing vector failed. When genomic DNA samples of putative His^+ Mut^+ transformants were transformed into *E. coli* , most frequently no plasmid was recovered. In the few cases where a plasmid was recovered, it contained no cDNA insert or the predicted product of the cDNA was not related to the yeast *PER* product. Finally, none of the recovered plasmids was able to retransform the original *per* mutant to Mut^+.

Attempts to recover cDNA inserts by PCR were also unsuccessful. Although the AOX5' and AOX3' primer combination could amplify the *AOX1* promoter in untransformed cells, they did not yield amplified cDNAs from transformed cells.

From these experiments, it appears that rat *PER* genes may not be able to functionally complement *P. pastoris per* mutants for growth on methanol. One possible variation of this functional complementation scheme that was not tried is to select for growth of *per* mutant transformants on oleic acid instead of methanol. A recent report showed that a fusion gene composed of approximately half of the *P. pastoris PAS8* gene and *PTS1R*, the human homologue of *PAS8*, was able to complement a *P. pastoris pas8* mutant for growth on oleate but not methanol (Dodt et al., 1995). Perhaps, for an unknown reason, partial restoration of peroxisome function is sufficient for β-oxidation pathway function but not for methanol-utilization pathway function.

The inability to directly clone mammalian *PER* cDNAs by functional complementation of yeast *per* mutants is disappointing since it would have provided an inexpensive and rapid means of isolating the genes responsible for Zellweger syndrome, a lethal peroxisomal biogenesis disorder. However, knowledge of yeast *PER/PAS* genes will still be an important tool for identifying human Zellweger genes and understanding their role in peroxisome biogenesis. Specifically, comparison of yeast *PER* products with those in the large and growing human cDNA database is resulting in the identification of human *PER* cDNA candidates.

Chapter Three

Investigations into human *PAF-1* homology with *Pichia pastoris PER6*

3.1 Introduction

The *PER6* gene encodes a 52-kD integral membrane protein (Per6p) that is predicted to span the membrane three times (Waterham et al., 1996). The protein contains a carboxy terminus approximately 100 amino acids long that is rich in acidic amino acids and is preceded by a cysteine-rich C_3HC_4 -type domain. On the basis of amino acid sequence similarity (32% identity, 49% similarity) and overall structural similarity (location of predicted membrane-spanning regions and C_3HC_4 motifs), Per6p is a strong candidate for the *P. pastoris* homologue of the human Zellweger gene product PAF-1 (Waterham et al., 1996).

The human PAF-1 protein is a 35-kD integral membrane protein (Shimozawa et al., 1992). Rat, mouse, and Chinese hamster homologues have also been cloned and characterized (Tsukamoto et al. 1991, Allen et al., 1994, Tsukamoto et al., 1994b, Thieringer et al., 1993, Wilson et al., 1994). All of the mammalian PAF-1s are very similar in sequence and structure including the presence and location of two putative membrane-spanning regions followed by a C_3HC_4 motif. Shimozawa identified two mutations in *PAF-1* in cell lines from one Zellweger syndrome complementation group (Shimozawa et al., 1992). One mutation produces a truncated protein lacking membrane-spanning and C_3HC_4 regions. The other mutation results in a substitution of threonine for one of the cysteines in the C_3HC_4 region. This suggests that the C_3HC_4 domain is essential for PAF-1 function. However, deletion studies indicated that the N-terminal portion of the protein, extending through the second membrane-spanning region, is essential for function, while the C_3HC_4 cysteine-rich region is not (Tsukamoto et al., 1994b).

The similarity between Per6p of *P. pastoris* and human PAF-1 is closest in the C_3HC_4 motif and membrane-spanning regions, although there is significant similarity

throughout most of the two proteins. However, there are distinct differences between the two proteins. PAF-1 does not contain an acid tail region which is present in Per6p. The N-terminus of Per6p also contains an additional potential membrane-spanning domain.

The purpose of this project is to determine if there is functional similarity as well as sequence similarity between the two proteins. This will be done by expressing the human *PAF-1* gene in a *P. pastoris per*6 mutant and determining whether the human PAF-1 can complement for the missing Per6p and restore functional peroxisomes. Toward this end, a human *PAF-1* cDNA was amplified by PCR and used to construct plasmids that express PAF-1 or selected fusions between PAF-1 and Per6p, or Per6p with portions deleted. Due to their peroxisome biogenesis defect, *per6* mutants cannot grow on media containing either methanol or oleate as the sole carbon source. Thus, as a first step in determining whether these constructs restore peroxisome biogenesis, we will determine the ability of the transformed *per6* mutants to grow on either of these carbon sources.

3.2 Methods and Materials

Table 3.1 *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
JC116	*per6 his4*	Waterham et al., 1995
***E. coli* strains**	**Genotype**	**Source or reference**
DH5α	F^-, ϕ80d*lac*ZΔM15, *end*A1, *rec*A1, *hsd*R17 (r_K^-, m_K^-), *sup*E44, *thi*-1, *gyr*A96, *rel*A1, Δ(*lac*ZYA-*arg*F), U169, λ^-	Promega, Madison, WI.
TB1	*araΔ(lac proAB) rpsL (ϕ80 lac*ZΔM15) *hsdR*	Johnston et al., 1986

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

3.2.1 Media and strains

P. pastoris and *Escherichia coli* strains used are listed in Table 3.1. Culturing media and conditions were as described in section 2.2.1. YNO medium was 0.1% oleic acid with 0.5% Tween 40 to solubilize oleate.

3.2.2 cDNA library

A HepG2 human hepatoma cDNA library was a gift from Chiron Corporation (Emeryville, CA). The library was constructed in the *S. cerevisiae-E. coli* shuttle vector pAB23-BXN. cDNA was inserted into the BstXI and NotI restriction sites of the vector. The library was transformed into and amplified in *E. coli* strain MC1061.

3.2.3 PCR amplifications

The polymerase chain reaction (PCR) was used to amplify the *PAF-1* gene from the human cDNA library described above, to screen total DNA extractions from transformed strains for the presence of a *PAF-1* cDNA clone, and to amplify portions of the *PER6* gene from the pHWO10/PER6 plasmid. Vent polymerase was used according to the protocol provided by the supplier (New England Biolabs, Beverly, MA). Reactions in a volume of 50 µl were overlain with 2.0 µl light mineral oil (Aldrich, Milwaukee, WI) to prevent evaporation. The reaction templates were either 50 ng of genomic DNA or 5 ng of plasmid (library) DNA (uncut and double stranded). 250 µM of each dNTP and 1 µM of each primer were added to the reactions which were carried out in the vent buffer provided by the polymerase supplier. The reactions were performed in a SingleBlock™ System thermocycler (Ericomp, Inc., San Diego, CA) using the following program: cycle 1, 94°C for 90 seconds; cycles 2-6, 94°C for 60 seconds, 45°C for 30 seconds, 72°C for 60 seconds; cycles 7-46, 94°C for 60 seconds, 60°C for 30 seconds, 72°C for 60 seconds; cycle 47, 72°C for 5 minutes. Amplified DNAs were extracted with PCA (25:24:1 ratio of phenol: chloroform: isoamyl alcohol), precipitated with 0.7 volume of 100% isopropanol and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.0) or water. Primers for *PAF-1* amplification were derived from sequences identical to the 5' end of the gene and complementary to the 3' end. Nucleotides encoding an EcoRI restriction site were added to the 5' ends of the primers to facilitate ligation of the PCR product into the EcoRI site of expression vectors. The 5' primer (5'HPAF-1) sequence was: ACCGAATTCATGGCTTCCAGAAAAGAG. The 3' primer (3'HPAF-1) sequence was CCCGAATTCCTAAAGGACATTCACTTC and contained one nucleotide change from the human *PAF-1* sequence that did not change the amino acid sequence.

A portion of *PER6* (between nucleotides 561 and 1504) and was amplified by PCR from the plasmid pHWO10/PER6 (see Figure 3.1 and section 3.2.4). Reaction components were as described above. The thermocycler program settings were: cycle 1, 94˚C for 90 seconds; cycles 2-41, 94˚C for 60 seconds, 55˚C for 30 seconds and 72˚C for 90 seconds; cycle 42, 72˚C for 5 minutes. The sense strand primer (PER6-1) was designed to add a BamHI restriction site followed by the gene sequence beginning at nucleotide number 561 (all positions are calculated from the ATG start): CGGGATCCATGCCTCTGAGTTAAAGCTCTTACTGGAAC. The anti-sense strand primer (3'P6T-2) was designed to introduce a translational stop codon (TAG), an AgeI site and a BamHI site, after nt 1504 of the *PER6* gene. Its sequence was: ATGGATCCTTACCGGTCTACAACGCGTCTTCCACATC.

All oligonucleotides were synthesized by the Center for Gene Research, Oregon State University, Corvallis, Oregon.

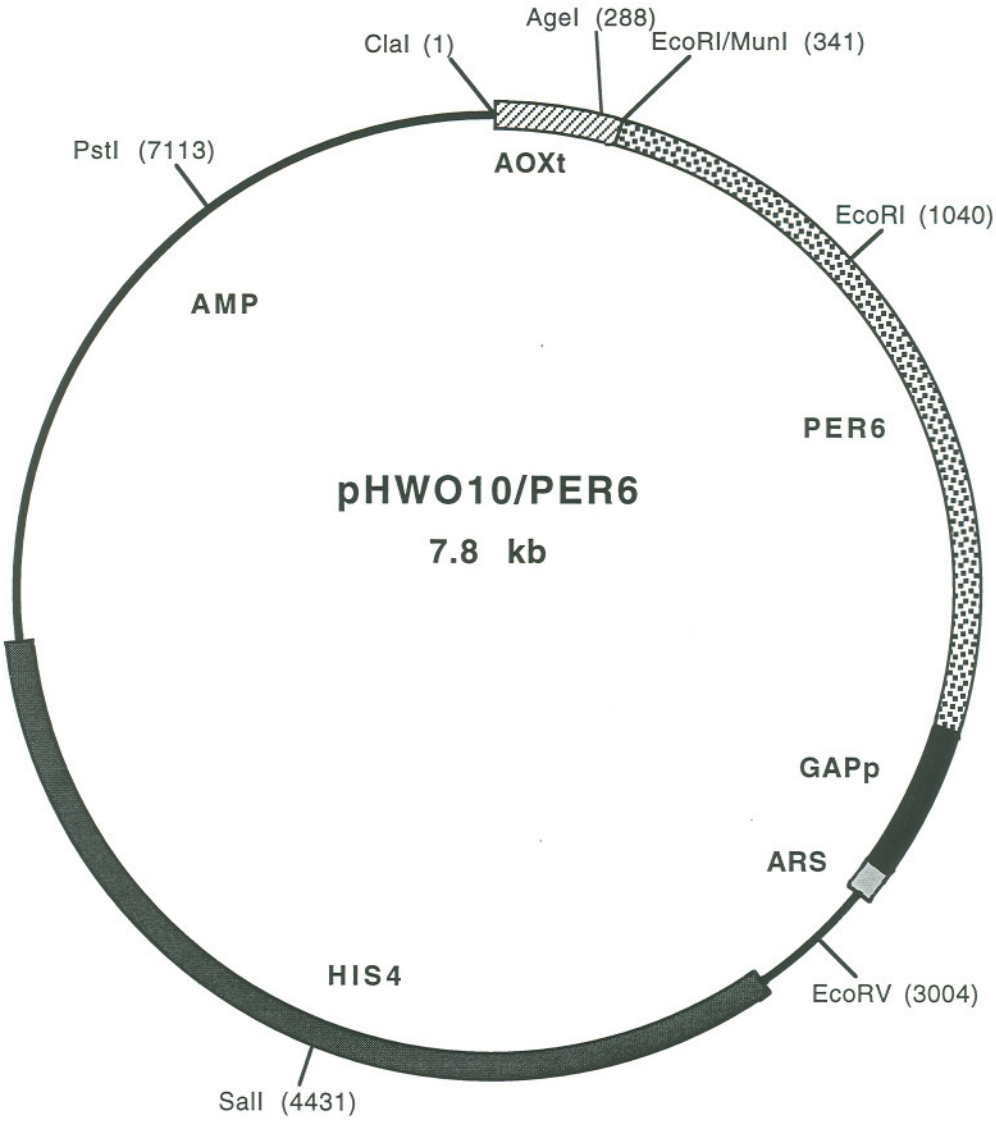

Figure 3.1. The pHWO10/PER6 plasmid. This plasmid was derived from the pHWO10 plasmid (Figure 3.3) and contains the *PER6* cDNA under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter (GAP_p) (See section 3.2.4).

3.2.4 Expression vectors

Three vectors were used in this study. PCR products were initially ligated into pBluescript II SK +/- (pBS, Stratagene, San Diego, CA). DNA fragments were then moved into either pHIL-A1 (Invitrogen, San Diego, CA) or pHWO10. To construct the pHWO10 plasmid, a DNA fragment containing the glyceraldehyde-3-phosphate dehydrogenase promoter (*GAPp*) was amplified by PCR. Primers were designed to include the native BamHI site on the 5' end of the promoter and an introduced EcoRI site on the 3' end, to facilitate insertion of the fragment into the pHIL-A1 plasmid. The pHIL-A1 plasmid was prepared to receive the *GAPp* fragment by digestion with BglII and EcoRI, thus releasing a fragment containing the *AOXp* and a portion of the ARS. This fragment was replaced with the *GAPp* fragment. This construction retained the EcoRI site but destroyed the BamHI/BglII site. Both pHIL-A1 and pHWO10 are shuttle vectors derived from pBR322 containing the ampicillin-resistance gene for selection in *E. coli* and the *P. pastoris HIS4* gene to facilitate selection in *P. pastoris* (Figures 3.2 and 3.3).

3.2.5 Oligonucleotide adaptor

Construction of a hybrid gene encoding PAF-1 fused with the Per6p acid tail (PAFAT) required an adaptor composed of two oligonucleotides: PAFP6-1 and PAFP6-2. The sequence of PAFP6-1 was: GGATGTGGAAGACGCGTTG. The PAFP6-2 sequence was: GATCCAACGCGTCTTCCACATCCTGCA. Annealing of these two produced the PAFP6 adaptor (Figure 3.7).

3.2.6 RNA (northern blot) hybridizations

P. pastoris cultures were grown overnight at 30°C in 10 ml YPD. Cells were harvested by centrifugation and resuspended in YNB (no carbon source) to OD_{600} of 10. This suspension was used to inoculate 20 ml of YNM and YNO to an OD_{600} of 0.8. YND cultures were inoculated to OD_{600} of 0.1. These cultures were incubated at 30°C for 6 hours and harvested by centrifugation. The resulting pellets were resuspended in 2 ml ice-cold RNA buffer (0.5 M NaCl, 200 mM, 10 mM EDTA, Tris-HCl, pH 7.5) and transferred to a cold minicentrifuge tube and kept on ice. Cells were briefly centrifuged at 4°C and the pellet was resuspended in 300 µl RNA buffer. These suspensions were quick-frozen in liquid nitrogen and stored at -70°C prior to RNA extraction. To extract RNA, cells were disrupted by adding 200 µl of acid-washed chilled glass beads and 300 µl PCA (RNA buffer-equilibrated) and vortexing the mixture for 2 minutes on the highest setting followed by centrifugation in a minicentrifuge on the highest setting for 1 minute at

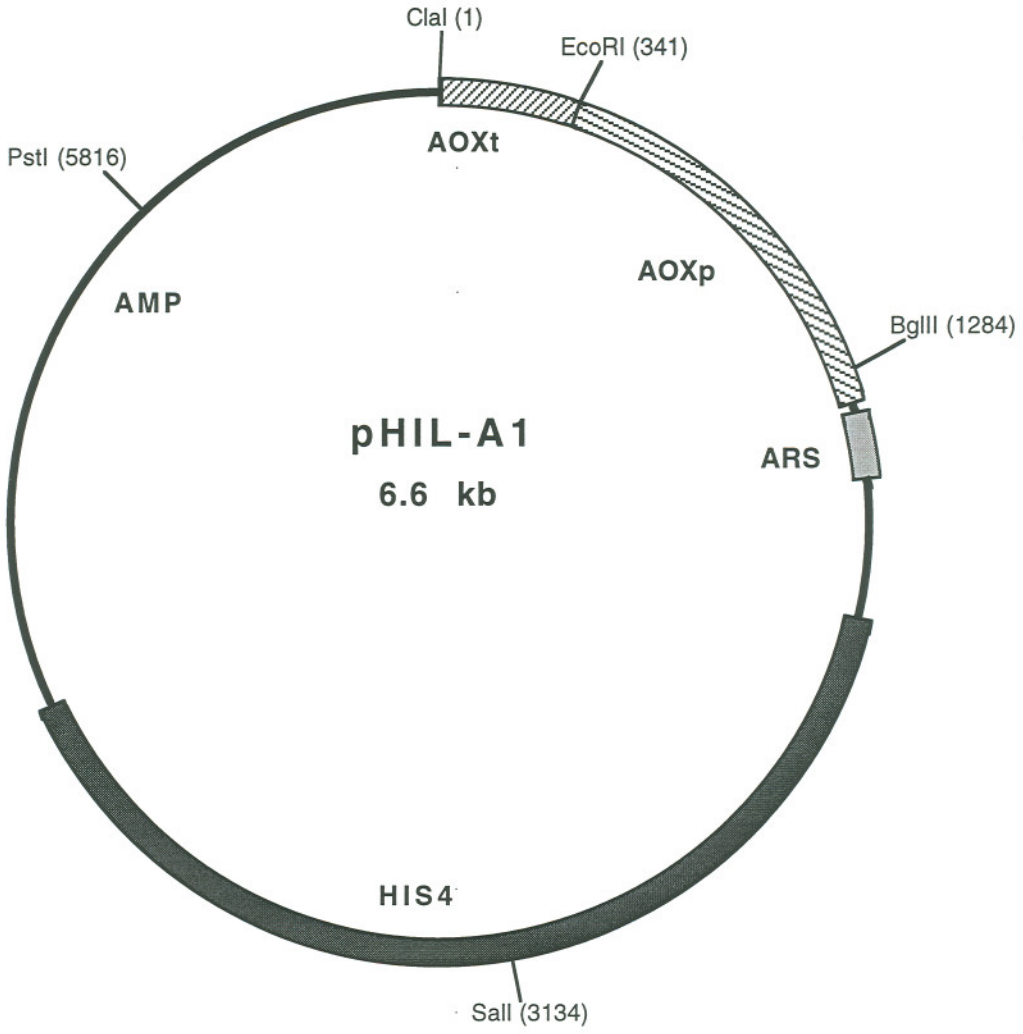

Figure 3.2. The pHIL-A1 plasmid. pHIL-A1 is a *P. pastoris -E. coli* shuttle vector derived from pBR322 and containing both an ampicillin resistance gene and the *P. pastoris HIS4* gene. The plasmid is designed for expression of foreign genes under the control of the methanol-regulated alcohol oxidase I promoter (AOX_p).

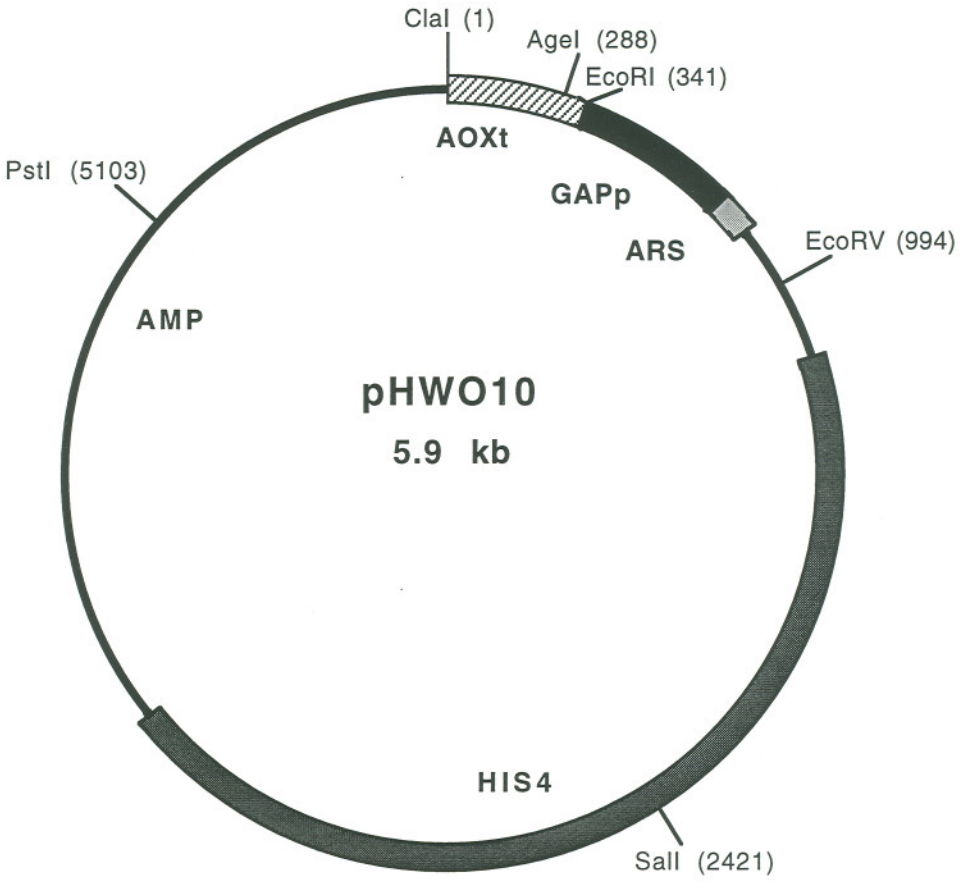

Figure 3.3 The pHWO10 plasmid. This plasmid is derived from the pHIL-A1 plasmid. The glyceraldehyde-3-phosphate dehydrogenase gene promoter (GAP_p) replaces the AOX_p and a portion of the ARS.

room temperature. The aqueous layer was transferred to a new tube and subjected to PCA extraction (using RNA buffer equilibrated PCA). RNA was precipitated by adding 750 µl of ice-cold 100% ethanol and placing the mixture at -20°C for approximately 30 minutes followed by centrifugation at 4°C on the highest setting. The pellet was washed by centrifugation in 70% ice-cold ethanol, dried, and resuspended in 20 µl water treated with DEPC (0.2 % diethyl pyrocarbonate).

RNA samples were separated by size on 1.5% agarose gels that contained 18% formaldehyde prepared in E buffer (0.02 M MOPS, 5 mM NaOAc, 5 mM EDTA, pH 7.0). Capillary transfer of RNA to nylon membranes was performed overnight using 10 × SSPE buffer (0.01 M Na_2EDTA, 1.5 M NaCl, 0.1 M NaH_2PO_4) as the transfer buffer. The prehybridization mix for RNA-DNA (northern) filter hybridizations contained 50% formamide, 1 × P (see section 2.2.6 for composition), 1 M NaCl, and 0.1 mg/ml denatured, sheared salmon sperm DNA. Filters were preincubated in heat-sealed bags with the prehybridization mix overnight at 42°C before addition of the radioactive probe.

DNA probes were made using the Amersham multiprime DNA labeling system kit (Amersham International, Amersham, UK) as described in section 2.2.6.

3.2.7 *E. coli* and *P. pastoris* transformation procedures

*P. pastoris per*6 competent cells were prepared according to the electroporation protocol provided by the manufacturer of the Electro Cell Manipulator™ 600 (BTX, Inc., San Diego, CA). Cells were grown at 30°C to an OD_{600} of 1.0 to 1.5 in 500 ml YPD medium and harvested by centrifugation. The pellet was resuspended in 100 ml YPD medium with 2.0 ml sterile 1M HEPES, pH 8.0. Sterile 1 M DTT (2.5 ml) was added, the mixture gently swirled and then incubated in a 30°C shaker for 15 minutes. The volume was increased to 500 ml with sterile water and then centrifuged at 4,000 × g for 5 minutes. Three successive washes followed. For the first wash, the pellet was resuspended in 500 ml of cold sterile H_2O and centrifuged at 5,000 × g for 5 minutes. The pellet was resuspended in 250 ml of sterile water and centrifuged as before. This pellet was resuspended in 20 ml of water and centrifuged again. This final pellet was resuspended in 0.5 ml of sterile, cold 1 M sorbitol. 40-µl aliquots were quick-frozen in liquid nitrogen and stored at -70 °C until use.

Prior to electroporation, plasmids (pHIL-A1 derivatives and pHWO10 derivatives) were linearized by digestion with SalI restriction enzyme which cuts these plasmids once in the *HIS4* gene.

For electroporation transformation, approximately 100 ng of plasmid DNA in less than 5 µl of TE buffer or water was added to a thawed 40-µl aliquot of prepared cells held on ice. The entire mixture was added to a BTX disposable cuvette P/N 620 (2 mm gap), inserted into the electroporation chamber and subjected to a 5-msec pulse using the electroporator. Immediately following electroporation, 1 ml cold 1 M sorbitol was added to the cuvette and gently mixed with the yeast cells. The mixture was then spread on YND plates and incubated at 30°C.

3.2.8 Plasmid constructions

3.2.8.1 Cloning of PCR-amplified *PAF-1*

The human *PAF-1* PCR product was purified from agarose gel using the Qiaex II gel extraction kit (Qiagen, Chatsworth, CA). It was then digested with EcoRI, PCA extracted, brought to 0.3 M NaOAc (pH 5.0) and precipitated by addition of a 0.7 volume of 100% isopropanol. Following a 15- to 20- minute centrifugation, the resulting pellet was washed with 70% ethanol, dried and resuspended in water or TE buffer.

A sample of PCR-amplified and EcoRI-digested *PAF-1* was cloned into three vectors: pBS, pHIL-A1 and pHWO10. Prior to ligation, each plasmid was digested with EcoRI and dephosphorylated with calf intestine alkaline phosphatase. Following PCA extraction and isopropanol precipitation these vectors were combined with T4 ligase, ligase buffer, and EcoRI-digested *PAF-1* and incubated overnight at room temperature (Sambrook et al., 1989). The following day, the ligation mixtures were transformed into DH5α cells. Ampicillin-resistant transformants were screened by restriction analysis for the presence and orientation of the *PAF-1* fragment in each plasmid. One of each type of correctly constructed plasmid was selected and a large stock prepared using the Qiagen Plasmid Prep kit (Qiagen, Chatsworth, CA). Stocks of pBS containing *PAF-1* in both orientations, pHIL-A1 and pHWO10 containing *PAF-1* in the correct orientation relative to the promoter were frozen at -20°C for use in subsequent experiments. These *PAF-1*-containing plasmids are diagrammed in Figures 3.4, 3.5 and 3.6.

3.2.8.2 Construction of the *PAF-1/PER6* hybrid gene

A hybrid gene was constructed that was designed to express most of PAF-1 followed by the acidic amino acid-rich C-terminus of Per6p. An oligonucleotide adaptor was designed to join *PAF-1* and *PER6* fragments (Figure 3.7). The 5' terminus of the adaptor was designed to be compatible with the PstI restriction site near the 3' terminus of

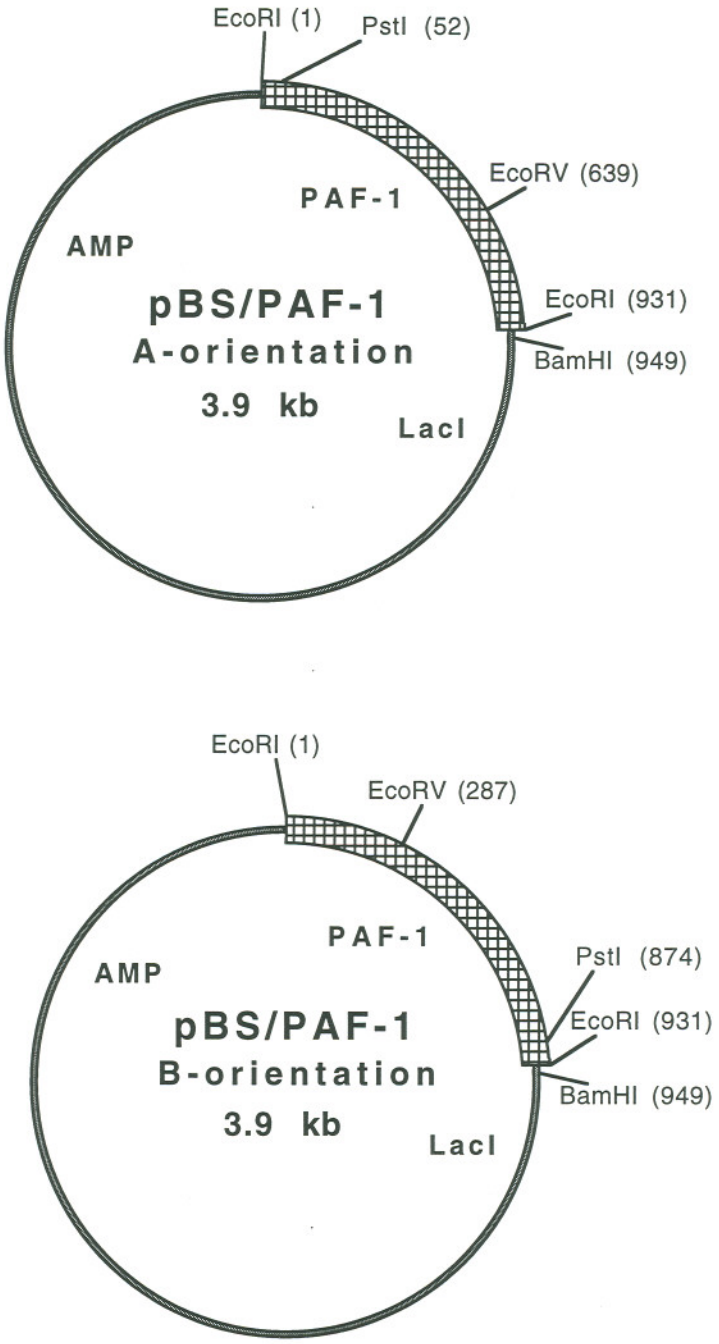

Figure 3.4. Human *PAF-1* cDNA in pBS. Human *PAF-1* cDNA was inserted into the pBS plasmid and maintained in two orientations relative to the *LacI* gene. Orientation B was used to construct the PAFAT hybrid (section 3.2.8.2).

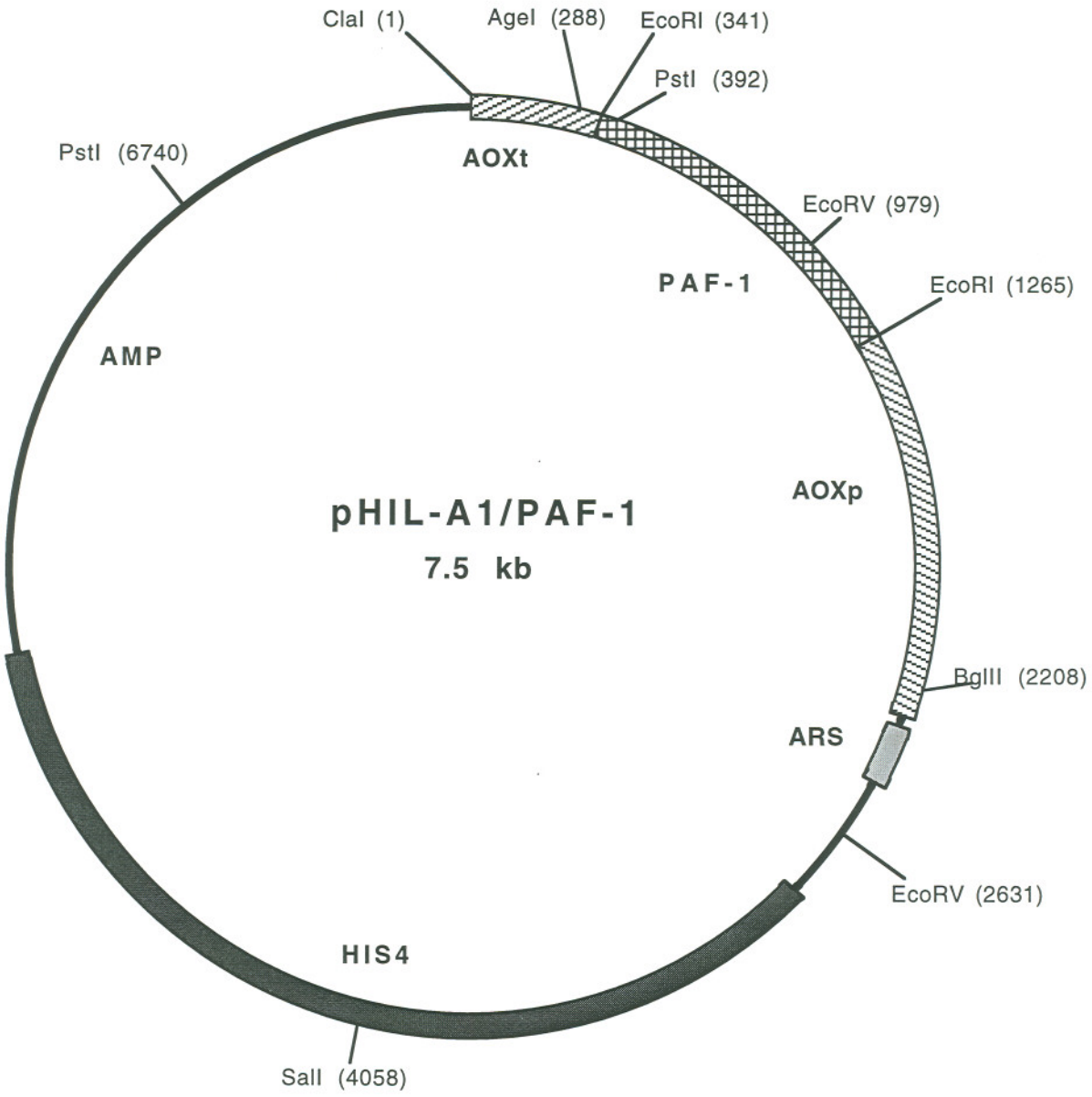

Figure 3.5. Human *PAF-1* cDNA in pHIL-A1. Human *PAF-1* cDNA was inserted into the pHIL-A1 plasmid (figure 3.2) under the control of the methanol-regulated *P. pastoris* alcohol oxidase I promoter (AOX_p).

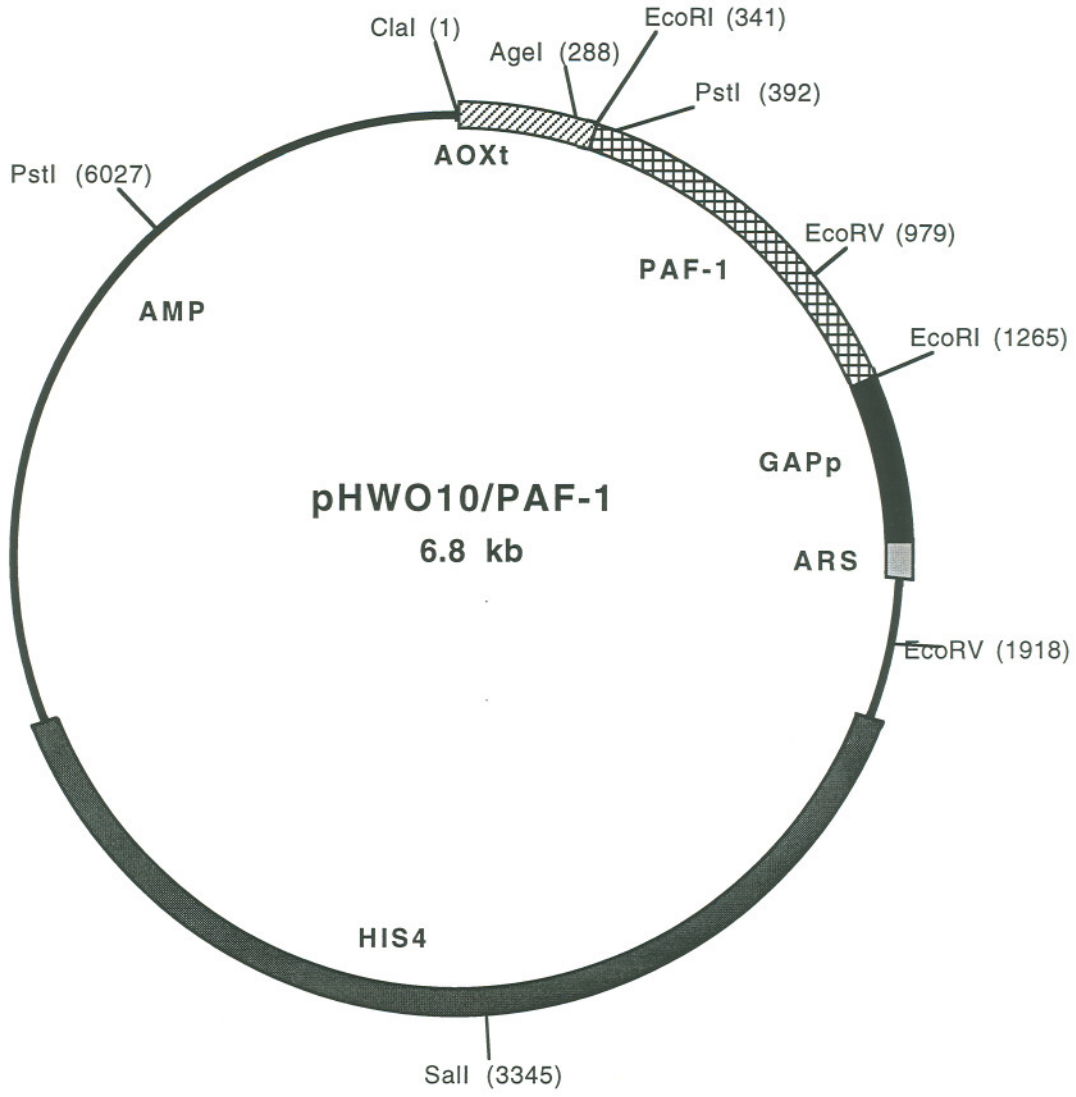

Figure 3.6. Human *PAF-1* cDNA in pHWO10. Human *PAF-1* cDNAwas inserted into the pHWO10 plasmid (figure 3.3) under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter (GAP_p).

PAF-1 (nt 872 from the ATG). Following this sequence, the oligonucleotide encoded four amino acids from the Per6p sequence (three of which are acidic) adjacent to a MluI restriction site. This MluI site was positioned to be in-frame with a MluI site near the 5' end of the *PER6* 3' region that encodes the acidic amino acid C-terminal domain. In order to facilitate ligation into an intermediate vector, a BamHI site was added to the 3' end of the adaptor.

A pBS vector containing *PAF-1* was digested with both PstI and BamHI restriction enzymes. The adaptor was ligated into this site, producing an intermediate vector containing the *PAF-1* gene followed by the adaptor. The PAF-1/adaptor fragment was removed from the intermediate vector by digestion with EcoRI and MluI and isolated from an agarose gel. To receive this fragment, the pHWO10 vector containing *PER6* was digested with EcoRI and MluI and the fragments separated by size on a 0.8% agarose gel by electrophoresis. The fragment containing the plasmid and sequences encoding acidic domain was purified from an agarose gel and ligated in the presence of the *PAF-1*/adaptor fragment. The final result was a pHWO10 vector capable of expressing human PAF-1 fused to the acid tail of Per6p (pHWO10/PAFAT, Figure 3.9). To verify the DNA sequences of the junctions in the pHWO10/PAFAT plasmid, the AOX 3', 5'PAF-1D and pGAP-1 (TCCTGACCCAAAGAC) primers were used for the sequencing reactions performed by the Center for Gene Research, Oregon State University, Corvallis, OR.

3.2.8.3 Construction of a *PER6* gene with sequences encoding the carboxy-terminal region deleted

PCR was used to amplify a region of *PER6* beginning with a BamHI site at nt 163 (A of ATG equals nt 1), and extending to the MluI site in *PER6* at nt 1099 . The PCR antisense primer included the MluI site followed by an in-frame stop codon (TAG), an AgeI site and a BamHI site. The PCR product was cut with BamHI, purified from 0.8% agarose gel and ligated into BamHI-digested pBS. This construct was then digested with BglII and AgeI, producing a fragment that encoded the C_3HC_4 region followed by the PCR- introduced stop codon. This fragment was ligated into the pHWO10/PER6 vector from which most of the *PER6* gene had been removed by digestion with BglII and AgeI (from the BglII site at nt 847 in *PER6* to the AgeI site in the *AOX* terminator region). The product of this fusion was a pHWO10 plasmid that expressed a PER6 product with the 81 carboxy-terminal amino acids deleted (see Figure 3.9). The sequences at the junctions in the pHWO10/TP6 plasmid were determined using the 3'AOX and pGAP-1 primers.

Figure 3.7. The adaptor used to construct the *PAF-AT* hybrid. The adaptor used to construct the *PAF-AT* hybrid between human *PAF-1* cDNA and the acid-tail-encoding region of the *P. pastoris PER 6* gene. Restriction site sequences are shown with arrows indicating cleavage sites. The 3-sided boxes indicate regions designed to match with ends of PstI/BamHI-digested pBS.

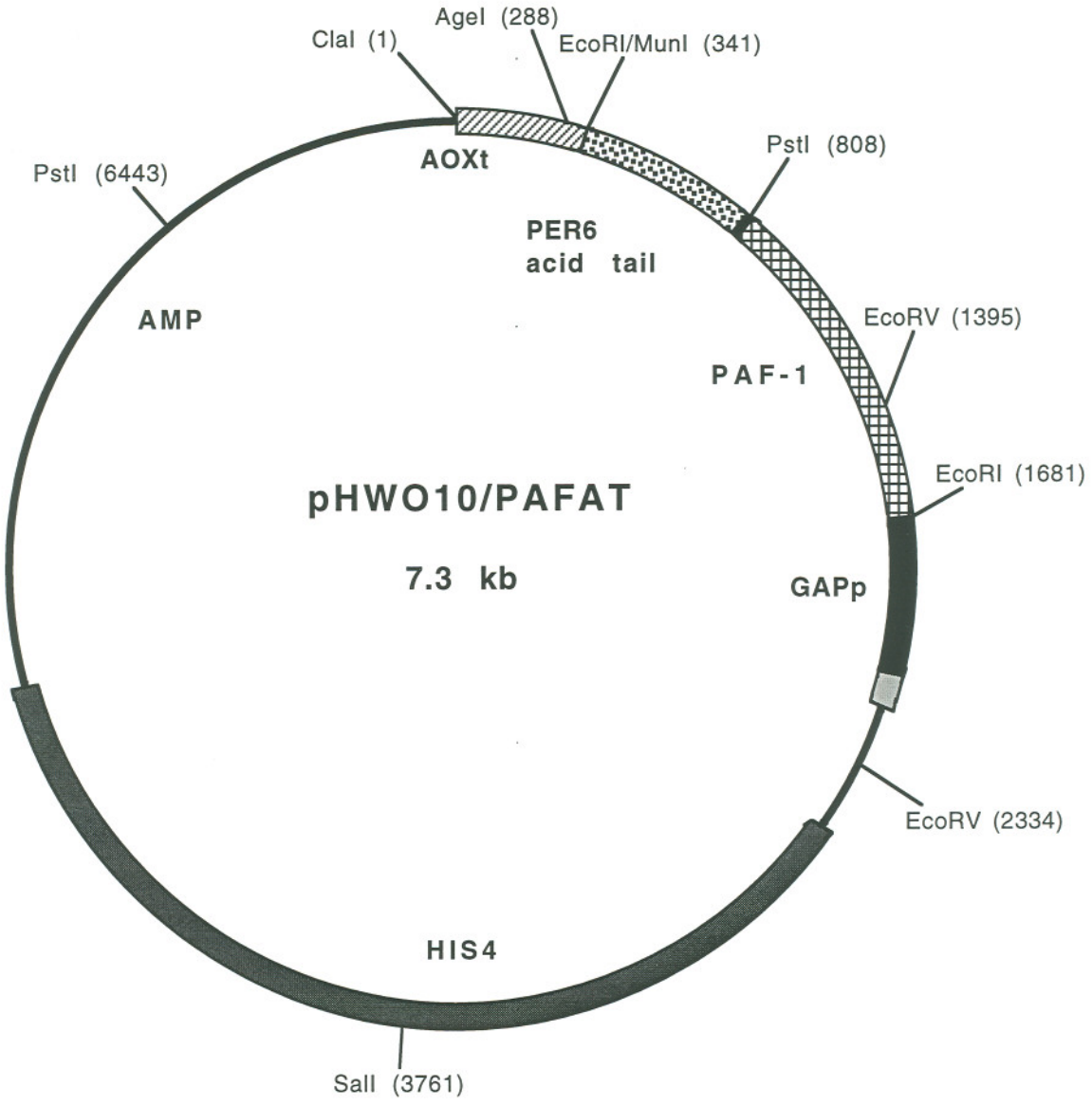

Figure 3.8. *PAF-AT* hybrid gene in pHWO10. After fusing human *PAF-1* cDNA with the acid-tail-encoding sequences of *P. pastoris PER6* (creating the "*PAFAT*" hybrid gene), *PAFAT* was inserted into the pHWO10 plasmid under the control of GAP_p .

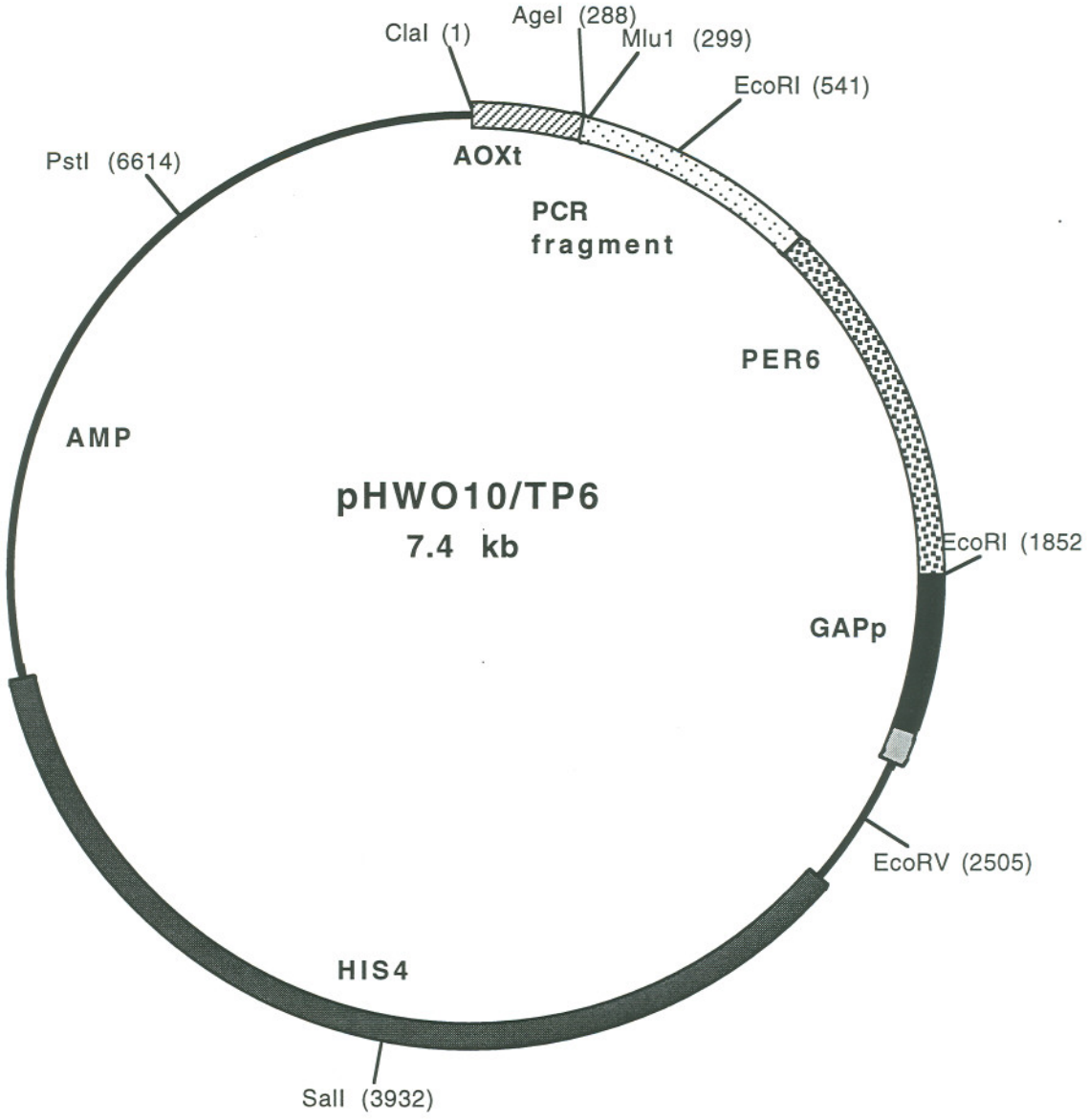

Figure 3.9. *TP6* in pHWO10. After deleting the acid-tail-encoding sequences of the *P. pastoris PER6* gene, this "truncated" version of *PER6* was inserted into the pHWO10 plasmid under the control of the GAP_p.

Sequencing reactions were performed by the Center for Gene Research, Oregon State University, Corvallis, OR.

3.2.9 DNA sequencing of the PCR-amplified *PAF-1* gene

The entire sequence of the *PAF-1* gene obtained by PCR and cloned into the pHIL-A1/PAF-1 plasmid was determined. Ten primers were designed to produce overlapping DNA sequence data from both DNA strands. These primers were: AOX5' (Invitrogen, San Diego, CA), which primes in the *AOX* promoter region of pHIL-A1; AOX3' (Invitrogen, San Diego, CA) which primes in the *AOX* terminator region of pHIL-A1; 5'PAF-1A, ATTCACCATCTACTCCA; 5'PAF-1B, GGAAAGTCAAGCAGTGT; 5'PAF-1C, TTCTCTTACCACTTATC; 5'PAF-1D, TAGTTTCTTATTTGACG; 3'PAF-1E, ATCCATGAAAGCACTGA; 3'PAF-1F, GTACAAACAGCATACCA; 3'PAF-1G, AAATACAGAATGAATAC; and 3'PAF-1H, CACTGGTGGCTAATGTA. AOX5' and those primers named with 5'PAF primed extensions of the sense strand. AOX3' and those primers named 3'PAF primed extensions of the antisense strand. Sequencing reactions were performed by the Center for Gene Research at Oregon State University.

3.2.10 Miscellaneous methods

P. pastoris genomic DNA extractions were performed as described in section 2.2.5.

Gel electrophoresis and DNA purification from those gels were performed as described in section 2.2.8.

Frozen glycerol stocks (100 µl) of $CaCl_2$-competent *E. coli* cells (DH5α) were prepared and transformed by heat shock as described in section 2.2.9.

3.3 Results

3.3.1 Cloning the human *PAF-1* gene by PCR amplification

The goal of these studies is to determine if the human *PAF-1* gene can functionally complement a *P. pastoris* mutant defective in *PER6*, the putative yeast homologue of *PAF-1*. As a first step, a DNA fragment encoding the human *PAF-1* gene was amplified by PCR from a cDNA library created from a human hepatoma cell line. The primers for the PCR reaction included an EcoRI restriction site to facilitate insertion of the PCR product into EcoRI sites of the plasmid vectors. The PCR reaction generated a DNA fragment approximately 900 bp long which was the expected size of *PAF-1* (Shimozawa et al., 1992).

Samples of the PCR product were ligated into three vectors: (1) pBS, a standard *E. coli* plasmid; (2) pHIL-A1, a *P. pastoris-E. coli* shuttle vector that expresses foreign genes under the control of the methanol-regulated alcohol oxidase I gene promoter (AOX_p) and (3) pHWO10, a *P. pastoris-E. coli* shuttle vector that expresses foreign genes under control of the constitutive glyceraldehyde-3-phosphate dehydrogenase gene promoter (GAP_p). The presence and orientation of the *PAF-1* fragment in each vector was confirmed by restriction analysis and by the DNA sequence of the vector-*PAF-1* gene junctions.

3.3.2 *PAF-1* PCR product did not complement *per6*

The pHIL-A1/PAF-1 and pHWO10/PAF-1 vectors (Figures 3.5 and 3.6) were transformed into *per6 P. pastoris* cells by electroporation and ten individual transformants were tested for ability to grow on methanol along with control *per6* strains that had been transformed with pHIL-A1 only and a *per6* strain transformed with a plasmid containing the wild-type *PER6* gene. Whereas six of the ten samples containing the wild-type *PER6* gene grew on YNM plates, none of the pHIL-A1 transformed strains grew. None of the pHIL-A1/PAF-1 or pHWO10/PAF-1 transformants grew on YNM plates.

The pHIL-A1/PAF-1 and pHWO10/PAF-1 transformed strains were also screened for their ability to grow in oleate (YNO) liquid medium. As shown in Figure 3.11, none of the transformants grew at a rate greater than the untransformed *per 6* mutant strain.

Northern blots of RNA from methanol-, oleate- and glucose-induced cells of selected pHIL-A1/PAF-1 and pHWO10/PAF-1 transformants were performed to ascertain if a *PAF-1* transcript was produced. A transcript of the expected size was indeed produced in methanol medium with a pHIL-A1/PAF-1 transformant.

In summary, although a *PAF-1* transcript was produced by the pHIL-A1/PAF-1 transformants, that transcript did not complement the *per6* mutation.

3.3.3 Sequencing of the *PAF-1* PCR product

The PCR reaction is known to introduce errors during amplification at a significant rate (approximately 1 per 10^4 bases amplified). Therefore, we determined the DNA sequence of the PCR-amplified *PAF-1* gene to examine it for possible sequence errors.

The *PAF-1* sequence revealed two single nucleotide deletions. The first deletion was at position 263 resulting in a frame shift that introduced an early stop codon that would produce an 80-amino-acid polypeptide rather than the wild-type 305-amino-acid polypeptide. A western blot of a fusion between PAF-1 and maltose binding protein produced a protein that was approximately the size expected for this frame shift mutation.

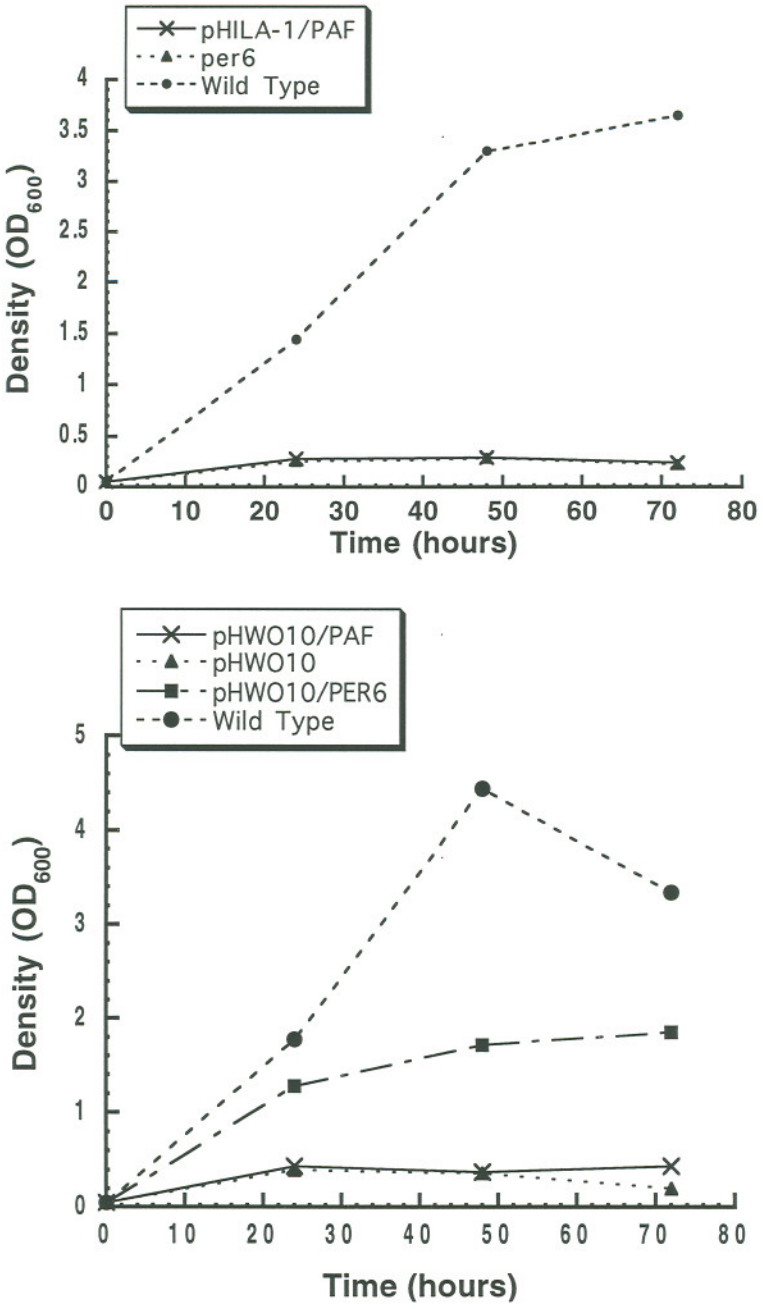

Figure 3.10. *PAF-1* transformant growth rates in oleate medium. Oleate-medium growth rates of *per6 P. pastoris* strains transformed with *PAF-1* compared with those wild-type, untransformed *per6* strains and *per6* strains transformed with the plasmid only (containing no cDNA insert). None of the cDNA transformed strains grew at a rate greater than untransformed *per6* cells.

The second deletion was at position 819 which introduced a frame shift that changed the amino acid sequence at the end of the C_3HC_4 motif, eliminating 2 of the cysteine residues of that region. Since this region may be necessary for the function of the protein (Shimozawa et al., 1992), such a frame shift could render the protein nonfunctional.

3.3.4 Expression of carboxy-terminus-deleted Per6p in *per6* mutants

Since PAF-1 appears to lack the acidic amino acid carboxy-terminus of Per6p, it was of interest to determine whether this acidic tail was essential for Per6p function, i.e., whether a *P. pastoris* strain that expressed Per6p without this acidic region as its only source of Per6p had functional peroxisomes. To examine this, a plasmid that expressed a truncated *PER6* gene was constructed and transformed into a *per6* mutant by electroporation. Twenty transformants were selected and examined for growth on methanol and oleate media. Three of these transformants displayed nearly wild-type growth rates. These results were possibly due to recombination between the point mutant *per6* host and the homologous region of the plasmid-borne truncated *PER6* gene (TP6) to regenerate a full-length wild-type *PER6* gene.

The other 17 transformants showed little or no growth on methanol relative to the *per6* control strain and therefore, it appears that the acidic amino acid carboxy-terminus of Per6p is important for Per6p function in *P. pastoris*. Since PAF-1 does not contain a comparable acidic amino acid region, this mammalian gene may not be able to functionally replace Per6p in *P. pastoris*.

3.4 Discussion and Future Experiments

The goal of this project was to clone and express the human *PAF-1* gene, the putative homologue of the *P. pastoris PER6* gene in a *per6* mutant host and to determine whether the human gene could functionally replace the yeast gene and restore peroxisome biogenesis. Numerous reports in the literature have described successful human/yeast cross-species complementation mostly involving soluble proteins or those that are monomeric. One exception to this rule is a CD protein involved in cell cycle control which interacts with a number of different proteins. Therefore, despite the dissimilarity between humans and yeast, the goal of this project is feasible.

As a first step, the human *PAF-1* gene was amplified by PCR from a human hepatoma cDNA library. Partial sequencing of the amplified fragment indicated that it was *PAF-1*. This *PAF-1* fragment was inserted into *P. pastoris* expression vectors and the

vectors transformed into a *P. pastoris per6* strain. Although the transformed *per6* strains synthesized a *PAF-1* message of the expected size, they did not grow on methanol, indicating that either the human PAF-1 protein could not substitute for a missing Per6p or that PAF-1 protein was not being synthesized.

To examine the second possibility more closely, the DNA sequence of the entire 918-bp PCR-amplified *PAF-1* gene was determined. Two differences from the wild-type *PAF-1* gene were identified. Both were deletions of a single T within a sequence of four or five T residues. These differences are presumably the result of mutations introduced during the cDNA synthesis step in library construction or during our PCR amplification step. Both *in vitro* polymerization reactions are known to be error prone. Since we used Vent ^{TM} polymerase in our PCR step which has 3' to 5' exonuclease activity and has an error rate of less than 1 in 10^{-4} bases, it is more likely that the error occurred during reverse transcriptase synthesis of cDNA.

Thus, it is necessary to obtain a *PAF-1* gene with the correct DNA sequence. A wild-type gene could be obtained by either correcting the sequence of the mutant *PAF-1* gene by *in vitro* mutagenesis, by isolating additional *PAF-1* cDNA fragments from the human liver library, or by obtaining the gene from another laboratory. From our experience, it is obvious that sequence verification is critical.

The one significant difference between PAF-1 and Per6p sequences is that PAF-1 appears to lack the carboxy-terminal region of approximately 100 amino acids present in Per6p. This region is strikingly rich in acidic residues. To determine if this acidic amino acid tail region is essential for Per6p function, plasmids were constructed which expressed derivatives of Per6p without this region. These plasmids were transformed into a *per6* mutant and the transformants examined for growth on methanol medium. Most (17 out of 20) could not grow, indicating that the deleted part of Per6p is essential for the function of this protein. However, a few of the transformants (3 out of 20) did grow on methanol. These were possibly due to integration of the vector at the mutant genomic *per6* locus. Such a recombination event could generate a wild-type *PER6* gene as long as the mutation in genomic *per6* allele was not located in the region encoding the acid tail amino acid residues that were deleted from the plasmid copy of *PER6*. To prevent a recombination event at the genomic *PER6* locus, experiments that involve the expression of portions of *PER6* should be performed in a *P. pastoris* host in which all of the *PER6* locus has been deleted. This strain is currently being constructed.

Because of the presence of mutations in our *PAF-1* gene clone, we were not able to answer the question of whether the human *PAF-1* gene can substitute for *PER6* in *P.*

pastoris. If so, it would confirm our hypothesis that these genes are homologues. Even if the human gene cannot substitute for the yeast gene, at least two interesting avenues of research could be pursued. *PAF-1* could be expressed in wild-type *P. pastoris* and the subcellular location of its product determined. If PAF-1 is targeted to and properly incorporated into the peroxisomal membrane, further experiments to determine the peroxisomal targeting signal(s) on PAF-1 could be performed. Such targeting studies would be more easily conducted in *P. pastoris* than in mammalian cells. In addition to the ease of manipulating a yeast relative to mammalian cells, *P. pastoris* does not express an endogenous PAF-1 as do mammals. Therefore, there should be no background PAF-1 protein to interfere with targeting studies in *P. pastoris.*

The second avenue of research would be to express Per6p-PAF-1 hybrid proteins in a *P. pastoris per6* mutant to determine whether substantial portions of PAF-1 can substitute for portions of Per6p. Logical portions of the genes to exchange include the amino-terminal region, the putative membrane-spanning regions, and the C_3HC_4 regions. Results would provide insight into which regions, if any, have been functionally conserved. Thus, experiments in which selected portions of *PAF-1* and *PER6* are exchanged and examined for complementation in *per6* seem reasonable.

References

Allen, L.-A. H., Hope, L., Raetz, C. R. H. and Thieringer, R. (1994). Genetic evidence supporting the role of peroxisome assembly factor (PAF)-1 in peroxisome biogenesis. *J. Biol. Chem.* **269:**11734-11742.

Becker, D. M. and Guarente, L. (1991). High-efficiency transformation of yeast by electroporation. *Methods Enzymol.* **194:** 182-187.

Brocard, C., Kragler, F., Simon, M. M., Schuster, T., and Hartig, A. (1994). The tetratricopeptide repeat-domain of the PAS10 protein of *Saccharomyces cerevisiae* is essential for binding the peroxisomal targeting signal- SKL. *Biochem. Biophys. Res. Comm.* **204:**1016-1022.

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.

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

de Hoop, M. J. and AB, G. (1992). Import of proteins into peroxisomes and other microbodies. *Biochem. J.* **286:**657-669.

de Wet, J. R., Wood, K. V., DeLuca, M., Helinski, D. R. and Subramani, S. (1987). *Mol. Cell. Biol.* **7**:725-737.

Dodt, G., Braverman, N., Wong, C., Moser, A., Moser, H. W., Watkins, P., Valle, D. and Gould, S. J. (1995). Mutations in the PTS1 receptor gene, *PXR1*, define complementation group 2 of the peroxisome biogenesis disorders. *Nature Genet.* **9:**115-125.

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

Erdmann, R. and Blobel, G. (1995). Giant peroxisomes in oleic acid-induced *Saccharomyces cerevisiae* lacking the peroxisomal membrane protein Pmp27p. *J. Cell Biol.* **128:**509-523.

Fong, H. K., Hurley, J. B., Hopkins, R. S., Miake-Lye, R., Johnson, M. S., Doolittle, R. F., and Simon, M. I. (1986). Repetitive segmental structure of the transducin beta subunit: homology with the CDC4 gene and identification of related mRNAs. *Proc. Natl. Acad. Sci. USA.* **83:**2162-2166.

Fransen, M., Brees, C., Baumgart, E., Vanhooren, J. C. T., Baes, M., Mannaerts, G. and van Veldhoven, P. P. (1995). Identification and Characterization of the putative human peroxisomal C-terminal targeting signal receptor. *J. Biol. Chem.* **270**:7731-7736.

Glover, J. R., Andrews, D. W. and Rachubinski, R. A. (1994). *Saccharomyces cerevisiae* peroxisomal thiolase is imported as a dimer. *Proc. Natl. Acad. Sci. USA.* **91:**10541-10545.

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

Goodman, J. M. (1985). Dihydroxyacetone is an abundant constituent of the methanol-induced peroxisome of *Candida boidinii. J. Biol. Chem.* **260:**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. 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., McCollum, D., Spong, A. P., Heyman, J. A. and Subramani, S. (1992). Development of the yeast *Pichia pastoris* as a model organism for a genetic and molecular analysis of peroxisome assembly. *Yeast* **8:**613-628.

Heupel, R. and Heldt, H. (1992). Transfer of metabolites between chloroplasts, mitochondria and peroxisomes. In Phylogenetic Changes in Peroxisomes of Algae--- Phylogeny of Plant Peroxisomes, ed. H. Stabenau, University of Oldenburg: Oldenberg, Germany: p.p.13-26.

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.

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:**665-673.

Lazarow, P. B., (1984). The peroxisomal membrane. Membrane structure & function. New York: Wiley. 5, 1-31.

Lazarow, P. B. (1989). Peroxisome biogenesis. *Curr. Opin. Cell Biol.* **1:**630-634

Lazarow, P. B. and de Duve, C. (1976). A fatty acyl-CoA oxidizing system in rat liver peroxisomes: enhancement by clofibrate, a hypolipidemic drug. *Proc. Natl. Acad. Sci. USA* **73**:2043-46.

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

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

Marshall, P.A., Krimkevich, Y. I., Lark, R. H., Dyer, J. M., Veenhuis, M. and Goodman, J. M. (1995). Pmp27 promotes peroxisomal proliferation. *J. Cell Biol.* **129:**345-355.

Marzioch, M., Erdmann, R., Veenhuis, M. and Kunau, W.-H. (1994). *PAS7* encodes a novel yeast member of the WD-40 protein family essential for import of 3-oxoacyl-CoA thiolase, a PTS2-containing protein, into peroxisomes. *EMBO J.* **13**:4908-4918.

McCammon, M. T., McNew, J. A., Willy, P. J. and Goodman, J. M. (1994). An internal region of the peroxisomal membrane protein PMP47 is essential for sorting to peroxisomes. *J. Cell Biol.* **124:**915-925.

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:**761-774.

McNew, J. A. and Goodman, J. M. (1994). An oligomeric protein is imported into peroxisomes *in vivo*. *J. Cell Biol.* **127:**1245-1257.

Mosser, J., Douar, A.-M., Sarde, C.-O., Kioschis, P., Feil, R., Moser, H., Poustka, A.-M., Mandel, J.-L. and Aubourg, P. (1993). Putative X-linked adrenoleukodystrophy gene shares unexpected homology with ABC transporters. *Nature* **361:**726-730.

Mosser, J., Lutz, Y., Stoeckel, M., Sarde, C., Kretz, C., Douar, A., Lopez, J., Aubourg, P. and Mandel, J. (1994). The gene responsible for adrenoleukodystrophy encodes a peroxisomal membrane protein. *Hum. Mol. Gen.* **3:**265-271.

Motley, A., Hettema, E., Distel, B. and Tabak, H. (1994). Differential protein import deficiencies in human peroxisome assembly disorders. *J. Cell Biol.* **125:**755-767.

Rhodin, J. (1954). *Correlation of Ultrastructural Organization and Functions in Normal and Experimentally Changed Proximal Convoluted Tubule Cells of the Mouse Kidney.* PhD. thesis. Karolinska Institute Aktiebolaget Godvil, 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., Liang, N. G., Soriano, E., Pericak-Vance, M. A., Haines, J., Rouleau, G. A., Gusella, J. S., Horvitz, H. R., and Brown, R. H., (1993). Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* **362:**59-62.

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

Santos, M. J., Imanaki, T., Shio, H., Small, G. M. and Lazarow, P. B. (1988). Peroxisomal membrane ghosts in Zellweger syndrome -- aberrant organelle assembly. *Science* **239:**1536-1538.

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* **225:**1132-1134.

Slawecki, M. L. , Dodt, G., Steinberg, S., Moser, A. B., Moser, H. W. and Gould, S. J. (1995). Identification of three distinct peroxisomal import defects in patients with peroxisome biogenesis disorders. *J. Cell Sci.* **108:**1817-1829.

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.

Subramani, S. (1992). Targeting of proteins into the peroxisomal matrix. *J. Memb. Biol.* **125**:99-106.

Subramani, S. (1993). Protein import into peroxisomes and biogenesis of the organelle. *Annu. Rev. Cell Biol.* **9**:445-478.

Tan, X., Waterham, H. R., Veenhuis, M. and Cregg, J. M. (1995). The *Hansenula polymorpha PER8* gene encodes a novel peroxisomal integral membrane protein involved in proliferation. *J. Cell Biol.* **128:**307-319.

Thieringer, R. and Raetz, C. R. H. (1993). Peroxisome-deficient Chineses hamster ovary cells with point mutations in peroxisome assembly factor-1. *J. Biol. Chem.* **268:**12631-12636.

Tolbert, N. E. (1981). Metabolic pathways in peroxisomes and glyoxisomes. *Annu. Rev. Biochem.* **50**:133-57.

Tsukamoto, T., Hata, S., Yokota, S., Miura, S., Fujiki, Y., Hijikata, M., Miyazawa, S., Hashimoto, T., and Osumi, T. (1994a). Characterization of the signal peptide at the amino terminus of the rat peroxisomal 3-ketoacyl-CoA thiolase precursor. *J. Biol. Chem.* **269:** 6001-6010.

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

Tsukamoto, T., Shimozawa, N. and Fujiki, Y. (1994b). Peroxisome assembly factor 1: nonsense mutation in a peroxisome-deficient Chinese hamster ovary cell mutant and deletion analysis. *Mol. Cell. Biol.* **14:**5458-5465.

van der Klei, I. J., Hilbrands, R. E., Swaving, G. J., Waterham, H. R., Vrieling, E. G., Titorenko, V. I., Cregg, J. M., Harder, W. and Veenhuis, M. (1995). The *Hansenula polymorpha PER3* gene is essential for the import of PTS1 proteins into the peroxisomal matrix. *J. Biol. Chem.* **270:**17229-17236.

Van der Leij, I., Franse, M. M., Elgersma, Y., Distel, B. and Tabak, H. F. (1993). PAS10 is a tetratricopeptide-repeat protein that is essential for the import of most matrix proteins into peroxisomes of *Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA* **90**:11782-11786.

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. and Harder, W. (1987). Metabolic significance and biogenesis of microbodies in yeast. *Peroxisomes in Biology and Medicine.* ed. H. D. Fahimi and H. Sies. Springer Verlag Berlin-Heidelberg.

Walton, P. A., Hill, P. E. and Subramani, S. (1995). Import of stably folded proteins into peroxisomes. *Mol. Biol. of the Cell* **6:**675-683.

Walton, P. A., Wendland, M., Subramani, S., Rachubinski, R. A. and Welch, W. J. (1994). Involvement of 70-kD heat shock proteins in peroxisomal import. *J. Cell Biol.* **125:**1037-1046.

Waterham, H. R., Titorenko, V. I., Swaving, G. J., Harder, W. and Veenhuis, M. (1993). Peroxisomes in the methyltrophic yeast *Hansenula polymorpha* do not necessarily derive from pre-existing organelles. *EMBO J.* **12:**4785-4794.

Waterham, H. R., de Vries, Y., Russell, K. A., Xie, W., Veenhuis, M. and Cregg, J. M. (1996). The *Pichia pastoris PER6* gene product is a peroxisomal integral membrane protein essential for peroxisome biogenesis and has sequence similarity to the Zellweger syndrome protein PAF-1. *Mol. Cell Biol.* **16:** 2527-2536.

Wiemer, E. A. C., Nuttley, W. M., Bertolaet, B. L., Li, X., Francke, U., Wheelock, M. J., Anne, U. K., Johnson, K. R. and Subramani, S. (1995). Human peroxisomal targeting signal-1 receptor restores peroxisomal import in cells from patients with fatal peroxisomal disorders. *J. Cell Biol.* **130**:51-56.

Wilson, G. N. and Bryant, D. D. (1994). Structure and expression of mammalian peroxisome assembly factor-1 (PMP35) genes. *Biochem. Med. Metabol. Biol.* **51:**140-148.

Zhang, J. W. and Lazarow, P. B. (1995). *PEB1 (PAS7)* in *Saccharomyces cerevisiae* encodes a hydrophilic, intra-peroxisomal protein that is a member of the WD repeat family and is essential for the import of thiolase into peroxisomes. *J. Cell Biol.* **129:**65-80.

Biographical Sketch

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