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The *PAL1* Gene Product Is a Peroxisomal ATP-binding Cassette Transporter in the Yeast *Saccharomyces cerevisiae*

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Abstract. The *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 *Saccharomyces cerevisiae* Ste6. The deduced *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 *PAL1* probe to isolated RNA and by expression of a *PAL1–lacZ* fusion, a *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 *pal1Δ* mutant grew normally on either glucose- or glycerol-containing media; however, unlike *PAL1*$^+$ cells (or the *pal1Δ* mutant carrying the *PAL1* gene on a plasmid), *pal1Δ* 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 Pal1 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 *PAL1* gene product because it was missing in *pal1Δ* cells and more abundant in *pal1Δ* cells expressing *PAL1* from a multicopy plasmid. The Pal1 polypeptide was highly enriched in the organellar 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 Pal1 was present, absent, or overexpressed. These findings demonstrate that Pal1 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)[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, 1992*a*), 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_6) fused to an

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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_6, six potential transmembrane segments; YP, rich medium.

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 TMS_6-NBF-TMS_6-NBF. Other well-studied members of this family include human transporters for antigen presentation (Tap) 1 and 2, which have the structure TMS_6-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 (Kuchler 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, 1992*b*) 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 Mdrs; 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, peroxisomal membrane protein of 70 kD (Pmp70) (Kamijo et al., 1990), and later, to adrenoleukodystrophy protein (ALDp), another protein also thought to be a component of peroxisomes (Mosser et al., 1993). Defects in both proteins are known to be responsible for human metabolic diseases. Defects in the locus on the X chromosome encoding ALDp cause the genetic disease, adrenoleukodystrophy (Mosser et al., 1993). Similarly, a subset of patients with the disorder, Zellweger's syndrome, have defects at the locus encoding Pmp70 (Gärtner 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 TMS_6-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 (*MAT***a**/*MATα ura3-52/ura3-52 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 trp1-1/trp1-1 ade2-1/ade2-1 can1-100/can1-100*) and its isogenic haploid derivatives, W303-1A (*MAT***a**) and W303-1B (*MATα*), 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 MMYO11 (*MATα ura3-52 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100*) (McCammon et al., 1990), obtained from Joel Goodman (Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX) and strain UTL-7A (*MATα ura3-52 trp1 leu2-3*) (Höhfeld 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 glycerol (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 precultured 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 agar YNO medium which contains 0.67% yeast nitrogen base without amino acids (Difco Laboratories, Inc., Detroit, MI), 0.05% yeast extract, 0.1% oleic acid, and 0.5% Tween 40 (Erdmann et al., 1989). To induce peroxisome biogenesis on a preparative scale, cells were grown to midexponential phase (A_{600nm} = 1) in YPGlc, collected by centrifugation, resuspended in a medium (YPOT) containing 0.5% Bacto-peptone (Difco Laboratories Inc.), 0.3% yeast extract, 0.15% oleate, and 0.015% Tween 40, buffered with potassium phosphate, pH 6.0 (Thieringer et al., 1991), and incubated with vigorous aeration for 18 h. DNA-mediated transformation of yeast cells (Ito et al., 1983), as well as sporulation and tetrad dissection (Sherman et al., 1986), were carried out using standard procedures.

Isolation of the PAL1 Gene

The *SSH2* PCR product described previously (Kuchler et al., 1992) was radiolabeled using the random primer method (Feinberg and Vogelstein, 1984) and hybridized to filter replicas of bacterial colonies containing a yeast genomic DNA library in a plasmid vector, pSB32 (gift of F. Spencer and P. Heiter, Johns Hopkins School of Medicine, Baltimore, MD), using standard methods (Sambrook et al., 1989). Five different plasmids were obtained that reproducibly hybridized strongly to this probe upon restreaking and retesting. Restriction endonuclease cleavage site mapping of these five plasmids (G1, G2, J1, J2, and K1), and comparison of their hybridization pattern to that obtained by Southern blot hybridization analysis of freshly isolated yeast genomic DNA using the same probe, revealed that the plasmids contained different-sized inserts, which nonetheless harbored a common and contiguous segment of *S. cerevisiae* genomic DNA. The region of the smallest insert from one of the plasmids (J2) that corresponded to the sequences homologous to the probe and its flanking DNA were subcloned into pBluescript (Stratagene, La Jolla, CA), and subjected to nucleotide sequence analysis on both strands using the dideoxynucleotide method (Sanger et al., 1977), α-^{35}S-dATP (Biggin et al., 1983), and Sequenase™ (United States Biochemical Corp., Cleveland, OH), as recommended by the supplier, using a combination of exonuclease III-generated deletions (Sambrook et al., 1989) and custom-synthesized oligonucleotide primers. The GenBank accession number for this sequence is L38491.

Construction of pal1 Null Mutations

A deletion–insertion allele (*pal1-Δ1::LEU2*) was constructed in the following manner. A 1.4-kb BglII–KpnI fragment (containing nucleotides 1574–2910; see Fig. 1) was excised from plasmid J2 and ligated into the corresponding sites in the vector, pGEM (Promega Biotec, Madison, WI), yielding plasmid pMV01. A 2.3-kb PvuII fragment containing the entire *LEU2* gene was excised from plasmid pJJ282 (Jones and Prakash, 1990), ligated into pMV01 that had been digested with XhoI (at nucleotide 1912) and EcoRI (at nucleotide 2531), and converted to blunt ends by incubation with the Klenow fragment of *Escherichia coli* DNA polymerase I, generating plasmid pES02. A 3.3-kb PvuII–NheI fragment containing the *pal1-Δ1::LEU2* construction was excised from pES02 and used for DNA-mediated transformation of strains W303, MMYO11, and UTL-7A, selecting for Leu prototrophy on SCGlc-Leu medium. Transplacement of the *PAL1* locus with the mutant allele on one homologue in the diploid strain W303 (and in spores derived from it), as well as in the MMYO11 and UTL-7A haploid strains, was confirmed by PCR analysis on DNA isolated from the Leu$^+$ transformants. One of the *MATa pal1-Δ1::LEU2* spores derived from dissection of a heterozygous *pal1-Δ1::LEU2/PAL1* derivative of strain W303 was designated ESY105 and used for most of the studies presented here.

Analysis of PAL1 Expression

Total RNA was extracted as previously described (Flick and Thorner, 1993) from cells of strain W303-1A grown either in YPGlc or in YPOT. Samples of the RNA (50 μg) were subjected to electrophoresis in a 1% agarose gel containing formaldehyde, transferred onto a nylon membrane (GeneScreen Plus; Stratagene) according to the manufacturer's instructions, hybridized to a 2.5-kb ScaI–EcoRV fragment of the *PAL1* sequence (nucleotides 658–3120) that had been labeled with ^{32}P using the random primer method (Feinberg and Vogelstein, 1984), and analyzed by autoradiography using x-ray film (X-AR; Eastman Kodak Co., Rochester, NY) and an intensifying screen (Cronex Lightning-Plus; DuPont Photo Products Div. x-ray, Burbank, CA).

To map the location of the 5′-end of the *PAL1* transcript, S1 nuclease protection analysis (Berk and Sharp, 1978) was used. Total RNA (100–200 μg) from strain W303-1A grown either in glucose or in oleate was coprecipitated with a 1.7-kb doubled-stranded *PAL1* DNA fragment (0.08 pmol) labeled with ^{32}P at one of its 5′-ends (5×10^6 cpm/pmol). This DNA fragment was generated by PCR using pES04 (see below) as the template, the standard M13 reverse primer (Stratagene), and a primer (prS1: 5′-CTGTAGCATTCATAAATTTGTTGTCATCGG-3′) corresponding to nucleotides 818–848 of the *PAL1* sequence, labeled by incubation with T4 polynucleotide kinase (United States Biochemical Corp.) and [γ-^{32}P]ATP, 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, 0.4 M NaCl, 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, 50% glycerol, 0.3 M sodium acetate, pH 4.6) and ddH_2O, digested with S1 nuclease (50 U) at 37°C for 30 min, extracted with phenol/chloroform, precipitated with ethanol, 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 boiled for 2 min and then applied to the wells of a polyacrylamide (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 prS1 as the primer) was subjected to electrophoresis side-by-side with the S1 protection products.

To construct a multicopy plasmid expressing *PAL1* from its own promoter, a 4.7-kb SphI fragment was excised from plasmid J2 and ligated into the SphI site of vector YEp352 (Hill et al., 1986), yielding plasmid pES03. To generate a plasmid expressing *PAL1* from the minimum promoter elements required for its proper regulation, 0.9 kb of the upstream region was removed from pES03 by digestion with SacI and religation, to yield plasmid pES04, which contains the entire *PAL1* open reading frame and only 1.25 kb of its 5′-flanking region.

To construct a *PAL1-lacZ* translational fusion, a 2.0-kb EcoRI–SalI fragment containing the 5′-flanking sequence and first 130 codons of the *PAL1* 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 SmaI, yielding plasmid pES01. To measure the level of expression from the *PAL1* promoter under different culture conditions, strain W303-1A carrying either the vector (pSEY101) or plasmid pES01 was grown in SCGlc, SCGal, SCGly, or SCOle. 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).

Preparation of Anti-Pal1 Antibodies

To generate a specific antigen, a 1.2-kb XhoI–EcoRV fragment containing the COOH-terminal 371 codons and the natural termination signals of the *PAL1* coding sequence were excised from pES03, converted to blunt ends with the Klenow fragment of *E. coli* DNA polymerase I and ligated in-frame to the coding sequence for the *Schistosoma japonicum* glutathione S-transferase (GST) in the vector, pGEX-3X (Pharmacia Fine Chemicals, Piscataway, NJ), which had been digested with SmaI, yielding plasmid pES05. *E. coli* strain BL21 was transformed with plasmid pES05 and expression of the GST–Pal1 fusion protein was induced according to the recommendations of the manufacturer. The GST–Pal1 chimera, which was found almost exclusively in the inclusion body fraction, was solubilized in 60 mM Tris-HCl, pH 6.8, 5% SDS, 1% β-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue, resolved by preparative-scale electrophoresis in 8% SDS-PAGE, visualized by staining with Coomassie brilliant blue dye, and electroeluted from the gel slices containing the band of appropriate molecular mass. Samples of the electroeluted GST–Pal1 fusion were injected into two adult female New Zealand White rabbits using complete Freund's adjuvant in the primary inoculation (0.5 mg of antigen) and incomplete Freund's adjuvant in the booster immunizations (0.1 mg of antigen), following standard procedures (Harlow and Lane, 1988). An antiserum from one of the rabbits (No. 1257) was used for the studies presented here.

Subcellular Fractionation by Differential Centrifugation

Cells were harvested by centrifugation from cultures (100 ml), grown either in YPGlc to midexponential phase (A_{600nm} = 1) or after transfer to YPOT medium to induce peroxisome biogenesis, as described above. The cells were washed by resuspension and recentrifugation in ice-cold H_2O, 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 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM benzamidine, 1 μg/ml pepstatin, 1 mM PMSF), 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 μg) 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 manufacturers instructions (Bio-Rad Laboratories, Richmond, CA).

Subcellular Fraction by Differential Solubilization

To determine whether Pal1 displayed the solubility characteristics expected of an integral membrane protein or a peripheral membrane-associated protein, samples of the resuspended pellet fraction (~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_2CO_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_2O, resuspended in 20 ml of 100 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 (2 mg/g wet wt of cells) for ~45 min at 30°C with gentle agitation. Lysis of the spheroplasts so generated, preparation of the crude organellar fraction, and velocity sedimentation of the organelles through a Nycodenz gradient (Sigma Chemical Co., St. Louis, MO) were performed essentially as described by Thieringer et al. (1991), with the following modifications. A linear 15–36% (wt/vol) Nycodenz gradient containing 0.25 M sucrose, 5 mM MES, pH 6.0, and 1 mM EDTA, was centrifuged in a rotor (SW40Ti; Beckman Instruments, Inc., Fullerton, CA) at 25,000 rpm for 3 h at 4°C. Gradient fractions (0.5 ml) were collected from the bottom of the tube, and samples of equal volume (50 μl) were resolved by SDS-PAGE and analyzed by immunoblotting.

Immunological Detection of Pal1

Proteins resolved by SDS-PAGE were transferred electrophoretically to nitrocellulose sheets (BA85, 0.45 mm; Schleicher & Schull Inc., Keene, NH), as described in detail elsewhere (Towbin et al., 1979). The resulting protein blots were blocked and incubated with the primary antibody (1:10,000 dilution of antiserum No. 1257) using standard procedures (Harlow and Lane, 1988). After washing, the remaining immune complexes on the filter were visualized using, as secondary antibodies, donkey anti-rabbit IgG antibodies coupled to HRP (1:5,000) and a commercial chemiluminescence detection system (ECL™; Amersham Corp., Arlington Heights, IL). A similar procedure was followed to analyze 3-oxoacyl-CoA thiolase and cytochrome *b2* on immunoblots, using 1:10,000 dilutions of the primary rabbit polyclonal antisera.

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_{600nm}/ml in 1.5 ml of 100 mM potassium phosphate, pH 7.5, buffer containing 1.2 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 first incubated with a 1:200 dilution of either affinity-purified anti-3-oxoacyl-CoA thiolase antibodies or anti-Pal1 antibodies for 1 h at room temperature. Affinity-purified anti-Pal1 antibodies were obtained by elution of antigen-specific antibodies from antigen (GST-Pal1) 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 every 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 100× 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; Göransson 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

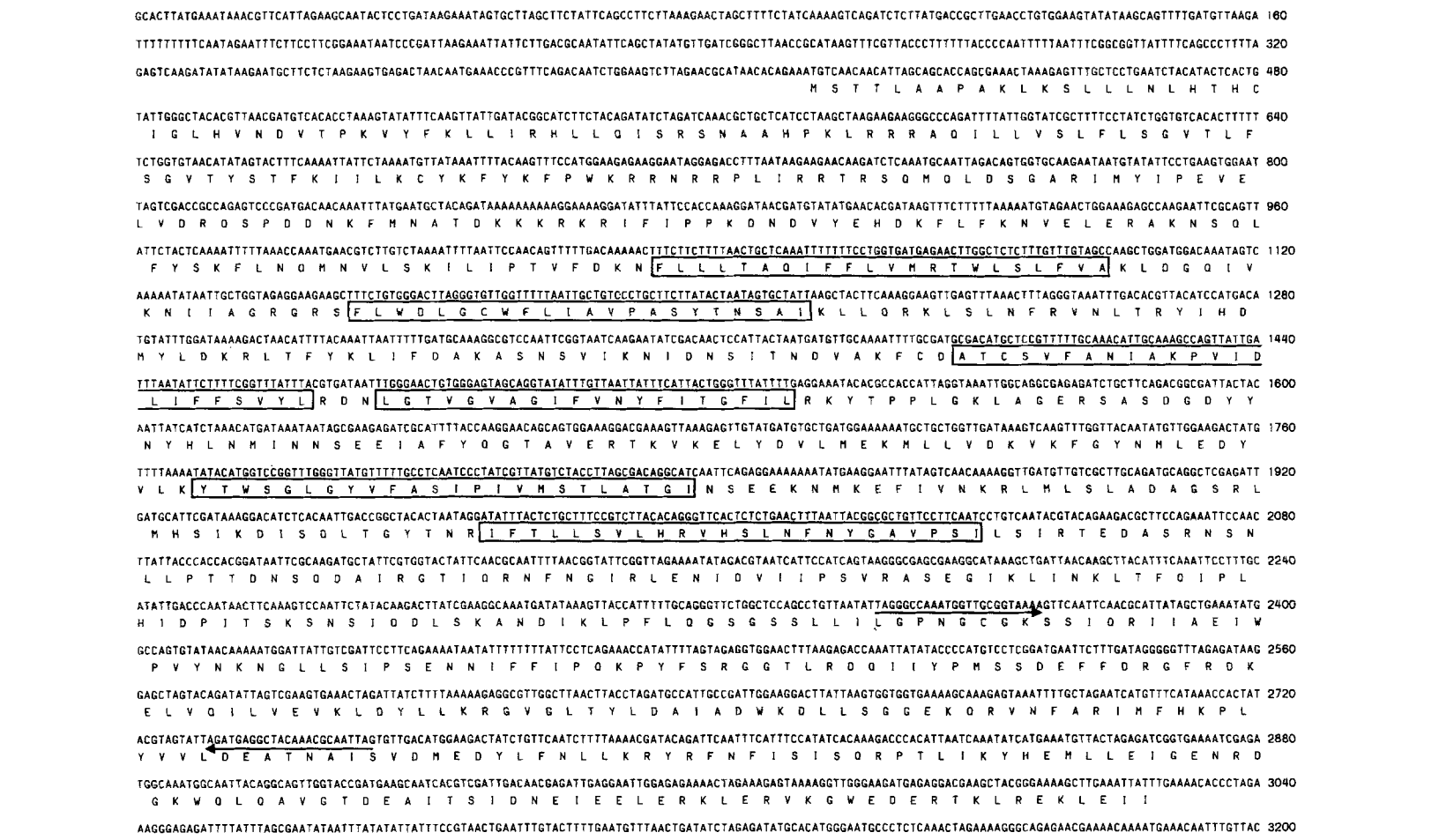
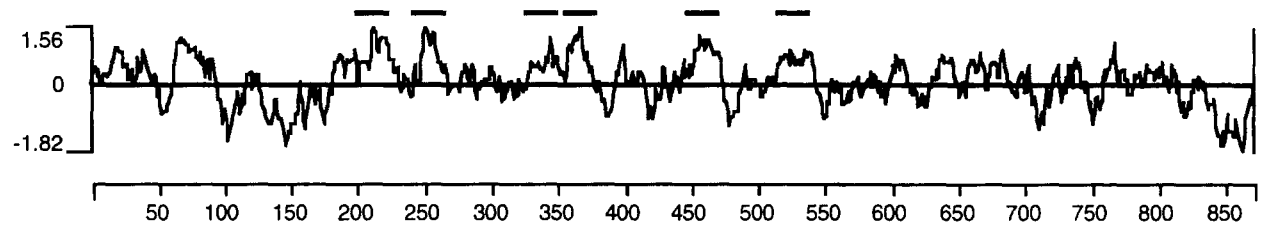


Figure 1. Nucleotide sequence and deduced amino acid sequence of the *PAL1* gene. The deduced amino acid sequence of the 870-residue *PAL1* open reading frame is shown beneath the corresponding nucleotide sequence. Numbers to the right indicate nucleotide positions, where the first base of the initiator ATG of the *PAL1* coding sequence is at +416. The sequences used to design the degenerate primers that generated the *SSH2* PCR product (Kuchler et al., 1992), which was, in turn, used to clone the genomic DNA fragment containing the *PAL1* gene, are underlined with arrows. The six strikingly hydrophobic segments within the *PAL1* gene product, as determined by hydropathy analysis (see Fig. 2 *a*), are boxed.

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 available databases were searched, the most homologous proteins found were the human peroxisomal proteins, ALDp and Pmp70. The degree of amino acid sequence identity between Pal1 and these two proteins (Fig. 2 *b*) is highest within the NBF domain (46 and 41%, respectively), but readily detectable outside of this region. Pal1 is somewhat less related (35% identity in the NBF domain) to a putative yeast Pmp70 homologue recently deposited in GenBank (accession No. L16958) as the result of the international effort to determine the nucleotide sequence of the entire *S. cerevisiae* genome (Fig. 2 *b*). In contrast, this same region of Pal1 shares only 25% amino acid sequence identity with the corresponding region in the first NBF domain of Ste6. Hydropathy analysis of both ALDp (Mosser et al., 1993) and Pmp70 (Gärtner et al., 1992) both predict that, like Pal1, these ABC transporters contain six hydrophobic segments followed by the hydrophilic, COOH-terminal, NBF-containing domain.

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 highly induced when the carbon source is a long chain fatty acid, like oleate (Veenhuis et al., 1987). If the *PAL1* gene product serves some role in peroxisome assembly or function, expression of the *PAL1* transcript 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 oleate medium, and total RNA was isolated from these cells. Equivalent amounts of RNA from each sample were fractionated by agarose gel electrophoresis, transferred to a filter, and hybridized with a *PAL1*-specific DNA probe. As anticipated, *PAL1* mRNA was not detectable in cells



b

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Pal1    LILGPNGCGKSSIQRIIAEIWPV-------YNKNGLL---SIPSEN---NIFFIPQKPYFSR-----GGTLRDQIIYPMS 703
ALDp    LITGPNGCGKSSLFRILGGLWPT-------YGGVLYK---PPP--Q---RMFYIPQRPYMSV------GSLRDQVIYPDS 562
Pmp70   LICGPNGCGKSSLFRVLGELWPL-------FGGRLTK---PER--R---KLFYVPQRPYMTL------GTLRDQVIYPDG 528
YKL174  LIIGPNGCGKSSLFRILGGLWPIRATPNKNHQSKLIM---PRRTVDRDCAIFYLPQRPYMGN-----RSTFREQIIYPDS 573
Ste6a   FIVGKSGSGKSTLSNLLLRFYDG-------YNGSISINGHNIQTID---QKLLIENITVVEQRCTLFNDTLRKNIL--LG 456

Pal1    SDEFFDR-----G----FRDKELVQILVEVKL--DYLLKRGVGL----------------------------TYLDAIAD 744
ALDp    VEDMQRK-----G----YSEQDLEAILDVVHL--HHILQREGGW----------------------------EA---MCD 600
Pmp70   REDQKRK-----G----ISDLVQKEYLDNVQL--GHILEREGGW----------------------------DS---VQD 566
YKL174  IEQFKERYHNDYD----LGDADLIKILQLLDL--EDLVTENMSLLLAQRTSKNDSQQLSTEDNQSPCAIKVRDAFSIVRN 647
Ste6a   STDSVRN-----ADCSTNENRHLIKDACQMALLDRFILDLPDGL----------------------------ETLIGTGG 503

Pal1    WKDLLSGGEKQRVNFARIMFHKPLYVVLDEATNAISVDMEDYLFNLLKRYRFNFISISQRPTLIKYHEMLLEIGE      819
ALDp    WKDVLSGGEKQRIGMARMFYHRPKYALLDECTSAVSIDVEGKIFQAAKDAGIALLSITHRPSLWKYHTHLLQFDG      675
Pmp70   WMDVLSGGEKQRMAMARLFYHKPQFALLDECTSAVSVDVEGYIYSHCRKVGITLFTVSHRKSLWKHHEYYLHMDG      641
YKL174  WSEELTIGVQQRLAMARMYYHKPKFAVLDECTSAVAPEMEQRMYENAQNFGISLISVCHRTSLWHFHNYLLKFDG      722
Ste6a   --VTLSGGQQQRVAIARAFIRDTPILFLDEAVSALDIVHRNLLMKAIRHWRKGKTTIILTHELSQIESDDYLYLM      576
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Figure 2. Characteristics of the *PAL1* gene product. (*a*) Hydropathy analysis of the Pal1 polypeptide. The algorithm of Hopp and Woods (1981), as supplied in the DNAStar™ sequence analysis package (DNASTAR, Inc., Madison, WI), was applied to the sequence of the predicted Pal1 protein. Prominent peaks representing contiguous stretches of primarily hydrophobic residues (see Fig. 1) are indicated by the solid bars. (*b*) The NBF domain of Pal1 is homologous to ALDp and Pmp70. The ABC domain of Pal1 was compared, using the multiple alignment algorithm of Hein (1990), with the corresponding regions in two human peroxisomal ABC transporters, ALDp (Mosser et al., 1993) and Pmp70 (Gärtner et al., 1992), with the *S. cerevisiae YKL741* gene product (Bossier et al., 1994), and with the first ABC domain of the *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. 3 *a*). The transcript of the essential yeast calmodulin gene (*CMD1*) (Davis et al., 1986), which served as an internal loading control, was expressed in both glucose- and oleate-grown cells, as expected. The *PAL1* transcript was ~3,000 nucleotides in length, as judged by its relative mobility with respect to size standards (Fig. 3 *a*), which is more than sufficient to encode the *PAL1* open reading frame (2610 nucleotides) plus both 5′- and 3′-untranslated regions and a polyA$^+$ tail.

To map the 5′-end of the *PAL1* mRNA and, thereby, definitively delimit the length of the *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. 3 *a*), protected products were only detected in the RNA isolated from oleate-grown cells (Fig. 3 *b*). 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 *PAL1* open reading frame is, in fact, the first Met codon encountered from the start of the mRNA (see Fig. 1). A sequence (TATATAA) 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. 3 *c*).

To further confirm these results and to obtain a semiquantitative estimate of the relative level of *PAL1* expression in various media, a construction was made in which the NH_2 terminus of the *E. coli lacZ* gene was fused in-frame to the first 130 amino acids of the *PAL1* coding sequence and ~1.6 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 *PAL1* promoter and its regulatory elements. A plasmid harboring this *PAL1–lacZ* translational fusion, and the parent vector (pSEY101), were introduced into wild-type cells by transformation. Cells carrying the plasmid (pES01) expressing the Pal1–LacZ fusion under control of the *PAL1* promoter, and the cells carrying the vector control, were then grown in media containing as carbon sources either

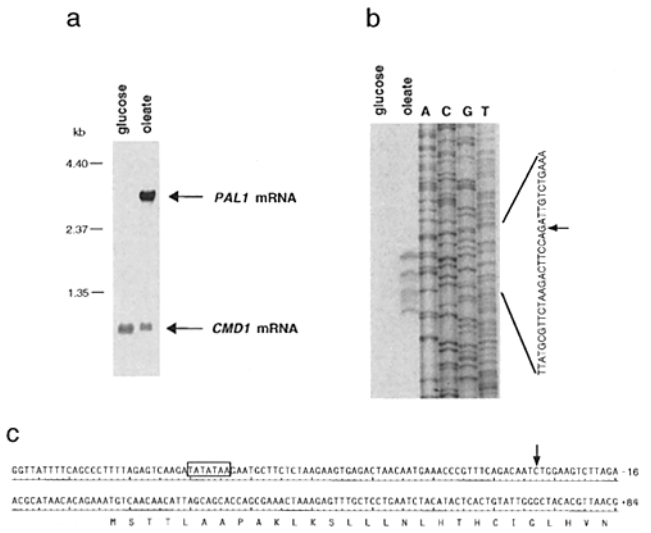

Figure 3. Analysis of *PAL1* mRNA expression. (*a*) Hybridization analysis. Total RNA was isolated from strain W303-1A grown either in YPGlc (*glucose*) or in YPOT (*oleate*), fractionated on a 1% agarose gel containing formaldehyde, transferred to a nitrocellulose filter, and hybridized simultaneously 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.

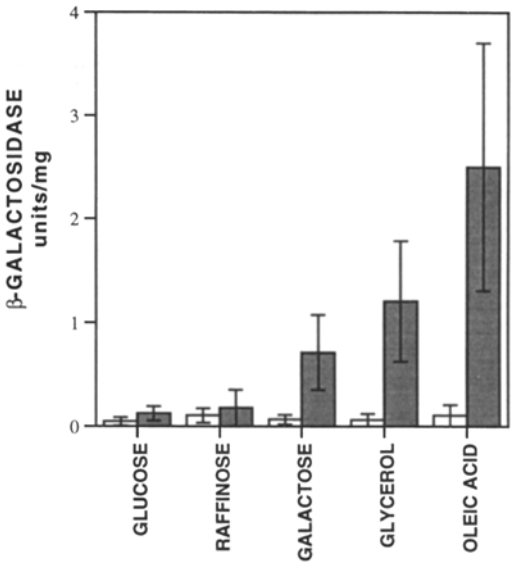

Figure 4. The *PAL1* promoter is derepressed on nonfermentable carbon sources. Cells of strain W303-1A carrying either a vector (pSEY101) containing a UAS-less *lacZ* gene or a plasmid (pES01) expressing a *PAL1–lacZ* translational fusion from the same vector were grown in either SCGlc (*glucose*), SCRaf (*raffinose*), SCGal (*galactose*), SCGly (*glycerol*), or SCOle (*oleic acid*). Glass bead lysates were prepared from triplicate samples and assayed for β-galactosidase activity, as described in *Materials and Methods.* Specific activity is given as units per milligram of total cell protein (where 1 unit is 1 nmol of *o*-nitrophenyl-β-D-galactoside cleaved per minute at 28°C). Error bars, standard deviation of the mean. W303-1A[pSEY101] (*open bars*) and W303-1A[pES01] (*shaded bars*).

glucose, raffinose, galactose, glycerol, or oleic acid. Samples of these cells were lysed and assayed, in triplicate, for their content of β-galactosidase activity (Fig. 4). In cells carrying the vector alone, enzyme activity was very low in all five media examined. In cells expressing the Pal1–LacZ fusion, β-galactosidase activity was lowest in glucose-containing medium, but slightly higher in media containing either galactose or raffinose (both of which are carbon sources known to cause less catabolite repression than glucose). Strikingly, enzyme activity was markedly elevated (at least 10-fold) in the cells expressing Pal1–LacZ that were grown in the oleate-containing medium, as expected. Interestingly, almost as high a level of β-galactosidase activity was observed when the cells expressing Pal1–LacZ were grown in glycerol-containing medium (Fig. 4). Elevated expression of other genes encoding peroxisomal functions in cells grown on a nonfermentable carbon source has been observed previously (Thieringer et al., 1991).

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 *pal1* null mutation (*pal1-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 *pal1-Δ1::LEU2/PAL1* heterozygotes were subjected to sporulation and tetrad dissection 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 on YPGly 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).

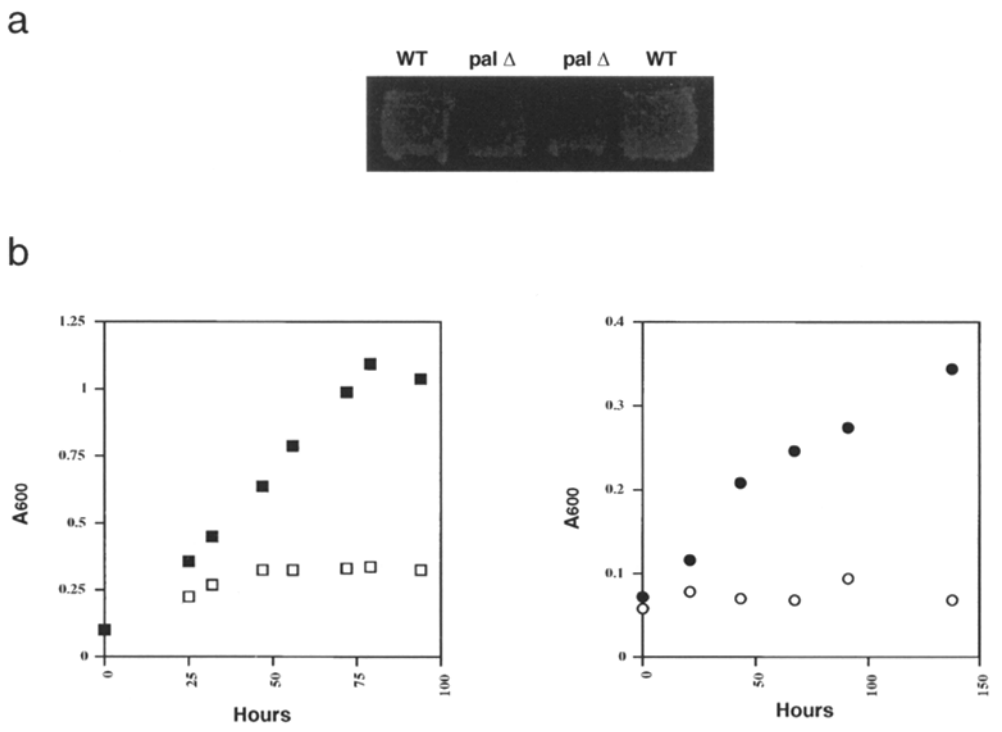

Figure 5. pal1 null mutants are unable to grow on oleic acid as sole carbon source. (*a*) Growth on solid medium. The four spores of a single tetrad derived by sporulation and dissection of a heterozygous *pal1-Δ1::LEU2/PAL1* derivative of diploid strain W303 were streaked as patches, grown to confluence on a YPGlc plate, and then replica-plated to a plate containing YNO medium and incubated for 7 d. The Leu$^-$ *PAL1*$^+$ spores (*WT*; *left* and *right*) and the *pal1-Δ1::LEU2* spores (*palΔ*; *center*) are indicated. (*b*) Growth on liquid medium. Cells of strain W303-1A and an otherwise isogenic *pal1-Δ1::LEU2* derivative (ESY105) (*left*), or ESY105 containing either vector alone (YEp352) or a plasmid (pES03) expressing the *PAL1* gene from its own promoter on the same vector (*right*), were precultured in SCGly, then inoculated into SCOle medium, and the A_{600nm} monitored over the time period indicated. W303-1A (*closed squares*), ESY105 (*pal1Δ*, *open squares*), ESY105[pES03-*PAL1*$^+$] (*closed circles*), and ESY105 [YEp352] (*open circles*).

In contrast to the ability of the *pal1-Δ1::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 *PAL1*$^+$ spores from the same tetrad were able to grow on the YNO medium (Fig. 5 *a*). To confirm that cells containing the *pal1-1Δ::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 $A_{600nm} \cong 0.05$. Unlike *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 *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 *PAL1* gene was required for the growth of strain W303 on oleate medium. Likewise, derivatives carrying the *pal1-1Δ::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 (Höhfeld 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 Pal1 Polypeptide

To monitor the subcellular distribution of the *PAL1* gene product, polyclonal antibodies were raised against a GST–Pal1 fusion protein containing the COOH-terminal 371 residues of Pal1 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 ~85 kD, as judged by its mobility relative to the migration of marker proteins of known molecular mass. This species represented the *PAL1* gene product because this protein was absent in *pal1-1Δ::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 *PAL1* on a multicopy plasmid (Fig. 6). There are several factors that may account for the discrepancy between the apparent molecular mass of ~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 Pal1 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 Pal1, 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 Pal1 may be subjected to other proteolytic processing events) during transit of the protein through the secretory pathway and its delivery to the peroxisome.

The PAL1 Gene Product Is a Component of the Peroxisome

As an initial means to determine the subcellular compart-

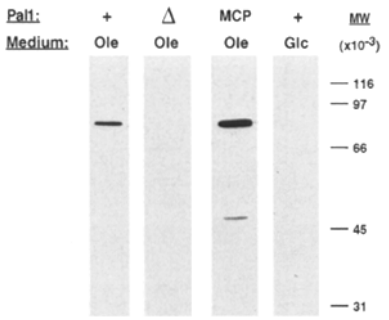

Figure 6. Immunological detection of Pal1. Cells of strain W303-1A (+), strain ESY105 (*pal1-Δ1::LEU2*) (*Δ*), or W303-1A carrying a multicopy plasmid (pES03) expressing *PAL1* from its own promoter (*MCP*) were grown, as indicated, in either SCGlc medium (*Glc*) or YPOT medium (*Ole*), lysed with glass beads, solubilized in SDS-PAGE sample buffer, subjected to SDS-PAGE on a 10% gel, transferred electrophoretically to a nitrocellulose filter, incubated with anti-Pal1 antibodies (antiserum No. 1257), washed, incubated with secondary antibodies conjugated to HRP, and visualized by chemiluminescence, all as described in *Materials and Methods.*

ment in which Pal1 resides, cell-free lysates were prepared from cultures of wild-type cells and a *pal1-Δ1::LEU2* mutant carrying either a multicopy plasmid (pES03) expressing *PAL1* or the vector alone (YEp352), grown either in YPGlc or shifted to YPOT medium to induce peroxisome biogenesis, and then subjected to differential centrifugation. After clarification by centrifugation at low speed (1,500 *g*), the extracts were subjected to centrifugation at 25,000 *g* to pellet the cellular organelles. Equivalent amounts of total protein from the initial clarified extracts and from the supernatant and pellet fractions of the 25,000 *g* sedimentation were separated by SDS-PAGE, transferred to a filter, and analyzed by incubation with the anti-Pal1 antibodies (Fig. 7, *upper panel*). As expected, Pal1 was not detectable in any fraction from wild-type cells grown in glucose or in the *pal1-Δ1::LEU2* mutant induced in oleate medium. In contrast, the Pal1 polypeptide was readily detectable in the whole extract of wild-type cells shifted to oleate medium and in the *pal1-Δ1::LEU2* cells expressing *PAL1* from the plasmid (Fig. 7, *upper panel*). Most significantly, the Pal1 protein was found exclusively in the organellar pellet fraction, even when overproduced from the multicopy plasmid. This pellet fraction clearly contained the majority of the peroxisomal material because the bulk of a soluble enzyme known to reside in the matrix of the peroxisome, 3-oxoacyl-CoA thiolase (product of the *FOX3/POT1* gene; Erdmann, 1989; Igual et al., 1991), also was found in the pellet fraction, as judged by immunoblotting with a specific antibody directed against this protein (Fig. 7, *lower panel*). Some of the 3-oxoacyl-CoA thiolase was found in the soluble fraction, suggesting that some of the peroxisomes were broken during the preparation. The leakiness of peroxisomes isolated under identical conditions has been noted by others (Thieringer et al., 1991).

To further delineate the subcellular compartment in which Pal1 resides, the organelles in the 25,000 *g* pellet were further resolved by velocity sedimentation in a Nycodenz density gradient. Samples were withdrawn from the bottom of the tube, and portions of each fraction were subjected to SDS-PAGE on three separate gels, which were each transferred to a filter and analyzed by incubation with an appropriate antibody. Pal1 was found almost exclusively in the denser fractions near the bottom of the gradient (Fig. 8, lanes *5* and *7*). The majority of the 3-oxoacyl-CoA thiolase also comigrated in the identical fractions, indicating that this material represented intact peroxisomes. Furthermore, the peroxisomes were well resolved from mitochondria because staining with specific antibodies directed against a known marker protein of the mitochondrial inner membrane, cytochrome *b2*, indicated that the majority of the mitochondria were found in the lightest density region of the gradient (Fig. 8, lanes *19* and *20*).

Pal1 Is an Integral Membrane Protein

The subcellular fractionation studies described immediately above demonstrated that Pal1 is associated with peroxisomes. Furthermore, hydropathy analysis (Fig. 2 *a*) predicted that Pal1p has the capacity to be a polytopic membrane protein. Indeed, unlike 3-oxoacyl-CoA thiolase, Pal1 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 Pal1 is a constituent of the peroxisomal membrane rather than a component of the internal matrix of the peroxisome. To determine whether Pal1 possesses the empirical characteristics dis-

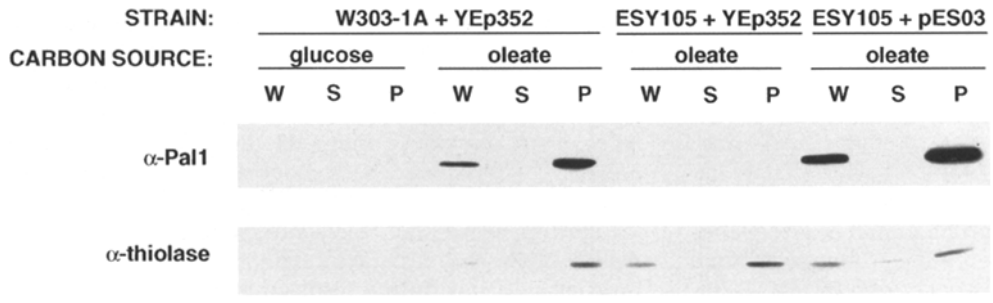

Figure 7. Subcellular localization of Pal1 by differential centrifugation. Cells of strain W303-1A carrying vector alone (YEp352), or strain ESY105 (*pal1-Δ1::LEU2*) carrying the same vector or a plasmid (pES03) expressing *PAL1* from the same vector, were grown in either YPGlc (*glucose*) or shifted 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-Pal1 antibodies (*α-Pal1*; *upper panel*) or anti-3-oxoacyl-CoA thiolase antibodies (*α-thiolase*; *lower panel*), as described in the legend to Fig. 6.

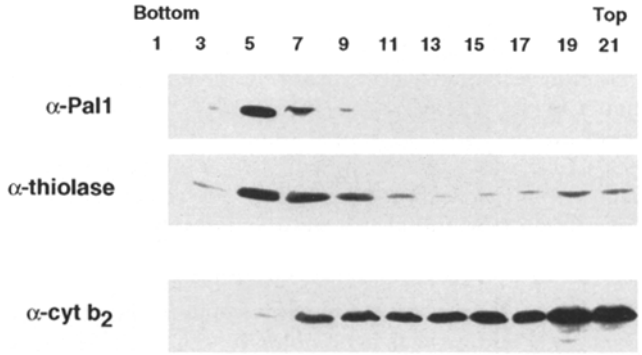

Figure 8. Subcellular localization of Pal1 by density gradient sedimentation. Cells of strain W303-1A were grown on YPGlc, shifted to YPOT for 18 h, converted to spheroplasts, lysed gently, and the 25,000 *g* organellar pellet fraction was prepared, as described in *Materials and Methods.* A sample (5 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 Pal1 (*upper panel*), 3-oxoacyl-CoA thiolase (*middle panel*), and cytochrome *b2* (*lower panel*), as described in the legend to Fig. 6.

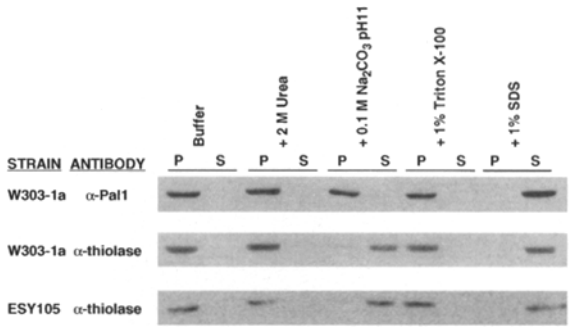

Figure 9. Pal1 behaves like an integral membrane protein. Samples (50 μg total protein) of the organellar pellet material, prepared from W3031-A and ESY105 (*pal1-Δ1::LEU2*) cells, as described in the legend to Fig. 7, were resuspended at 4°C in either lysis buffer alone (*buffer*) or lysis buffer containing, as indicated, 2 M urea, 0.1 M Na_2CO_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 Pal1 or thiolase by immunoblotting, as described in the legend to Fig. 6.

played by other integral membrane proteins, the ability of various conditions and treatments to solubilize Pal1 from the organellar pellet were examined. For this purpose, equivalent amounts of protein from the 25,000 *g* pellet material were resuspended at 4°C in either lysis buffer alone or the same buffer containing either 2 M urea, 0.1 M Na_2CO_3, pH 11, 1% Triton X-100, or 1% SDS. After incubation on ice for 30 min, the solutions were subjected to sedimentation at 100,000 *g*. The content of Pal1 in the resulting supernatant and pellet fractions was assessed by SDS-PAGE and immunoblotting (Fig. 9). Treatments known to be effective in stripping proteins peripherally associated with membranes (2 M urea and alkaline Na_2CO_3) or in lysing the peroxisome (alkaline Na_2CO_3) were totally ineffective in releasing Pal1 into the soluble fraction. In contrast, the peroxisomal matrix protein, 3-oxoacyl-CoA thiolase, was effectively released into the soluble fraction upon alkaline Na_2CO_3 treatment of the organellar pellet. The only treatment tested that effectively extracted Pal1 into the soluble fraction was treatment with the strong anionic detergent, SDS (Fig. 9). Thus, Pal1 behaves like an intrinsic membrane protein.

Peroxisome Assembly Is Unaffected by Absence or Overproduction of Pal1

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 organellar 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 that disrupt the biogenesis of peroxisomes. In certain of these *pas* mutants (Peroxisome Assembly–defective), 3-oxoacyl-CoA thiolase is mislocalized to the cytosol (Erdmann et al., 1989; Hohfeld et al., 1991). Because we found that Pal1 is a member of the ABC transporter family (Figs. 1 and 2 *b*), some members of which translocate polypeptides (Kuchler and Thorner, 1992*b*), and because we demonstrated that Pal1 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 *PAL1* gene product. Like Pal1, 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 organellar pellet fraction from cells lacking or overproducing Pal1 was not detectably different from that observed in wild-type cells (Fig. 7). By this criterion, *PAL1* function did not appear to be required either for the biogenesis of sedimentable peroxisomes or for the import of 3-oxoacyl-CoA thiolase into the peroxisome. The possibility that, in the *pal1Δ* 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_2CO_3 released 3-oxoacyl-CoA thiolase with equal efficiency from *PAL1*$^+$ and *pal1-Δ1::LEU2* mutant cells (Fig. 9).

Likewise, when the organellar material isolated from *PAL1*$^+$ cells, *pal1-Δ1::LEU2* mutants or *PAL1*-overexpressing cells was further resolved by velocity sedimentation on Nycodenz density gradients, the migration profiles of mitochondrial cytochrome *b2*, peroxisomal 3-oxoacyl-CoA thiolase, and two other soluble enzymes found in the matrix of the peroxisome (acyl-CoA oxidase and the mul-

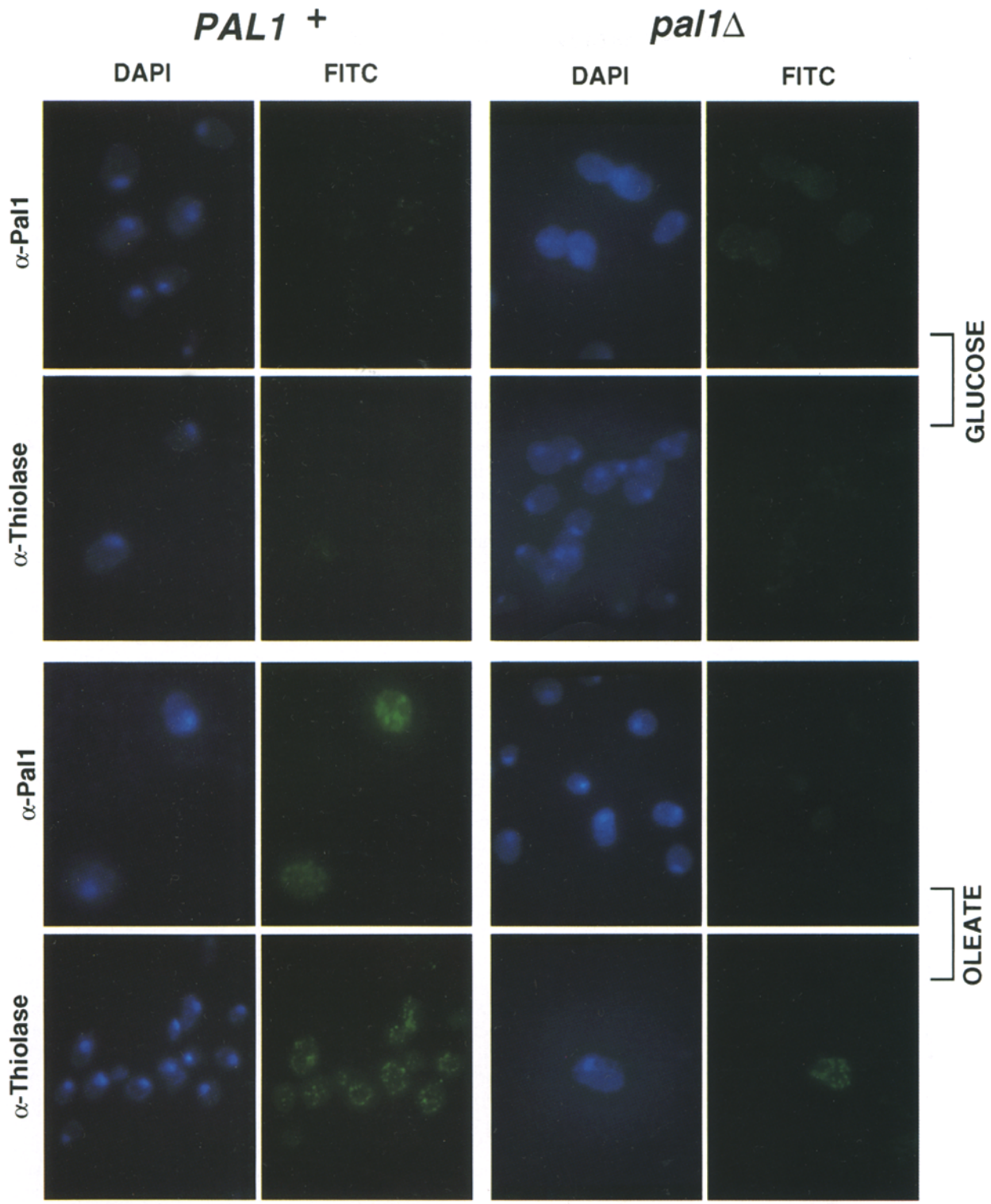

Figure 10. Localization of 3-oxoacyl-CoA thiolase to peroxisomes in *PAL1*$^+$ and *pal1Δ* mutant cells. Strain W303-1A (*PAL1*$^+$) and ESY105, its otherwise isogenic *pal1-Δ1::LEU2* derivative (*pal1Δ*), were grown either on YPGlc (*glucose*) or shifted to YPOT (*oleate*) to induce the synthesis of 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 MMYO11 and an otherwise isogenic *pal1-1Δ::LEU2* derivative were kindly performed by James McNew and Joel P. Goodman

(Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX).

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 Pal1 and 3-oxoacyl-CoA thiolase in either *PAL1*$^+$ cells and or *pal1-Δ1::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 *pal1-Δ1::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 Pal1 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 *pal1-Δ1::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 Pal1 (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. Pal1 possesses the TMS_6-NBF organization displayed by certain human ABC transporters, like Tap1 and Tap2, rather than the TMS_6-NBF-TMS_6-NBF organization displayed by Ste6, Mdr1, and cystic fibrosis transmembrane conductance regulator. The Pal1 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 Pal1 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, *PAL1* expression occurs under the same conditions that induce peroxisome biogenesis. Moreover, *pal1* 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, Pal1 comigrates with a known marker of the peroxisomal matrix, 3-oxoacyl-CoA thiolase. Finally, both Pal1 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 Pal1 in peroxisomal function remains unclear. The quantitative association of Pal1 with the particulate organellar material, the sedimentation of Pal1 nearly exclusively with the dense membrane fraction, and the inability of Pal1 to be solubilized by anything other than a strong detergent indicate that, as predicted by hydropathy analysis of its sequence, Pal1 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 Pal1 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, Pal1 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 Pal1 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 Pal1 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 Pal1 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 *pal1* null mutants to metabolize oleic acid was due to the requirement of Pal1 for the import of some essential peroxisomal enzyme. However, *pal1* 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 NH_2 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 *pal1* 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, 1992*a*), ions, drugs (reviewed in Göttesman and Pastan, 1993), and lipids (Reutz and Gros, 1994). Thus, the nature of the substrate transported by Pal1 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 Pal1 in the peroxisomal membrane, which is in progress, may help elucidate the direction of substrate transport and the role of Pal1 in peroxisomal function.

It is noteworthy that, unlike the peroxisomes in yeast *pal1* 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 (Mosser et al., 1993). Thus, it remains 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 peroxisome. 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, Pal1 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 *pal1* mutants and human X-linked adrenoleukodystrophy are rather similar, and because Pal1 and ALDp share a high level of amino acid sequence identity, further study of the *PAL1* gene product and its role in peroxisome 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 NH_2-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 *pxa1* and *pal1* 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 *pal1Δ* 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 *pxa1Δ* cells by electron microscopy.

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