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The \textit{PAL1} Gene Product Is a Peroxisomal ATP-binding Cassette Transporter in the Yeast \textit{Saccharomyces cerevisiae}

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Abstract. The \textit{PAL1} gene was isolated using PCR and degenerate oligonucleotide primers corresponding to highly conserved amino acid sequence motifs diagnostic of the ATP-binding cassette domain of the superfamily of membrane-bound transport proteins typified by mammalian multidrug resistance transporter 1 and \textit{Saccharomyces cerevisiae} Ste6. The deduced \textit{PAL1} gene product is similar in length to, has the same predicted topology as, and shares the highest degree of amino acid sequence identity with two human proteins, adrenoleukodystrophy protein and peroxisomal membrane protein (70 kD), which are both presumptive ATP-binding cassette transporters thought to be constituents of the peroxisomal membrane. As judged by hybridization of a \textit{PAL1} probe to isolated RNA and by expression of a \textit{PAL1-lacZ} fusion, a \textit{PAL1} transcript was only detectable when cells were grown on oleic acid, a carbon source which requires the biogenesis of functional peroxisomes for its metabolism. A \textit{pal1\Delta} mutant grew normally on either glucose- or glycerol-containing media; however, unlike \textit{PAL1}\textsuperscript{+} cells (or the \textit{pallA} mutant carrying the \textit{PAL1} gene on a plasmid), \textit{pallA} cells were unable to grow on either a solid medium or a liquid medium containing oleic acid as the sole carbon source. Antibodies raised against a chimeric protein in which the COOH-terminal domain of Pall was fused to glutathione S-transferase specifically recognized a protein in extracts from wild-type cells only when grown on oleic acid; this species represents the \textit{PAL1} gene product because it was missing in \textit{pallA} cells and more abundant in \textit{pallA} cells expressing \textit{PAL1} from a multicopy plasmid. The Pall polypeptide was highly enriched in the organelar pellet fraction prepared from wild-type cells by differential centrifugation and comigrated upon velocity sedimentation in a Nycodenz gradient with a known component of the peroxisomal matrix, 3-oxoacyl-CoA thiolase. As judged by both subcellular fractionation and indirect immunofluorescence, localization of 3-oxoacyl-CoA thiolase to peroxisomes was unchanged whether Pall was present, absent, or overexpressed. These findings demonstrate that Pall is a peroxisome-specific protein, that it is required for peroxisome function, but that it is not necessary for the biogenesis of peroxisomes or for the import of 3-oxoacyl-CoA thiolase (and at least two other peroxisomal matrix proteins).

ATP-binding cassette (ABC)\textsuperscript{1} transporters (Higgins et al., 1986), or traffic ATPases (Ames et al., 1992), are a superfamily of membrane-bound proteins whose structure and function have been highly conserved from eubacteria to mammals. ABC transporters catalyze the ATP-dependent transmembrane translocation of a wide variety of substances, which include amino acids, peptides, proteins, sugars, oligosaccharides (reviewed in Higgins, 1992; Kuchler and Thorner, 1992a), man-made drugs (reviewed in Gottesman and Pastan, 1993), and lipids (Reutz and Gros, 1994). The structure of these transporters is comprised of modular components. In eukaryotes, these modular units consist of a domain containing six potential transmembrane segments (TMS\textsubscript{6}) fused to an

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\textsuperscript{1} Abbreviations used in this paper: ABC, ATP-binding cassette; ALDp, adrenoleukodystrophy protein; Gal, galactose; Glc, glucose; Gly, glycerol; GST, glutathione S-transferase; Mdr, multidrug resistance transporter (1 and 3); NBF, nucleotide-binding fold; Ole, oleate; Pmp70, peroxisomal membrane protein of 70 kD; Raf, raffinose; SC, synthetic complete medium; Tap, transporters for antigen presentation (1 and 2); TMS\textsubscript{6}, six potential transmembrane segments; YP, rich medium.

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ABC domain, which contains a particular type of nucleotide-binding fold (NBF). The arrangement of these modular units can vary, however. Among the best characterized ABC transporters in humans are multidrug resistance transporter (Mdr) 1 (Chen et al., 1986) and cystic fibrosis transmembrane conductance regulator (Riordan et al., 1989), which have the structure TMS6-NBF-TMS6-NBF. Other well-studied members of this family include human transporters for antigen presentation (Tap) 1 and 2, which have the structure TMS6-NBF but are thought to form both homo- and heterodimers (Monaco, 1992; Kelly et al., 1992; Spies et al., 1992).

Among the first ABC transporters to be identified in eukaryotes, and the first whose physiological substrate was defined, was the STE6 gene product of the yeast Saccharomyces cerevisiae, which is a dedicated transporter responsible for the release of the peptide mating pheromone, a-factor (Kucher et al., 1989; McGrath and Varshavsky, 1989). Ste6 is similar in structure and overall topology to mammalian Mdr1. In fact, when expressed in ste6Δ cells, both human Mdr1 (Kuchler and Thorner, 1992b) and its mouse counterpart (Mdr3) can mediate a-factor export (Kuchler et al., 1992; Raymond et al., 1992). As one means to discern how broad a spectrum of ABC transporters exists in a given organism and to be able to apply genetic analysis to address the functions of these proteins, we previously described a search for genes encoding additional ABC transporters in the S. cerevisiae genome using PCR and degenerate oligonucleotide primers corresponding to two sequence motifs highly conserved between the NBF domains of Ste6 and those of other ABC transporters (Kuchler et al., 1992). In this way, we isolated multiple PCR products that corresponded to at least two novel genes, initially designated SSH1 and SSH2 (Sterile-Six Homologs) (Kuchler et al., 1992; Göransson and Thorner, 1993). The SSH1 fragment was most similar to mammalian Mdr3; the corresponding gene and yet another highly related homolog were cloned and characterized independently by another group (Dean et al., 1994), and designated MDL1 and MDL2 (Mdr-Like transporters). Likewise, this same PCR approach was used recently to identify a mitochondrial ABC transporter (Leighton and Schatz, 1995).

The SSH2 fragment was of special interest because the segment of the NBF domain contained therein possessed highest similarity, first, to a presumed ABC transporter associated with mammalian peroxisomes, peroxisomial membrane protein of 70 kD (Pmp70) (Kamiyo et al., 1990), and later, to adrenoleukodystrophy protein (ALDp), another protein also thought to be a component of peroxisomes (Görtner et al., 1992). Both diseases lead to aberrant peroxisomal function and are fatal. Unlike individuals afflicted with Zellweger’s syndrome, patients with X-linked adrenoleukodystrophy possess apparently intact peroxisomes that appear to contain only a single enzyme deficiency. Both ALDp and Pmp70 possess the TMS6-NBF structure and are much more closely related to each other than to other ABC transporters. The substrates of these two molecules and their specific roles in peroxisome biogenesis and function have remained elusive, however.

Peroxisomes are single membrane-bound organelles found ubiquitously in eukaryotic cells (van den Bosch et al., 1992). These compartments contain no DNA or ribosomes and, thus, all of the constituent proteins of the peroxisomal matrix must be imported posttranslationally from the cytosol. Studies in a large number of laboratories have demonstrated that there are several distinct routes by which different classes of peroxisomal components are delivered (reviewed in Aitchison, 1992; Subramani, 1993; Lazarow, 1993; Purdue and Lazarow, 1994). However, detailed understanding at the molecular level of the mechanisms involved in the biogenesis and proliferation of peroxisomes is still lacking. In yeast, peroxisomes are the sole site of the β-oxidation of fatty acids and are required for growth on medium containing unsaturated fatty acids (such as oleate) as sole carbon source. Mutants defective in peroxisome function and peroxisomal protein import have been isolated in several different yeast species, including S. cerevisiae (Erdmann et al., 1989; Van der Leij et al., 1992; Zhang et al., 1993; Elgersma et al., 1993).

As one approach for beginning to determine the role of proteins like ALDp and Pmp70 in peroxisome synthesis and function, we isolated the gene corresponding to the SSH2 PCR fragment we described previously (Kuchler et al., 1992; Göransson and Thorner, 1993). Here we report the complete nucleotide sequence and deduced amino acid sequence of this gene, which we have renamed PAL1 (Peroxisomal ABC Transporter-Like protein). We then used genetic, biochemical, and cytological methods to investigate whether the PAL1 gene product is produced in cells in which peroxisome biogenesis is required, whether the Pal1 protein is a constituent of the peroxisome, whether Pal1 is essential for peroxisomal function in vivo, and, finally, whether Pal1 is necessary either for peroxisome assembly or for the import of three proteins known to be components of the peroxisomal matrix.

Materials and Methods

Yeast Strains, Culture Conditions, and Genetic Techniques

The S. cerevisiae diploid strain W303 (MATaMATa ura3-52/ura3-52 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 trpl-I/trpl-I ade2-1/ade2-1 can1-100) and its isogenic haploid derivatives, W303-1A (MATa) and W303-1B (MATa), were used for most of the studies because we observed that it is able to grow on oleate as sole carbon source, as described here. In addition, two other S. cerevisiae stocks reported to grow well on oleate, strain MMY011 (MATa ura3-52 leu2-3,112 his3-11,15 trpl-1 trpl-1 ade2-1 ade2-1 can1-100) (McCammon et al., 1990), obtained from Joel Goodman (Department of Pharmacology, University of Texas Southwestem Medical Center, Dallas, TX) and strain UTL-7A (MATa ura3-52 trpl leu2-3) (Höfßeld et al., 1991), obtained from Suresh Subramani (Department of Biology, University of California at San Diego, La Jolla, CA), were used in certain experiments. Cultures were grown at 30°C in either rich (YP) or synthetic complete (SC) medium (Sherman et al., 1986) containing either glucose (Glc), galactose (Gal), raffinose (Raf), or glycero1 (Gly) as the carbon source and lacking one or more amino acids, as required for the maintenance of plasmids and/or integrated markers. Both a liquid and a solid medium were used to test for growth on oleic acid at 30°C. To assess the ability to grow in liquid medium, cells were preincubated in SC containing
5% glycerol (SCGlY) and then inoculated into a medium (SCOle) containing 0.1% oleate and 0.05% Tween 40. To assess growth on solid medium, cells were grown on agar plates containing YPGlc and replica-plated onto plates containing 0.1% oleate and 0.05% Tween 40. To assess growth on solid medium, cells were grown to mid-exponential phase (A600 nm = 1) in YPGlc, collected by centrifugation, resuspended in a medium (YPOT) containing 0.5% Bacto-peptone (Difco Laboratories, Detroit, MI), 0.3% yeast extract, 0.15% oleate, and 0.015% Tween 40, buffered with potassium phosphate, pH 6.0 (Thieringer et al., 1991), and then digested with EcoRI to remove the label from the 5'-end of the irrelevant strand. The RNA-DNA coprecipitate was resolubilized in 50 μl hybridization buffer (80% formamide, 1 mM EDTA, 40 mM Pipes, pH 6.6), heated to 80°C for 10 min, then cooled slowly to 50°C, and incubated at that temperature for 18 h. After hybridization, the mixture was diluted to a final volume of 300 μl by addition of 30 μl of 10 × S1 nuclease buffer (1 M NaCl, 10 mM zinc acetate, 0.5% glycerol, 0.3 M sodium acetate, pH 4.6) and 10μl of 32P-labeled with 32P at one of its 5'-ends (5 × 106 cpm/pmol). This DNA fragment was generated by PCR using pES04 (see below) as the template, and the resulting products resuspended in 8 μl of electrophoresis buffer (80% formamide, 10 mM NaOH, 1 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanole FF). Before loading, the samples (4 μl) were heated for 2 min to the wells and competed with polyethyleneimine (5%) sequencing gel containing 7 M urea. To determine the precise length of the longest protected fragment, a standard DNA sequencing ladder of the same PAL1 DNA segment (using ps1 as the primer) was subjected to electrophoresis side-by-side with the S1 protection products.

To construct a multicopy plasmid expressing PALI from its own promoter, a 4.7-kb Sphi fragment was excised from plasmid J2 and ligated into the Sphi site of vector YEp52 (Hill et al., 1986), yielding plasmid pES03. To generate a plasmid expressing PALI from the minimum promoter elements required for its proper regulation, 0.9 kb of the upstream region was removed from pES03 by digestion with SalI and religation, to yield plasmid pES04, which contains the entire PALI open reading frame and only 1.25 kb of its 5'-flanking region. To construct a PALI-lacZ translational fusion, a 2.0-kb EcoRI-Sall fragment containing the 5'-flanking sequence and first 130 codons of the PALI gene was excised from pES03, converted to flush ends by treatment with mung bean nuclease, and ligated into plasmid pSEY101 (Douglas et al., 1984) that had been digested with Smal, yielding plasmid pES01. To measure the level of expression from the PALI promoter under different culture conditions, strain W303-1A carrying either the vector (pSEY101) or plasmid pES01 was grown in SCGlc, SCOal, SCGly, or YEp52. Samples of each culture, in triplicate, were lysed with glass beads and assayed for β-galactosidase activity (Miller, 1972) using methods described in detail elsewhere (Rose et al., 1981; Hagen and Sprague, 1984).
Subcellular Fractionation by Differential Centrifugation

Cells were harvested by centrifugation from cultures (100 ml) grown either in YPGlc to midexponential phase (A_{600} = 1) or after transfer to YPOT medium to induce peroxisome biogenesis, as described above. The cells were washed by resuspension and centrifugation in ice-cold H_{2}O, resuspended in 0.2 ml ice-cold lysis buffer (10 mM Hepes, pH 7.8, 1 mM EDTA, 10% glycerol) containing a battery of protease inhibitors (1 pg/ml aprotonin, 1 pg/ml leupeptin, 1 mM benzamidine, 1 pg/ml pepstatin, 1 mM PMSE), and lysed by six 30-s periods of vigorous vortex mixing with an equal volume of glass beads (0.45-0.6 mm diam) with intermittent 30-s periods of cooling on ice. After removal of the glass beads, the whole cell lysate was clarified by centrifugation at 1,500 g to remove unbroken cells and large cellular debris. The clarified crude extract was then subjected to centrifugation at 25,000 g. Samples of the resulting supernatant and pellet fractions containing equivalent amounts of total protein (~50 pg) were solubilized in SDS-PAGE sample buffer (Laemmli, 1970) containing 5% SDS, boiled for 5 min, resolved by SDS-PAGE, and analyzed by immunoblotting (see below). Protein concentration was determined by the method of Bradford (1976), using a commercial assay kit according to the manufacturer's instructions (Bio-Rad Laboratories, Richmond, CA).

Subcellular Fraction by Differential Solubilization

To determine whether Pall displayed the solubility characteristics expected of an integral membrane protein or a peripheral membrane-associated protein, samples of the resuspended pellet fractions (~50 µg total protein) were incubated on ice for 30 min in the following solutions: lysis buffer (control), 2 M urea, 0.1 M Na_{2}CO_{3}, pH 11, 1% Triton X-100, or 1% SDS. After the incubation, the solutions were subjected to centrifugation at 100,000 g, and then samples of the resulting supernatant and pellet fractions were resolved by SDS-PAGE, as described immediately above, and analyzed by immunoblotting. Before electrophoresis, protein in the supernatant fractions was collected by precipitation with 10% TCA and then resuspended in gel sample buffer.

Preparation of Peroxisomes

Cultures (1-liter) were pregrown in YPGlc and then shifted into YPOT, as described above. Cells were harvested by centrifugation, washed twice with ice-cold H_{2}O, resuspended in 20 ml of 0.1 mM Tris-HCl, pH 7.5, 50 mM EDTA, 10 mM β-mercaptoethanol, and incubated at 30°C for 20 min with gentle agitation. The treated cells were collected by centrifugation, resuspended in the same volume of spheroplasting buffer (20 mM potassium phosphate, pH 7.4, 1.2 M sorbitol), and then incubated with Zymolyase 100T (Seikagaku America Inc., Rockville, MD) at 37°C for 30 min with gentle shaking. Aliquots (10 µl) of fixed cells were then placed in the wells of poly-L-lysine-coated microscope slides and permeabilized with methanol and acetone. After rinsing and blocking with BSA-PBS (10 mg/ml BSA in 100 mM potassium phosphate buffer, pH 7.4, 0.9% NaCl), cells were probed with 1:200 dilution of either affinity-purified anti-3-oxoacyl-CoA thiolase antibodies or anti-Pall antibodies for 1 h at room temperature. Affinity-purified anti-Pall antibodies were obtained by elution of antigen-specific antibodies from antigen (GST-Pall) immobilized onto a nitrocellulose filter (Smith and Fisher, 1984). Secondary antibodies (1:200 FITC goat anti-rabbit IgG antibodies; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) were applied for 30 min at room temperature, followed by a 2-min incubation with 1 mg/ml 4,6-diamino-2-phenylindole. After each treatment, the slides were washed by incubation for 3 min in BSA-PBS at least five times. Cells were examined with an Optiphot epifluorescence microscope (Nikon Inc., Garden City, NY) using an 100x objective and photographed using color slide film (Kodak Ektachrome Elite 400; Eastman Kodak Co.).

Detection of Proteins In Situ by Indirect Immunofluorescence

Yeast cells were visualized by indirect immunofluorescence essentially as described (Pringle et al., 1991). Cells were grown in either YPGlc or YPOT, fixed in 4% formaldehyde for 1 h at room temperature, and resuspended at a final concentration of 10 A_{600} ml in 1.5 ml of 100 mM potassium phosphate, pH 7.5, 2 M sorbitol and 30 mM β-mercaptoethanol. Cell walls were removed by digestion with 20 µg/ml Zymolyase 100T (Seikagaku America Inc., Rockville, MD) at 37°C for 30 min with gentle shaking. Aliquots (10 µl) of fixed cells were then placed in the wells of poly-L-lysine-coated microscope slides and permeabilized with methanol and acetone. After rinsing and blocking with BSA-PBS (10 mg/ml BSA in 100 mM potassium phosphate buffer, pH 7.4, 0.9% NaCl), cells were incubated with 1:200 dilution of either affinity-purified anti-3-oxoacyl-CoA thiolase antibodies or anti-Pall1 antibodies for 1 h at room temperature. Affinity-purified anti-Pall1 antibodies were obtained by elution of antigen-specific antibodies from antigen (GST-Pall1) immobilized onto a nitrocellulose filter (Smith and Fisher, 1984). Secondary antibodies (1:200 FITC goat anti-rabbit IgG antibodies; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) were applied for 30 min at room temperature, followed by a 2-min incubation with 1 mg/ml 4,6-diamino-2-phenylindole. After each treatment, the slides were washed by incubation for 3 min in BSA-PBS at least five times. Cells were examined with an Optiphot epifluorescence microscope (Nikon Inc., Garden City, NY) using an 100x objective and photographed using color slide film (Kodak Ektachrome Elite 400; Eastman Kodak Co.).

Results

Isolation and Sequence Analysis of the PAL1 Gene

The most highly conserved elements in the NBF domains of ABC transporters are the so-called Walker A and B motifs also found in other classes of ATPases (Walker et al., 1982; Traut, 1994). Using PCR with degenerate primers based on the A and B motifs found in Ste6, Mdr1, and other related ABC transporters, PCR products thought to define two novel yeast genes were obtained (Kuchler et al., 1992; Gönnanson and Thorner, 1993). Indeed, as described in Materials and Methods, using the cloned SSH2 PCR product generated previously (Kuchler et al., 1992) as the hybridization probe, we were able to isolate several plasmids from a yeast genomic DNA library that contained an authentic and contiguous segment of the S. cerevisiae genome. Nucleotide sequence analysis of this entire region revealed a larger open reading frame encoding a gene, now designated PAL1 (Peroxisomal ABC Transporter-Like protein) (Fig. 1). Assuming that the most upstream-in-frame ATG is the initiator Met codon, the deduced PAL1 gene product is an 870-residue polypeptide with a calculated molecular weight of 100,038. As determined by hybridization of an internal fragment of the PAL1 coding sequence to whole yeast chromosomes resolved by orthogonal field gel electrophoresis (generously provided by Gary Anderson, Department of Plant Biology, University of California, Berkeley, CA) and to an ordered array of yeast genomic fragments in a bacteriophage lambda vector collectively containing the entire S. cerevisiae genome (generously provided by Linda Riles, Maynard Olson, and Mark Johnston, Department of Genetics, Washington University School of Medicine, St. Louis, MO), the PAL1 locus is situated on the left arm of chromosome XVI and located near the PEP4 gene (data not shown).

Hydropathy analysis (Fig. 2 a) of the deduced protein using the algorithm of Hopp and Woods (1981) predicts
that PAL1 contains at least six segments of sufficient length (20-27 residues) and sufficiently high hydrophobicity that these sequences could serve as transmembrane elements, followed by a large (330-residue) hydrophilic region at the COOH terminus, which contains the NBF domain. When equivalent amounts of RNA from each sample were fractionated by agarose gel electrophoresis, transferred to PAL1-specific nitrocellulose filters, and hybridized, the only detectable transcript was the PAL1-specific transcript. This suggests that the PAL1 transcript might be expected to serve some role in peroxisome assembly or function. As anticipated, an open reading frame (ORF) was found within the nucleotide sequence of the PAL1 gene. The deduced amino acid sequence of the 870-residue PAL1 open reading frame is shown beneath the corresponding nucleotide sequence. Numbers to the right indicate nucleotide positions on the deduced amino acid sequence.

**The PAL1 Gene Is Expressed Only When Peroxisomes Are Required**

Gene products required for the function of peroxisomes in yeast have been shown to be repressed when the growth medium contains glucose as the carbon source and induced when the carbon source is a long chain fatty acid, like oleate (Veenhuis et al., 1987). If the gene product serves some role in peroxisome assembly or function, it might be expected to follow this same pattern. To determine the effect of carbon source on PAL1 expression, a wild-type strain (W303-1A) was grown either in glucose medium or induced in 1.3% ethanol for 5 days before total RNA extraction and Northern analysis (Fig. 1A).
Figure 2. Characteristics of the \textit{PAL1} gene product. (a) Hydrophypanalysis of the Pall polypeptide. The algorithm of Hopp and Woods (1981), as supplied in the DNASAR\textsuperscript{TM} sequence analysis package (DNASTAR, Inc., Madison, WI), was applied to the sequence of the predicted Pall protein. Prominent peaks representing contiguous stretches of primarily hydrophobic residues (see Fig. 1) are indicated by the solid bars. (b) The NBF domain of Pall is homologous to ALDp and Pmp70. The ABC domain of Pall was compared, using the multiple alignment algorithm of Hein (1990), with the corresponding regions in two human peroxisomal ABC transporters, ALDp (Mossor et al., 1993) and Pmp70 (G{"a}rtner et al., 1992), with the \textit{S. cerevisiae} YKL741 gene product (Bossier et al., 1994), and with the first ABC domain of the \textit{S. cerevisiae} STE6 gene product (Kuchler et al., 1989). White on black letters, identities. Dashes, single-residue gaps introduced to optimize the alignment.

grown in glucose, but was readily detectable in cells grown in oleate medium (Fig. 3a). The transcript of the essential yeast calmodulin gene (\textit{CMD1}) (Davis et al., 1986), which served as an internal loading control, was expressed in both glucose- and oleate-grown cells, as expected. The \textit{PAL1} transcript was \sim 3,000 nucleotides in length, as judged by its relative mobility with respect to size standards (Fig. 3a), which is more than sufficient to encode the \textit{PAL1} open reading frame (2610 nucleotides) plus both 5'- and 3'-untranslated regions and a polyA\textsuperscript{+} tail.

To map the 5'-end of the \textit{PAL1} mRNA and, thereby, definitively delimit the length of the \textit{PAL1} open reading frame, total RNA from strain W3031-A was annealed to an end-labeled DNA probe (which spanned from 1,250 nucleotides upstream of the putative initiator Met codon to 432 nucleotides downstream of this codon) and subjected to S1 nuclease digestion. Consistent with the RNA hybridization analysis (Fig. 3a), protected products were only detected in the RNA isolated from oleate-grown cells (Fig. 3b). Moreover, the 5'-end of the largest protected fragment corresponded to a position 29 bases upstream of the first base of the putative initiator codon; thus, the most upstream in-frame ATG in the deduced \textit{PAL1} open reading frame is, in fact, the first Met codon encountered from the start of the mRNA (see Fig. 1). A sequence (TATAAA) that could serve as a potential site for binding the TATA box–binding protein for the initiation of transcription lies 57 bp upstream from the transcriptional start site (Fig. 3c).

To further confirm these results and to obtain a semiquantitative estimate of the relative level of \textit{PAL1} expression in various media, a construction was made in which the NH\textsubscript{2} terminus of the \textit{E. coli} \textit{lacZ} gene was fused in-frame to the first 130 amino acids of the \textit{PAL1} coding sequence and \sim 0.6 kb of its 5'-flanking region, which, based on the structure of other yeast genes (Guarente, 1984), should be more than sufficient to contain the entire \textit{PAL1} promoter and its regulatory elements. A plasmid harboring this \textit{PAL1--lacZ} translational fusion, and the parent vector (pSEY101), were introduced into wild-type cells by transformation. Cells carrying the plasmid (pES01) expressing the Pall--LacZ fusion under control of the \textit{PAL1} promoter, and the cells carrying the vector control, were then grown in media containing as carbon sources either
Figure 3. Analysis of PAL1 mRNA expression. (a) Hybridization analysis. Total RNA was isolated from strain W303-1A grown either in YPGlc (glucose) or in YPOT (oleate), fractionated on a 1% agarose gel containing formaldehyde, transferred to a nitrocellulose filter, and hybridized simultaneously to PAL1 and CMD1-specific DNA probes, as described in Materials and Methods. Migration positions of commercial RNA size standards are indicated. (b) S1 nuclease protection. Total RNA, isolated as described in a, was annealed exhaustively with the complementary strand of an end-labeled DNA probe spanning the 5’-end of the PAL1 open reading frame and then digested S1 nuclease, as described in Materials and Methods. The resulting protected DNA fragments were fractionated on a polyacrylamide sequencing gel alongside a standard nucleotide sequence ladder for the corresponding region of PAL1 DNA, as described in Materials and Methods. Arrow, position of the 5’-most nucleotide of the longest protected DNA fragment. (c) Transcriptional start site of the PAL1 gene. Arrow, 5’-end of the PAL1 mRNA as determined in b. Boxed nucleotides, potential TATA box-binding protein binding site for the initiation of transcription.

PAL1 Is Required for Peroxisomal Function

Although PAL1 was clearly expressed under conditions where cells generate peroxisomes, to determine if PAL1 is required for the function of peroxisomes, a pall null mutation (pall-Δ1::LEU2) was constructed. In this deletion-insertion allele, 206 codons within the NBF domain were deleted and replaced with the LEU2 gene. Linear DNA fragments containing this construction were used for transformation of a diploid strain (W303) on glucose medium. Leu⁺ transformants in which the mutant allele had replaced the PAL1 locus by homologous recombination on one copy of chromosome XVI were identified by PCR analysis using primers corresponding to PAL1 sequences flanking the site of marker insertion. The pall-Δ1::LEU2/PAL1 heterozygotes were subjected to sporulation and tetrads dissected on glucose medium. The majority of tetrads yielded four viable spores, in which Leu⁺:Leu⁻ segregated 2:2, indicating that the PAL1 gene is not necessary for either germination or vegetative growth on glucose medium, in agreement with its lack of expression on glucose-containing medium. Furthermore, all four spores were able to grow onYPGly medium at 25, 30, and 35°C, indicating that PAL1 function is not required for growth on this nonfermentable carbon source, despite the fact that PAL1 is expressed at a significant level on glycerol-containing medium (Fig. 4). To confirm the genetic constitution of the spores, PCR was conducted using the same primers as mentioned above and DNA isolated from each of the four spores as the template. PCR products of the expected size were obtained from the wild-type (Leu⁺) and mutant (Leu⁻) spores (data not shown).
null mutants are unable to grow on oleic acid as sole carbon source. (a) Growth on solid medium. The four spores of a single tetrad derived by sporulation and dissection of a heterozygous pall-\(\Delta_{1}:\text{LEU2/PAL1}\) derivative of diploid strain W303 were streaked as patches, grown to confluence on a YPGlc plate, and then replica-plated to a plate containing YNO medium and incubated for 7 d. The Leu\(^+\)\(\text{PAL1}^{+}\) spores (WT; left and right) and the pall-\(\Delta_{1}:\text{LEU2}\) spores (palA; center) are indicated. (b) Growth on liquid medium. Cells of strain W303-1A and an otherwise isogenic pall-\(\Delta_{1}:\text{LEU2}\) derivative (ESY105) (left), or ESY105 containing either vector alone (YEp352) or a plasmid (pES03) expressing the \(\text{PAL1}\) gene from its own promoter on the same vector (right), were precultured in SCGly, then inoculated into SCOle medium, and the \(A_{600\text{nm}}\) monitored over the time period indicated. W303-1A (closed squares), ESY105 (palA, open squares), ESY105[pES03-PAL1\(^+\)] (closed circles), and ESY105 [YEp352] (open circles).

In contrast to the ability of the \(\text{pall-}\Delta_{1}:\text{LEU2}\) spores to grow on glucose- and glycerol-containing medium, these cells were unable to grow on plates of YNO medium, even when replicated as patches from a YPGlc plate, whereas the isogenic \(\text{PAL1}^{+}\) spores from the same tetrad were able to grow on the YNO medium (Fig. 5 a). To confirm that cells containing the \(\text{pall-}\Delta_{1}:\text{LEU2}\) allele were unable to utilize oleic acid as sole carbon source, such a strain (ESY105) was pregrown in liquid SCGly medium to late exponential phase and then inoculated into SCOle at an \(A_{600\text{nm}}\approx 0.05\). Unlike \(\text{PAL1}^{+}\) cells, the mutant strain was unable to propagate in liquid SCOle medium, even after 100 h (Fig. 5 b, left); however, when a functional \(\text{PAL1}\) gene was reintroduced into strain ESY105 on a multicopy plasmid (pES03), the ability of the cells to grow on the oleate-containing medium was restored, whereas the same cells carrying the control vector were still unable to propagate in oleate medium (Fig. 5 b, right). Thus, a functional \(\text{PAL1}\) gene was required for the growth of strain W303 on oleate medium. Likewise, derivatives carrying the \(\text{pall-}\Delta_{1}:\text{LEU2}\) mutation of two other yeast stocks known to grow reasonably well on oleate as sole carbon source, MMYO11 (McCammon et al., 1990) and UTL-7A (Höhlfeld et al., 1991), were constructed in a similar fashion and were also found to be incapable of growth on oleate-containing medium (data not shown).

Immunological Detection of the Pall Polypeptide

To monitor the subcellular distribution of the \(\text{PAL1}\) gene product, polyclonal antibodies were raised against a GST-Pall fusion protein containing the COOH-terminal 371 residues of Pall expressed in bacteria, as described in Materials and Methods. When extracts were prepared from oleate-induced wild-type cells, resolved by SDS-PAGE and analyzed by immunoblotting (Fig. 6), this antiserum specifically recognized a single major band with an apparent molecular mass of \(\sim 85\) kD, as judged by its mobility relative to the migration of marker proteins of known molecular mass. This species represented the \(\text{PAL1}\) gene product because this protein was absent in \(\text{pall-}\Delta_{1}:\text{LEU2}\) cells shifted to oleate medium, was undetectable in wild-type cells grown on glucose medium, and was greatly overproduced in oleate-grown cells carrying \(\text{PAL1}\) on a multicopy plasmid (Fig. 6). There are several factors that may account for the discrepancy between the apparent molecular mass of \(\sim 85\) kD determined by mobility on SDS-PAGE and the predicted molecular weight of 100,000 based on the deduced amino acid sequence. One likely possibility is that the highly basic nature of the Pall polypeptide (\(pI = 9.6\)) causes aberrantly rapid migration upon SDS-PAGE, since the converse has been observed for proteins with highly acidic domains (Benton et al., 1994). Alternatively, since Pall, like Pmp70 (Kamijo et al., 1990) and ALDp (Mosser et al., 1993), contains a seventh strikingly hydrophobic segment near its NH\(_2\) terminus (Fig. 2 a), this element may represent a signal sequence that is cleaved (or Pall may be subjected to other proteolytic processing events) during transit of the protein through the secretory pathway and its delivery to the peroxisome.

The \(\text{PAL1}\) Gene Product Is a Component of the Peroxisome

As an initial means to determine the subcellular compart-
Figure 6. Immunological detection of Pall. Cells of strain W303-1A (+), strain ESY105 (pall-Δ1::LEU2) (Δ), or W303-1A carrying a multicopy plasmid (pES03) expressing PAL1 from its own promoter (MCP) were grown, as indicated, in either SCGlc medium (Glc) or YPOT medium (Ole), lysed with glass beads, solubilized in SDS-PAGE sample buffer, subjected to SDS-PAGE on a 10% gel, transferred electrophoretically to a nitrocellulose filter, incubated with anti-Pall antibodies (antiserum No. 1257), washed, incubated with secondary antibodies conjugated to HRP, and visualized by chemiluminescence, all as described in Materials and Methods.

Figure 7. Subcellular localization of Pall by differential centrifugation. Cells of strain W303-1A carrying vector alone (YPE352), or strain ESY105 (pall-Δ1::LEU2) carrying the same vector or a plasmid (pES03) expressing PAL1 from the same vector, were grown in either YPGlc (glucose) or shifted to YPOT (oleate). Total cell lysates were prepared by glass bead lysis and then clarified by low speed centrifugation. The resulting clarified whole cell extract (W) was then subjected to centrifugation at 25,000 g, producing a soluble supernatant fraction (S) and a particulate organellar pellet fraction (P). Samples (50 μg) of each fraction were resolved by SDS-PAGE and analyzed by immunoblotting with either anti-Pall antibodies (α-Pall; upper panel) or anti-3-oxoacyl-CoA thiolase antibodies (α-thiolase; lower panel), as described in the legend to Fig. 6.

Pall1 is an Integral Membrane Protein

The subcellular fractionation studies described immediately above demonstrated that Pall is associated with peroxisomes. Furthermore, hydropathy analysis (Fig. 2a) predicted that Pallp has the capacity to be a polytopic membrane protein. Indeed, unlike 3-oxoacyl-CoA thiolase, Pall was found only in the particulate fraction of cell extracts (Fig. 7) and sedimented almost exclusively (>90%) with the dense membrane fraction (Fig. 8). Taken together, these findings suggested that Pall is a constituent of the peroxisomal membrane rather than a component of the internal matrix of the peroxisome. To determine whether Pall possesses the empirical characteristics dis-
Peroxisome Assembly Is Unaffected by Absence or Overproduction of Pall

3-Oxoacyl-CoA thiolase is a soluble enzyme that is imported into the yeast peroxisome posttranslationally, apparently directed by an NH$_2$-terminal targeting sequence (Erdmann, 1994). As shown here, this protein was found predominantly in the organelar pellet of a 25,000 g sedimentation (Fig. 7) and colocalized in density gradients with other peroxisomal marker proteins (Fig. 8; data not shown). Mutations are known to disrupt the biogenesis of peroxisomes. In certain of these pas mutants (Peroxi-

some Assembly-defective), 3-oxoacyl-CoA thiolase is mislocalized to the cytosol (Erdmann et al., 1989; Hohfeld et al., 1991). Because we found that Pall is a member of the ABC transporter family (Figs. 1 and 2 b), some members of which translocate polypeptides (Kuchler and Thorner, 1992b), and because we demonstrated that Pall is necessary for growth on oleate (Fig. 5), a function requiring the synthesis of intact peroxisomes, it was possible that the defect in peroxisome function was due to the lack of import of proteins critical to the assembly and function of the peroxisome. Therefore, it was of interest to determine whether the distribution of 3-oxoacyl-CoA thiolase was altered when cells either lacked or overproduced the PALL gene product. Like Pall, 3-oxoacyl-CoA thiolase is only expressed in cells grown on oleic acid medium. As judged by subcellular fractionation by differential centrifugation, the distribution of 3-oxoacyl-CoA thiolase in the organelar pellet fraction from cells lacking or overproducing Pall was not detectably different from that observed in wild-type cells (Fig. 7). By this criterion, PALL function did not appear to be required either for the biogenesis of sedi-

mentable peroxisomes or for the import of 3-oxoacyl-CoA thiolase into the peroxisome. The possibility that, in the pallA mutant cells, 3-oxoacyl-CoA thiolase is only adventitiously associated with peroxisomes because it becomes "trapped" during its translocation through the peroxisomal membrane was eliminated by the demonstration that lysis of peroxisomes using alkaline Na$_2$CO$_3$ released 3-oxoacyl-CoA thiolase with equal efficiency from PALL and pallA::LEU2 mutant cells (Fig. 9).

Likewise, when the organelar material isolated from PALL+ cells, pallA::LEU2 mutants or PALL-overexpressing cells was further resolved by velocity sedimentation on Nycodenz density gradients, the migration profiles of mitochondrial cytochrome b$_2$, peroxisomal 3-oxoacyl-CoA thiolase, and two other soluble enzymes found in the matrix of the peroxisome (acyl-CoA oxidase and the mul-

Figure 8. Subcellular localization of Pall by density gradient sedimentation. Cells of strain W303-1A were grown on YPGlc, shifted to YPOT for 18 h, converted to spheroplasts, lysed gently, and the 25,000 g organellar pellet fraction was prepared, as described in Materials and Methods. A sample (5 mg total protein) of the pellet material was layered onto a 15%-36% Nycodenz gradient containing 0.25 M sucrose. After centrifugation for 3 h at 100,000 g, fractions (0.5 ml) were collected from the bottom of the tube. Portions (one-tenth vol) of every second fraction were resolved by SDS-PAGE on three separate gels and analyzed by immunoblotting with antibodies directed against Pall (upper panel), 3-oxoacyl-CoA thiolase (middle panel), and cytochrome b$_2$ (lower panel), as described in the legend to Fig. 6.

Figure 9. Pall behaves like an integral membrane protein. Samples (50 μg total protein) of the organellar pellet material, prepared from W303-1A and ESY105 (pallA::LEU2) cells, as described in the legend to Fig. 7, were resuspended at 4°C in either lysis buffer alone (buffer) or lysis buffer containing, as indicated, 2 M urea, 0.1 M Na$_2$CO$_3$, pH 11, 1% Triton X-100, or 1% SDS. After incubation for 30 min on ice, the solutions were subjected to centrifugation at 100,000 g. The resulting supernatant (S) and pellet (P) fractions were resolved by SDS-PAGE and analyzed for their content of Pall or thiolase by immunoblotting, as described in the legend to Fig. 6.
Figure 10. Localization of 3-oxoacyl-CoA thiolase to peroxisomes in PAL1+ and pal1Δ mutant cells. Strain W303-1A (PAL1+) and ESY105, its otherwise isogenic pal1-Δ1::LEU2 derivative (pal1Δ), were grown either on YPGlc (glucose) or shifted to YPOT (oleate) to induce the synthesis of peroxisomes. Samples of the cultures were then fixed, affixed to slides, permeabilized, and stained with a DNA dye (DAPI), with primary antibodies directed against either Pal1 or 3-oxoacyl-CoA thiolase, as indicated, and with secondary anti-rabbit IgG antibodies conjugated to fluorescein (FITC), as described in detail in Materials and Methods.

tifunctional β-oxidation protein), were indistinguishable from those observed in wild-type cells (data not shown). Analysis of acyl-CoA oxidase (product of the POX1 gene; Dmochowska et al., 1990) and the multifunctional β-oxidation protein (product of the FOX2 gene; Hiltunen et al., 1992) in isolated peroxisomes prepared from MYO11 and an otherwise isogenic pal1-Δ1::LEU2 derivative were kindly performed by James McNew and Joel P. Goodman.
To further confirm that the assembly of morphologically recognizable peroxisomes, and the localization of 3-oxoacyl-CoA thiolase to those structures, was not perturbed by the absence of the PAL1 gene product, indirect immunofluorescence was used to examine the distribution of both PalI and 3-oxoacyl-CoA thiolase in either PAL1+ cells and or palI-D1::LEU2 mutant cells either grown on glucose medium or shifted to oleate medium to induce peroxisome biogenesis. Only a very low level of nonspecific background staining was seen in glucose-grown cells and in oleate-grown palI-D1::LEU2 mutant cells. As expected, in wild-type cells, intensely stained vesicular bodies representing peroxisomes were only detectable by immunofluorescence in oleate-grown cells, and these compartments contained both PalI and 3-oxoacyl-CoA thiolase (Fig. 10). In agreement with the results of subcellular fractionation by differential centrifugation and density gradient sedimentation, approximately the same number of brightly stained vesicular bodies as found in normal cells were also observed in the palI-D1::LEU2 mutant cells, and, at this level of resolution, appeared to contain as much 3-oxoacyl-CoA thiolase as the peroxisomes from normal cells, even though these bodies lacked any detectable PalI (Fig. 10).

**Discussion**

The *S. cerevisiae* PAL1 gene was isolated by virtue of its homology to the NBF domains of other previously characterized members of the superfamily of ABC transporters. PalI possesses the TMS-N-BF organization displayed by certain human ABC transporters, like Tap1 and Tap2, rather than the TMS-N-BF-TMS-N-BF organization displayed by Ste6, Mdr1, and cystic fibrosis transmembrane conductance regulator. The PalI polypeptide is strikingly more similar to two human peroxisomal membrane proteins, ALDp and Pmp70, than it is to any other protein, including Ste6 and other yeast ABC transporters. Indeed, the genetic, biochemical, and cytological evidence presented here demonstrates that PalI is a peroxisomal protein. The PAL1 gene is expressed at a high level on oleate-containing medium, but not expressed when the medium contains fermentable carbon sources. Because peroxisomes are required for the metabolism of oleate, PALI expression occurs under the same conditions that induce peroxisome biogenesis. Moreover, palI null mutants in three different *S. cerevisiae* strain backgrounds are unable to utilize oleic acid as a carbon source, establishing that the PAL1 gene product is required for proper peroxisomal function. Furthermore, as judged by fractionation using either differential centrifugation or velocity sedimentation in a density gradient, PalI comigrates with a known marker of the peroxisomal matrix, 3-oxoacyl-CoA thiolase. Finally, both PalI and 3-oxoacyl-CoA thiolase are localized to vesicular bodies (presumably peroxisomes) that are present in yeast cells only when grown on oleate, as judged by indirect immunofluorescence with specific antibodies.

Nonetheless, the role of PalI in peroxisomal function remains unclear. The quantitative association of PalI with the particulate organellar material, the sedimentation of PalI nearly exclusively with the dense membrane fraction, and the inability of PalI to be solubilized by anything other than a strong detergent indicate that, as predicted by hydropathy analysis of its sequence, PalI is likely to be a polytopic integral membrane protein of the peroxisomal envelope. In this regard, it is interesting to note that the six strikingly hydrophobic segments recognized by the analysis method of Hopp and Woods (or that of Kyte and Doolittle) are also strongly predicted to be segments of pure β-sheet by the several available algorithms for prediction of secondary structure (Chou and Fasman, 1978; Garnier et al., 1978) available in the DNAStar sequence analysis package (DNASTAR, Inc., Madison, WI). In addition, four of the six hydrophobic segments contain at least one potentially charged residue. Once placed in the hydrocarbon interior of the membrane, these segments may assume an α-helical conformation (Steitz and Engelman, 1981; Jones et al., 1990). On the other hand, the transmembrane segments of PalI may cross the bilayer and associate with each other as extended chains, similar to what has been observed for the hydrophobic segments of the porin proteins which reside in the outer membrane of Gram-negative bacteria (Nikaido, 1992).

Furthermore, PalI may need to form homooligomers, or a heterooligomer, to constitute a functional transporter, in analogy to the heterodimeric Tap1/Tap2 transporter in the ER membrane of macrophages and other professional antigen-presenting cells (Kelly et al., 1992; Spies et al., 1992). An *S. cerevisiae* gene (YKL741) encoding a protein with homology to PAL1, ALDp, and Pmp70 was identified recently on the left arm of chromosome XI (Bossier et al., 1994). It remains a formal possibility that PalI associates with YKL741 to form a heterooligomer, and experiments are currently under way to address this issue. Given the nature of its putative transmembrane segments, if PalI oligomerizes (with itself and/or with YKL741), the lateral associations between subunits may involve salt bridges between the charged residues that lie within the otherwise hydrophobic segments because such electrostatic interactions would be favored in the apolar environment of the lipid bilayer.

The fact that PalI appears to be an integral membrane protein and is a member of the ABC family of transporters suggests that it is responsible for the translocation of some molecule into or out of the peroxisome. It was tempting to speculate that the inability of palI null mutants to metabolize oleic acid was due to the requirement of PalI for the import of some essential peroxisomal enzyme. However, palI null mutants contain peroxisomes that appear to be intact and able to accumulate 3-oxoacyl-CoA thiolase and two other enzymes (acyl-CoA oxidase and the multifunctional β-oxidation protein) of the peroxisomal matrix, as judged by biochemical and immunocytochemical criteria. Each of these three proteins is targeted to and imported into the peroxisome by a discrete mechanism (Lazarow, 1993; Purdue and Lazarow, 1994). The multifunctional β-oxidation protein (Hiltunen et al., 1992) is an example of a protein that contains the first type of peroxisomal targeting signal (PTS1) that was recognized, namely the tripeptide sequence, -SKL, at its COOH terminus (Gould et al., 1989). Import of 3-oxoacyl-CoA thiolase is mediated via a different type of peroxisomal targeting signal (PTS2) at its NH2 terminus (Erdmann, 1994). Acyl-CoA oxidase
import has been best studied in another yeast species, Candida tropicalis, where its import appears to involve yet another class of peroxisomal targeting signal (PTS3) located internally in the polypeptide chain (Small et al., 1988). Mutants of S. cerevisiae have been isolated that are defective in peroxisomal function because they fail to import one or several classes of peroxisomal proteins or are defective in peroxisomal function because they fail to assemble morphologically identifiable peroxisomes (Erdmann et al., 1989; Van der Leij et al., 1992; Zhang et al., 1993; Elgersma et al., 1993; Lazarow, 1993). Because pall mutants clearly have peroxisomes and import all three known types of matrix proteins, the PAL1 gene product represents a new class of peroxisomal protein required for the function of the peroxisome.

Different ABC transporters mediate the transport of a wide variety of substrates, including amino acids, peptides, proteins, sugars, oligosaccharides (reviewed in Higgins, 1992; Kuchler and Thorner, 1992a), ions, drugs (reviewed in Göttesman and Pastan, 1993), and lipids (Reutz and Gros, 1994). Thus, the nature of the substrate transported by Pall remains to be defined. It has been reported recently that S. cerevisiae contains an ABC transporter (product of the ATM1 gene) that is associated with the mitochondrion and required for normal cell growth (Leighton and Schatz, 1995). Although neither the function of this transporter nor its substrate was elucidated, it was speculated, based on the luminal orientation of the NBF domain, that Atm1 may function to transport peptides out of the mitochondria as part of a communication link between the mitochondrion and the expression of nuclear genes required for the assembly of this organelle. In contrast, protease "shaving" experiments suggest that the NBF domain of Pmp70 faces the cytosol (Kamijo et al., 1990). Determination of the orientation of Pall in the peroxisomal membrane, which is in progress, may help elucidate the direction of substrate transport and the role of Pall in peroxisomal function.

It is noteworthy that, unlike the peroxisomes in yeast pall mutants, human patients with Zellweger's syndrome exhibit peroxisomes with multiple enzyme defects and, in some cases, lack identifiable peroxisomes altogether (Moser et al., 1991). Some cases of Zellweger's syndrome have been attributed to genetic defects in the locus that encodes Pmp70 (Gärtner et al., 1992). In marked contrast, X-linked adrenoleukodystrophy is characterized by the presence of intact peroxisomes, which reportedly contain only a single enzyme deficiency, namely a lack of peroxisomal lignoceroyl-CoA ligase (also known as very-long-chain-fatty acyl-CoA synthetase) (Lazo et al., 1988). Nonetheless, the cause of X-linked adrenoleukodystrophy is mutation of the locus that encodes ALDp, an ABC transporter (Moser et al., 1993). Thus, remians possible that ALDp may be required for the import of very-long-chain-fatty acyl-CoA synthetase into the peroxisome. On the other hand, there is some dispute in the literature about whether very-long-chain-fatty acyl-CoA synthetase is actually localized to the peroxisome (van den Bosch et al., 1992); and, thus, it remains unclear why this enzyme is absent in patients suffering from X-linked adrenoleukodystrophy. Alternatively, ALDp may be required for the transport of very long chain fatty acids into the peroxi-

some. When ALDp is defective, the resulting lack of very long chain fatty acids in the peroxisome might destabilize very-long-chain-fatty acyl-CoA synthetase (if the enzyme is indeed localized to the peroxisome), or the resulting overaccumulation of very long chain fatty acids in the cytosol may inactivate very-long-chain-fatty acyl-CoA synthetase (if the enzyme is actually in the cytosol).

Like ALDp in humans, Pall in yeast is not required for the assembly of peroxisomes and is not necessary for the import of at least three different classes of peroxisomal matrix proteins, but is nonetheless required for peroxisomal function. Because the phenotypes of yeast pall mutants and human X-linked adrenoleukodystrophy are rather similar, and because Pall and ALDp share a high level of amino acid sequence identity, further study of the PAL1 gene product and its role in peroxisomal function may yield valuable insight into the pathology of X-linked adrenoleukodystrophy and may further our understanding of the biology of the peroxisome, a somewhat enigmatic organelle.

While our paper was under review, a publication appeared describing the isolation of an S. cerevisiae gene (designated PXA1) that appears to represent the same locus as PAL1 (Shani et al., 1995). However, several of our results differ in significant ways from the findings reported in this other study. First, we found that the longest continuous open reading frame is 112 residues longer at its NH2-terminal end than that reported by Shani et al. (1995); moreover, we demonstrated that this longer open reading frame is encoded in the PAL1 mRNA. Second, presumably because the 5'-end of the gene was truncated in the constructs studied by these other workers, they were unable to examine regulation of the expression of the gene in vivo and were forced to fuse the gene to a heterologous promoter to drive its expression. In contrast, we were able to demonstrate that expression of either the endogenous or a plasmid-borne PAL1 gene under control of its natural promoter is tightly regulated by both glucose catabolite repression and by oleic acid induction. Third, when we fused the shorter open reading frame reported by Shani et al. (1995) to the GAL1 promoter, the resulting protein produced was markedly smaller, as judged by SDS-PAGE (data not shown), than the authentic PAL1 gene product expressed from its native promoter, which we have described here. Nonetheless, our results are in substantial agreement with certain of the other findings reported by Shani et al. (1995). In particular, both pxal and pall null mutants are unable to grow on oleate as sole carbon source, even though the mutants appear to possess peroxisomes and the peroxisomes apparently contain known peroxisomal constituents. Also, in agreement with our conclusion that peroxisomal biogenesis appears normal in pallΔ mutants, which was based on differential centrifugation, velocity density gradient sedimentation, and indirect immunofluorescence, Shani et al. (1995) reported that morphologically intact peroxisomes were observed in pxalΔ cells by electron microscopy.

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