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# A Novel Cytochrome $b_5$-like Domain Is Linked to the Carboxyl Terminus of the *Saccharomyces cerevisiae* Δ-9 Fatty Acid Desaturase*

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**Cytochrome $b_5$ is an amphipathic mobile membrane protein that is predominantly located at the endoplasmic reticulum surface. It is an essential component of a number of membrane-bound redox systems. In animal and fungal cells cytochrome $b_5$ is thought to be an electron donor for sterol modifying enzymes and fatty acid desaturases. Disruption of the Saccharomyces cytochrome $b_5$ gene, however, yielded cells that had no nutritional requirement for either sterols or unsaturated fatty acids. Expression of sterol and fatty acid-modifying genes was increased in the cytochrome $b_5$-disrupted cells, however, suggesting that cytochrome $b_5$ may play some nonessential role in these functions. Unsaturated fatty acids in yeast are formed by Ole1p, an oxygen-dependent Δ-9 fatty acid desaturase that is an intrinsic endoplasmic reticulum membrane protein. Although the yeast Δ-9 fatty acid desaturase does not appear to require cytochrome $b_5$, introduction of the rat liver stearoyl-CoA desaturase gene into an *ole1*-disrupted, cytochrome $b_5$-disrupted yeast strain revealed that this enzyme specifically requires cytochrome $b_5$ to function. Comparison of the coding sequences of the yeast and rat desaturase genes showed that the yeast protein contains a 113-amino acid carboxyl-terminal extension not found in the rat enzyme. That extension has regions of strong homology to cytochrome $b_5$, particularly in the heme binding and electron transfer motifs. Truncation or disruption of the desaturase cytochrome $b_5$-like domain in cells that contain the wild type diffusible $b_5$ produced unsaturated fatty acid auxotrophy, suggesting that the cytochrome $b_5$-like domain of Ole1p plays an essential role in the desaturase reaction.**

Cytochrome $b_5$ is a ubiquitous eukaryotic protein that appears to be an essential component of a number of endoplasmic reticulum-linked redox enzyme systems. Its function has been indicated in the modification of xenobiotic substances by cytochromes P450 (1) and lipogenic enzyme systems that include fatty acid desaturation (2, 3), sterol biosynthesis (4) and fatty acid elongation (5). Its ability to function in such diverse enzymatic reactions is apparently due to its structure, in which the large hydrophilic catalytic domain is linked to a short carboxyl-terminal hydrophobic peptide sequence that serves as a membrane anchor. This allows the heme protein to diffuse laterally across the membrane surface and orient its heme moiety with its electron donor and acceptors in a manner that allows for rapid electron transfer. A recent study of cytochrome $b_5$ suggests that it has a number of highly dynamic surfaces that may, in part, allow it to interact with many different substrates (6).

Evidence for the requirement of a heme-containing electron donor in lipogenic systems comes from the requirement for sterols and unsaturated fatty acids in heme-deficient yeast mutants (7). However, Truan *et al.* (8) indicated that disruption of cytochrome $b_5$ does not produce a nutritional requirement for sterols. In this paper we show that disrupted cytochrome $b_5$ does not affect the production of unsaturated fatty acids. This, and the observation that heme-deficient yeast mutants require both sterols and unsaturated fatty acids, suggests that a minor cytochrome $b_5$ isoform, or an alternative heme-containing electron donor plays a role in these essential reactions.

In the yeast *Saccharomyces*, the *OLE1* gene encodes the microsomal Δ-9 fatty acid desaturase (9). In animal and fungal cells monounsaturated fatty acids are aerobically synthesized from saturated fatty acids by this intrinsic, membrane-bound desaturase. A double bond is inserted between the 9- and 10-carbons of palmitoyl (16:0) and stearoyl (18:0) CoA to form palmitoleic (16:1) and oleic (18:1) acids. In the proposed reaction mechanism electrons are transferred from NADH-dependent cytochrome $b_5$ reductase, via the heme-containing cytochrome $b_5$ molecule, to the Δ-9 fatty acid desaturase (3). It had previously been shown that the rat liver desaturase, driven by the *OLE1* promoter and containing the first 27 amino acids of Ole1p, will rescue an *OLE1*-disrupted strain (9). Cells containing this chimeric gene were observed to have equivalent growth rates and only modest fatty acid compositional changes to wild type cells. This further indicated that the rat liver desaturase efficiently interacts with the yeast redox system. The apparent nonrequirement for cytochrome $b_5$ in fatty acid desaturation and sterol biosynthesis in gene-disrupted strains of yeast led us to examine the role of cytochrome $b_5$ in the expression and function of yeast endoplasmic reticulum lipogenic systems. This paper indicates that the *Saccharomyces* fatty acid desaturase is a modular protein that contains a carboxyl-terminal cytochrome $b_5$-like domain. Similar domains are absent from the cytochrome $b_5$-dependent rat liver desaturase and from yeast cytochrome P450 sterol biosynthetic enzymes. However, cytochrome $b_5$ appears to play some role in the regulation of these endoplasmic reticulum-based electron transport systems, as disruption of the cytochrome $b_5$ gene causes significant changes in the level of gene expression in the cytochrome P450-like *ERG11* gene and in the fatty acid desaturase gene.

## MATERIALS AND METHODS

### *Strains and Media and Recombinant DNA Methods*

The *Saccharomyces cerevisiae* strains used in this study were derived from W303 1a and 1b (Table I). Standard yeast genetics methods were

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TABLE I
*S. cerevisiae strains used in this study*

| Strain | Genotype | Source |
|---|---|---|
| W303–1a | *MATa, CYTb5, OLE1, leu2–3, leu2–112, trp1–1, can1–100, ura3–1, ade2–1, his3–11, his3–15* | L. Neigeborn |
| W303–1b | *MATα, CYTb5, OLE1, leu2–3, leu2–112, trp1–1, can1–100, ura3–1, ade2–1, his3–11, his3–15* | L. Neigeborn |
| DTY-10a | *MATa, CYTb5, OLE1, leu2–3, leu2–112, TRP1, can1–100, ura3–1, ade2–1, his3–11, his3–15* | This laboratory |
| DTY-11a | *MATα, CYTb5, OLE1, leu2–3, leu2–112, trp1–1, can1–100, ura3–1, ade2–1, HIS3* | This laboratory |
| AMY-1a | *MATa, cytb5Δ::LEU2, OLE1, TRP1, can1–100, ura3–1, ade2–1, his3–11, his3–15* | This laboratory |
| AMY-1α | *MATα, cytb5Δ::LEU2, OLE1, trp1–1, can1–100, ura3–1, ade2–1, HIS3* | This laboratory |
| AMY-2a | *MATa, CYTb5, ole1(ΔBstEII)::LEU2, TRP1, can1–100, ura3–1, ade2–1, his3–11, his3–15* | This laboratory |
| AMY-2α | *MATα, CYTb5, ole1(ΔBstEII)::LEU2, trp1–1, can1–100, ura3–1, ade2–1, HIS3* | This laboratory |
| AMY-3α | *MATα, CYTb5, ole1(ΔHpa)::LEU2, trp1–1, can1–100, ura3–1, ade2–1, HIS3* | This laboratory |
| AMY-4α | *MATα, CYTb5, ole1(Δb5:BsmFI-PacI)::LEU2, trp1–1, can1–100, ura3–1, ade2–1, HIS3* | This laboratory |
| AMY-5α | *MATα, cytb5Δ::LEU2, ole1(ΔBstEII)::LEU2, ura3–1, ade2–1* | This laboratory |

used for mating, sporulation, complementation, and construction of strains bearing the appropriate mutations (10). Cell growth conditions and growth medium for *E. coli* and yeast have been previously described (11). Yeast were transformed by electroporation (Life Technologies, Inc. cell porator) according to the manufacturer's instructions. Standard molecular biological techniques were used as described in Refs. 12 and 13.

### *Plasmid Construction*

*Yeast Cytochrome $b_5$*—The yeast cytochrome $b_5$ gene (YSCYb5, GenBank™ accession number L22494) was cloned by PCR[1] from DTY-10a genomic DNA, prepared as described by (13). PCR primers (Table II) were designed to allow independent amplification of the promoter region (primers AGM 1 and 4), the coding sequence (AGM 3 and 2), and the full-length gene (AGM 1 and 2). PCR products were subcloned using the pCRII TA cloning kit (Invitrogen) and confirmed by sequenase sequencing (U.S. Biochemical Corp.).

A gene disruption fragment was produced by removing a 282-base pair *Hin*dIII fragment that included both promoter and 5′ protein coding sequences from the 1.4-kb YSCYb5 gene. A *Hin*dIII-linkered *Kas*I-*Hpa*I *LEU2* fragment from YEP351 (14) was inserted within the region created by the *Hin*dIII digestion. An *Xba*I-*Xho*I fragment was used for the linear transformation of DTY-10a and -11a, generating yeast strains AMY-1a and AMY-1α (Fig. 1 and Table I).

*Yeast Stearoyl-CoA Desaturase (OLE1)*—To create the various *OLE1* gene disruptions, the blunt YEp351 *LEU2* (14) gene was ligated into either the YEp352/OLE 4.8 or pBS/OLE1.5 (15) plasmids. The *Hpa*I-deleted YEp352/OLE 4.8 removes the entire coding sequence of *OLE1* (AMY-3α). The *Bst*EII deletion of pBS/OLE1.5 makes a construct in which *OLE1* is disrupted but its $b_5$ domain remains intact (AMY-2a and AMY-2α). Conversely a *Bsm*FI/*Pac*I deletion of pBS/OLE1.5 (OLEΔ$b_5$) removes an internal 100 base pairs of the cytochrome $b_5$ -like domain, leaving the "desaturase" domain intact (AMY-4α) (Fig. 2).

*Rat Liver Stearoyl-CoA Desaturase*—The preparation of multiple copy plasmids bearing the rat liver stearoyl-CoA desaturase gene have been previously described (11).

*GALI Expression*—For overexpression of *OLE1* and *OLE*Δ$b_5$ in yeast, *Bam*HI fragments of pBS/OLE1.5 and pBS/OLEΔ$b_5$ were ligated into a single copy plasmid, containing the galactose (*GALI*) promoter (YCp-*GAL*), linearized with *Bam*HI.

### *Northern and Southern Blot Analysis*

Total yeast RNA was isolated using the methods of Schmitt (16) and Herrick (17). Equal amounts (10 μg) of total RNA were resolved using 1% formaldehyde gels (13). DNA was isolated and resolved as described in Ref. 13. Both RNA and DNA were transferred to a Zeta Probe™ membrane (Bio-Rad) using a vacuum blotter (Bio-Rad model 785). Prehybridization, hybridization with [α-$^{32}$P]dATP-labeled probe, and washing were carried out according to the manufacturer's instructions. Either *PGK1* or the *L32A* gene were used as internal standards. Blots were quantified by phosphor image analysis (Molecular Dynamics).

### *Preparation of Radiolabeled Probes*

DNA fragments were labeled with [α-$^{32}$P]dATP (DuPont NEN) using the PROBE-EZE (5 Prime → 3 Prime) random primer labeling kit.

[1] The abbreviations used are: PCR, polymerase chain reaction; kb, kilobase(s); *OLE1, S. cerevisiae* Δ-9 fatty acid desaturase; *ERG11, S. cerevisiae* cytochrome P450, lanosterol 14 α-demethylase; *HMG1, S. cerevisiae* 3-hydroxy-3-methylglutaryl coenzyme A reductase; *PGK1, S. cerevisiae* phosphoglycerate kinase.

TABLE II
*PCR primers used to isolate YSCYb5*

| Primer | Sequence 5′–3′ |
|---|---|
| AGM001 (forward) | GTAATATTTGCTCAATTCGGTG |
| AGM002 (reverse) | TACTTCTATGCGATATAGTAGC |
| AGM003 (forward) | CAATGATCACTAAAGTTTACAG |
| AGM004 (reverse) | CTTTAGTGATCATTGTTTG |

Unincorporated nucleotides were removed using Sephadex G-50 spin columns (5 Prime → 3 Prime).

## RESULTS AND DISCUSSION

*Cloning and Disruption of Cytochrome $b_5$*—The yeast cytochrome $b_5$ promoter and coding sequences were cloned using the PCR primers (Table II) and a strategy outlined in Fig. 1. Disruptions of the wild type gene in strains DTY-10a and -11a were made by transforming cells with a linear DNA fragment of the cloned gene, in which part of the protein coding sequence was replaced by the *Saccharomyces LEU2* gene. Strains containing the disrupted cytochrome $b_5$ gene did not exhibit a phenotype and were found to grow on synthetic medium that did not contain either sterol or fatty acid supplements. There were no significant differences in growth rates or differences in unsaturated fatty acid levels and fatty acid compositions between wild type and cytochrome $b_5$-disrupted cells (data not shown).

From earlier studies of heme biosynthetic mutants (7) it is known that a heme-containing electron donor is required for the formation of ergosterol and unsaturated fatty acids in *Saccharomyces*. It was therefore expected that if cytochrome $b_5$ was required for fatty acid desaturation and sterol biosynthesis, disruption of cytochrome $b_5$ would either knockout or adversely affect the level of these components within the yeast cell. Both Southern and Northern analysis (Figs. 3 and 4) were performed to confirm that the disrupting DNA fragment replaced the authentic cytochrome $b_5$ gene in the transformed cells. The Southern blot of *Eco*RI-cut genomic DNA prepared from DTY-10a and AMY-1a (Table I) was probed with a [α-$^{32}$P]dATP-labeled 1.4-kb cytochrome $b_5$ DNA fragment (Fig. 3). This analysis confirmed that there was a single copy of the cytochrome $b_5$ gene in DTY-10a and that this had been disrupted with the *LEU2* marker in AMY-1a. The *LEU2* gene contains a *Eco*RI site and therefore gives two bands on the Southern blot.

*Disruption of YSCY$b_5$ Alters the Expression of Lipogenic Redox Enzyme Genes*—A Northern blot of RNA from wild type and cytochrome $b_5$-disrupted cells was probed with both the 0.5-kb coding sequence of cytochrome $b_5$ and a 0.8-kb *OLE1* gene fragment (Fig. 4). This shows the absence of a cytochrome $b_5$ message in the disrupted strain. Quantitative analysis of mRNA levels by phosphor imaging, using the *Saccharomyces* ribosomal protein *L32A* gene as an internal control, indicated that the *OLE1* message is increased in the cytochrome $b_5$-

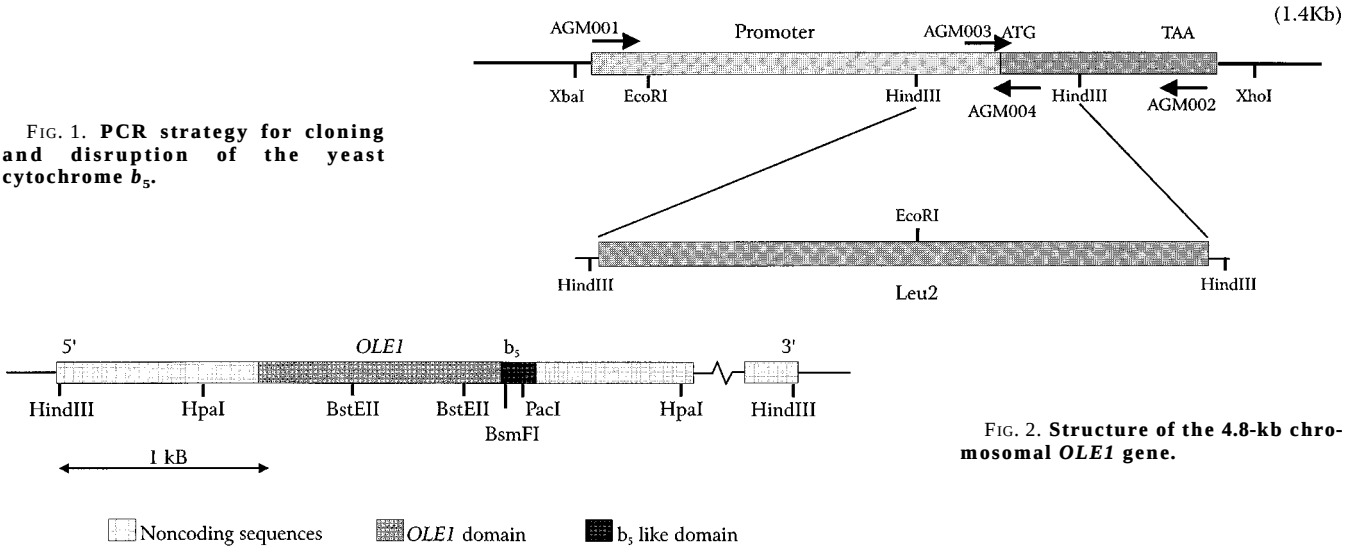


FIG. 1. **PCR strategy for cloning and disruption of the yeast cytochrome $b_5$.**

FIG. 2. **Structure of the 4.8-kb chromosomal *OLE1* gene.**

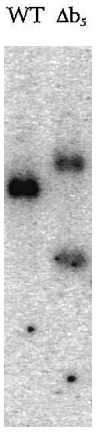


FIG. 3. **Southern blot analysis of cytochrome $b_5$-disrupted yeast.** Genomic DNA isolated from wild type strain DTY10a and strain AMY-1a were cut with *Eco*RI. The resulting blots were probed with the 1.4-kb DNA fragment that encompasses the cytochrome $b_5$ gene. A unique *Eco*RI site within the *LEU2* gene gives rise to two fragments of the predicted molecular size for insertion within the cytochrome $b_5$ locus.

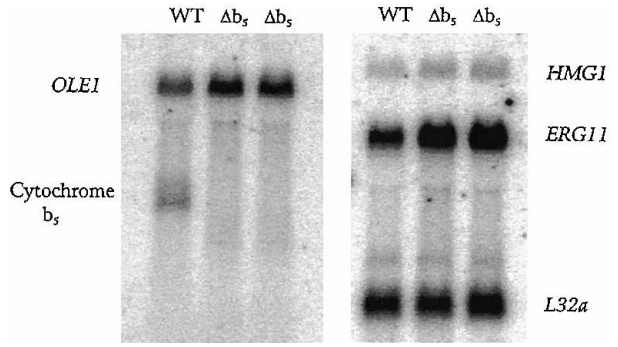


FIG. 4. **Northern blot analysis of wild type (DTY10a) and two independent cytochrome $b_5$ disruptants (AMY-1a and -1$\alpha$).** *Left panel*, phosphor images of RNA blots probed with radiolabeled *OLE1* and cytochrome $b_5$ DNA fragments. *Right panel*, blots probed with radiolabeled DNA fragments from *HMG1* (encoding HMG CoA reductase, a sterol biosynthetic enzyme), *ERG11* (a cytochrome P450 sterol demethylase), and ribosomal protein *L32A*.

disrupted strain, suggesting that it is compensating for the loss of the redox protein. To examine the effect of the loss of the cytochrome $b_5$ on sterol biosynthesis, the blot was stripped and reprobed with *ERG11*, *HMG1*, and *L32A* gene fragments. Erg11p is a cytochrome P450 sterol demethylase involved in the latter steps of sterol biosynthesis and is a potential enzyme that might require cytochrome $b_5$. Hmg1p is the dominant isoform of the two yeast HMGCoA reductase enzymes. It is a highly regulated enzyme in isoprenoid biosynthesis and is considered to be an important regulatory control point in sterol metabolism. Analysis of the resulting blots indicated that the relative levels of *OLE1*, *ERG11*, and *HMG1* transcripts, as compared with *L32A*, increased 1.8-, 3-, and 3-fold respectively in the cytochrome $b_5$-disruptant strains. However, this does not explain how these enzymes continue to function in the absence of cytochrome $b_5$.

Previous enzyme reconstitution experiments by Strittmatter *et al.* (3, 18) had indicated that mammalian fatty acid desaturase enzymes have a specific requirement for cytochrome $b_5$ as an electron donor. The *Saccharomyces* Δ9 fatty acid desaturase was initially thought to be similar in function to its homologous mammalian enzyme, given a well established heme requirement for the formation of unsaturated fatty acids (19, 20). Based on those observations, disruption of cytochrome $b_5$ in a haploid yeast strain should cause the cells to become unsaturated fatty acid auxotrophs. The prototrophy for unsaturated fatty acids in the cytochrome $b_5$-disrupted strain, however, suggested that the microsomal cytochrome $b_5$ is not essential for fatty acid desaturation. The observations that heme-deficient mutants of yeast are auxotrophic for unsaturated fatty acids indicate that the desaturase reaction requires a heme-containing molecule. In light of these findings there must be a previously unidentified alternative electron donor to the desaturase.

*Expression of the Rat Δ-9 Desaturase in Yeast Requires the Saccharomyces Cytochrome $b_5$*—Given that the enzyme reconstitution experiments of the mammalian enzymes were performed with purified cytochrome $b_5$ and desaturase enzymes in artificial liposomes raises the question of whether those enzymes might also respond to this alternative electron donor. Stukey *et al.* (9) demonstrated that the rat Δ-9 desaturase gene complements a strain carrying the disrupted *OLE1* gene and is therefore able to accept electrons from a yeast redox system. To determine whether the rat desaturase was able to function in the absence of microsomal cytochrome $b_5$, the *OLE1* gene-disrupted strain, AMY-2$\alpha$, was crossed with the cytochrome $b_5$-disrupted AMY-1a strain. The resulting diploid was subsequently sporulated, and the co-segregation of the *LEU2* markers with the requirement for unsaturated fatty acids was used to identify haploid strains (AMY-5$\alpha$) in which both the desaturase and cytochrome $b_5$ genes were disrupted. Plasmids con-

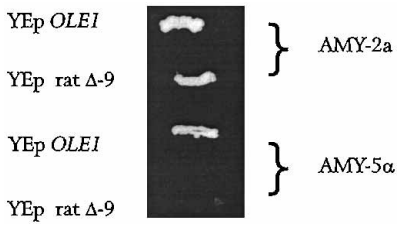


FIG. 5. **Multiple copy plasmids containing either the intact *OLE1* gene (YEp *OLE1*) or the rat stearoyl CoA-desaturase coding sequences fused to the *OLE1* promoter and its first 27 amino acids (YEp rat Δ-9) were transformed into an *OLE1*-disrupted strain, AMY-2a (*top half*) and *OLE1*, cytochrome $b_5$-disrupted strain AMY-5α (*bottom half*).** Cells were streaked on fatty acid-free synthetic complete agar containing 2% glucose as the carbon source. Plates were incubated at 30 °C for 4 days.

taining either the rat Δ-9 desaturase (under the control of the yeast *OLE1* promoter) and the YEp352/4.8*OLE1*, which contains the yeast desaturase gene (9), were transformed into the *OLE1*-disrupted (AMY-2a) and doubly disrupted strains (AMY-5α). Fig. 5 shows that both the rat and the yeast desaturase genes compliment strain AMY-2a, which contains a disrupted chromosomal *OLE1* gene and the wild type cytochrome $b_5$ gene. However, only the yeast desaturase gene compliments the strain in which chromosomal copies of the desaturase and cytochrome $b_5$ are disrupted. The rat desaturase gene fails to rescue the cells in the absence of the yeast microsomal cytochrome $b_5$. This means that *OLE1* and the ratΔ-9 desaturase differ in their interaction with the yeast redox system. *OLE1* does not require microsomal cytochrome $b_5$ and can receive electrons from an alternative electron donor. The rat desaturase requires the yeast cytochrome $b_5$ but does not have access to this alternative electron donor.

*Comparison of the Mammalian and Saccharomyces Desaturase Proteins*—Previously Stukey *et al.* (9) had compared the hydropathy profiles of the yeast and rat desaturases and observed two long hydrophobic domains, each capable of spanning the membrane twice (Fig. 6). These were common to both desaturases and were in identical positions with respect to regions of high identities between the enzymes. This gives a model in which the two hydrophobic domains anchor the molecule to the endoplasmic reticulum (9), while three hydrophilic domains, which are shown to contain the conserved histidine residues (H($X_{3\text{ or }4}$)H and two regions of H($X_{2\text{ or }3}$)HH), reside on the cytoplasmic face of the endoplasmic reticulum membrane (21). The distance between these conserved histidine motifs and the end of the previous hydrophobic domain is relatively conserved in all membrane-bound desaturases and thus thought to constitute an active site consisting of a coordinated diiron-oxo moiety that is assembled near the membrane surface (21, 22). It has been shown that mutation of any of these histidines results in loss of desaturase activity (21). Alignment of the rat and *OLE1* amino acids shows 34% identity and 64% similarity (Fig. 6). However, there is a noticeable difference in the size of the two desaturases, with *OLE1* having a 113-amino acid carboxyl-terminal extension. Analysis of this region reveals significant homology (26% identity, 46% similarity) to cytochrome $b_5$ (Fig. 7). This cytochrome $b_5$-like domain contains the conserved EHPGG($X_{10}$)DAT($X_{9-11}$)HS motif that corresponds to a heme-binding pocket (Fig. 7).

*Comparison of OLE1 Cytochrome $b_5$-like Domain with the Native Autonomous Cytochrome $b_5$*—There is a striking conservation of residues in critical regions of the Ole1p cytochrome $b_5$-like domain to those previously identified by x-ray diffraction analysis of the autonomous mammalian cytochrome $b_5$ (Fig. 8). Residues 428–488 of *OLE1* correspond to residues 21–78 from the bovine heme protein. These form a crevice that contains the heme group. The walls of this crevice are formed by two roughly antiparallel α-helices, with a floor of β-pleated sheets (23). Histidines 446 and 471 of *OLE1* correspond to the histidines 36 and 63 that bind to the heme iron group in the bovine enzyme. Furthermore, *OLE1* leucine 453 and phenylalanine 464 are identities that correspond to bovine residues 46 and 58. In the bovine enzyme these appear to form strong π-π interactions that rigidly hold the conformation of the heme-linked histidines with the iron atom in the heme group. Unlike other cytochrome $b_5$ proteins, the homologous *OLE1* motif is not linked to a carboxyl-terminal, membrane-anchoring, hydrophobic tail. Its orientation to the membrane (and to the active site of the desaturase) is apparently anchored by the transmembrane sequences associated with the "desaturase domain" of this protein.

A significant difference between the cytochrome $b_5$ domain of Ole1p and other cytochrome $b_5$ proteins is the number of acidic residues located in the region that corresponds to the bovine cytochrome $b_5$ residues 36–83. This area consists of the heme-binding pocket and residues that appear to be involved in the protein donor/acceptor interaction sites for a number of cytochrome $b_5$-mediated electron transfer reactions. Mammalian cytochrome $b_5$ proteins have 12 acidic residues within this region. By comparison plant cytochrome $b_5$s have 13, the autonomous *Saccharomyces* cytochrome $b_5$ has 11, and Ole1p has only 5 corresponding acidic residues. In diffusible cytochrome $b_5$ proteins, the surface residues in this region appear to be part of a highly dynamic face of the protein that must adapt its conformation to interact with numerous substrates (6). By contrast, the Ole1p cytochrome $b_5$-like domain presumably has only one electron-accepting substrate and therefore may not need to form as many charge-pair interactions to dock with the desaturase domain.

*Disruption of the Cytochrome $b_5$-like Motif of OLE1*—To test whether the cytochrome $b_5$ motif of *OLE1* was required for desaturase function, a *Bsm*FI-*Pac*I deletion (Fig. 2) within the *Ole1p* coding sequence was made so that 100 base pairs of the presumptive heme binding domain were removed (AMY-4a). The 3′ coding sequences following this deletion remain in frame, producing a 484-amino acid polypeptide consisting of the 410 $NH_2$-terminal residues of the desaturase domain, the $NH_2$-terminal 8 residues of the putative cytochrome $b_5$ domain, and 66 residues of the Ole1p carboxyl-terminal cytochrome $b_5$ domain. This gene was placed under the control of the *GAL1* promoter in a single copy centromere-containing vector. Transformation of this vector into the AMY-3α *OLE1* gene-disrupted strain did not repair the unsaturated fatty acid auxotrophy under galactose-induced conditions, even in the presence of the native cytochrome $b_5$ gene. By comparison, a plasmid containing the complete *OLE1* coding sequence under *GAL1* control repairs the requirement for unsaturated fatty acids in the same strain when grown in galactose and fails to repair the requirement when the *GAL1* promoter is repressed by glucose.

To create a chromosomal disruption within the cytochrome $b_5$ domain of *OLE1*, the vector carrying the *Bsm*FI-*Pac*I deletion was recut at the *Bsm*FI site (which was not destroyed) to allow insertion of the *LEU2* gene. A linear DNA fragment of this construct was used to transform the wild type DTY-10a strain. Expression of this gene in the resulting transformants (AMY-4α) theoretically produces a 458-amino acid polypeptide consisting of the amino-terminal 410-residue "desaturase domain" of *OLE1*, the $NH_2$-terminal 8 residues of the cytochrome $b_5$-like domain, and an additional 50 residues derived from bases in the upstream promoter region of *LEU2*. This disruption of the native *OLE1* gene also produced transformants that were auxotrophic for unsaturated fatty acids in cells that also

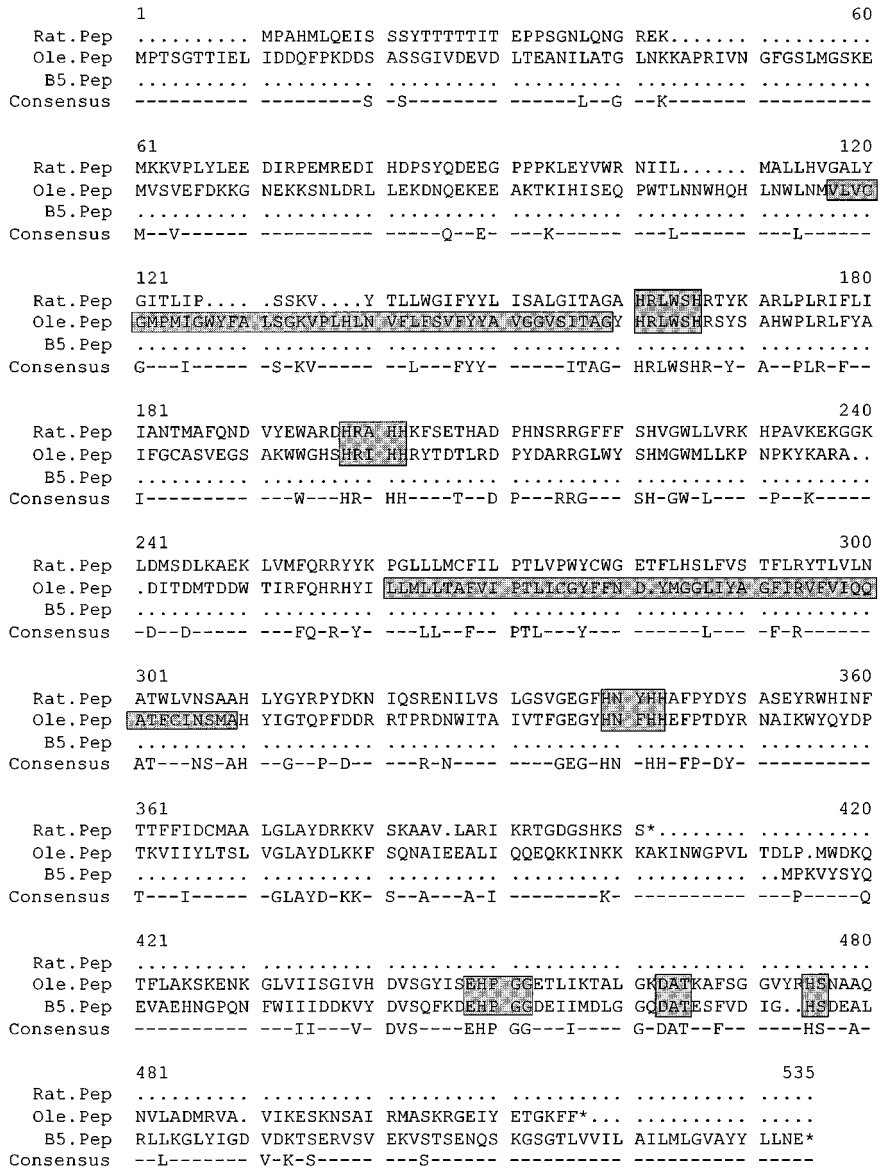


FIG. 6. **Pileup diagram of complete protein coding sequences of the yeast desaturase, Ole1p, the rat stearoyl-CoA desaturase, and the yeast cytochrome $b_5$.** *Shaded* regions in *line groups 3*, *4*, and *6* of the comparison show the location of conserved histidine motifs associated with the diiron-oxo moieties in the desaturase regions of the yeast and rat proteins. Yeast cytochrome $b_5$ aligns only with the carboxyl-terminal region of Ole1p starting at residue 413. *Shaded regions* in *line group 8* indicate the position of conserved amino acids that correspond to a heme-binding pocket.

contain a functional native cytochrome $b_5$ gene.

To determine if these modified strains were, in fact, synthesizing the appropriate *OLE1*-encoding mRNAs, Northern blot analysis was performed on cells containing the native *OLE1* gene (DTY-11a), *OLE1*-disrupted cells (AMY-3$\alpha$) containing plasmids bearing either the native *OLE1* gene or the cytochrome $b_5$ motif-disrupted form of *OLE1* under the control of the *GAL1* promoter, and cells containing the chromosomal form of *OLE1* with a disrupted cytochrome $b_5$ motif (AMY-4$\alpha$). We have previously shown that unsaturated fatty acids repress *OLE1* expression at the level of transcription and mRNA stability (24, 25). Because unsaturated acids are required for growth of cells that contain the disrupted forms of *OLE1*, cells were first grown to a density of $2 \times 10^7$/ml in the presence of 0.5 mM 16:1 and 0.5 mM 18:1 (in the repressed state). The cells were then washed and incubated in fatty acid-free medium to induce expression of the modified genes. Phosphor image analysis of the blots using the *Saccharomyces PGK1* gene as an internal control indicated that under these conditions the mRNA from the wild type *OLE1* gene was induced 18-fold following transfer to fatty acid-free medium. Messenger RNA from the chromosomal truncated *OLE1* gene was induced from undetectable levels in the repressed state to approximately ¼ that seen with the wild type gene under derepressed conditions (Fig. 9). That mRNA was at a significantly lower molecular weight than the wild type. Messenger RNAs produced under control of the *GAL1* promoter that encoded either the native *OLE1* protein coding sequence or the cytochrome $b_5$ motif-disrupted form of the protein were expressed in both unsaturated fatty acid-fed and washed cells.[2] In derepressed cells, those mRNA levels were, respectively, 81 and 45% of the wild type species. We were unable to determine if the proteins produced by these constructs are expressed. Attempts to detect native Ole1p using antibodies generated against the $NH_2$-terminal and COOH-

[2] The *OLE1* transcripts produced under the control of the *GAL1* promoter are not repressed in the presence of unsaturated fatty acids due to the absence of promoter elements involved in unsaturated fatty acid repression of *OLE1* transcription (Choi, J.-Y., Stukey, J., Hwang, S.-Y., and Martin, C. E., submitted for publication). Native *OLE1* transcripts are also regulated by unsaturated fatty acids at the level of mRNA stability. Elements of the *OLE1* 5′-untranslated region of those mRNAs essential for that regulation have been replaced by the *GAL1* mRNA 5′-untranslated region (C. Gonzalez, unpublished data).

```
                1                                                  50
      Rat.Pep   ...MAEQSDK DVKYYTLEEI QKHKDSKSTW VILHHKVYDL TKFLEEHPGG
   Rabbit.Pep   ...MAAQSDK DVKYYTLEEI KKHNHSKSTW LILHHKVYDL TKFLEEHPGG
    Human.Pep   ...MAEQSDE AVKYYTLEEI QKHNHSKSTW LILHHKVYDL TKFLEEHPGG
   Bovine.Pep   ...MAEESSK AVKYYTLEEI QKHNNSKSTW LILHYKVYDL TKFLEEHPGG
     Rice.Pep   ...MSNDNKK .V..YTLEEV AKHNSKDDCW LIIGGKVYNV SKFLEDHPGG
 Tobacco.Pep    MIIMGGE.TK .V..FTLAEV SQHNNAKDCW LVISGKVYDV TKFLDDHPGG
Brassica.Pep    ...MASE.KK .V..LGFEEV SQHNKTKDCW LIISGKVYDV TPFMDDHPGG
    Yeast.Pep   ...MP....K .V..YSYQEV AEHNGPQNFW IIIDDKVYDV SQFKDEHPGG
     Ole1.Pep   ...M...WDK Q....TF..L AKSKENKG.L VIISGIVHDV SGYISEHPGG
    Consensus   ---MA----K -V--YTLEE- -KHN--K--W LII--KVYDV TKFLEEHPGG

                51                                                 100
      Rat.Pep   EEVLREQAGG DATENF.EDV .GHSTDAREL SKTYIIGELH ......PDDR
   Rabbit.Pep   EEVLREQAGG DATENF.EDV .GHSTDAREL SKTFIIGELH ......PDDR
    Human.Pep   EEVLREQAGG DATENF.EDV .GHSTDAREM SKTFIIGELH ......PDDR
   Bovine.Pep   EEVLREQAGG DATENF.EDV .GHSTDAREL SKTFIIGELH ......PDDR
     Rice.Pep   DDVLLSSTGK DATDDF.EDV .GHTTTARAM MDEYYVGDID ..TSTIP.AR
 Tobacco.Pep    DEVLLSATGK DATDDF.EDV .GHSSSARAM LDEYYVGDID ..SATIP.TK
Brassica.Pep    DEVLLSSTGK DATNDF.EDV .GHSDTARDM MEKYYIGEID ..SSTVPATR
    Yeast.Pep   DEIIMDLGGQ DATESF.VDI .GHSDEALRL LKGLYIGDVD KTSERVSVEK
     Ole1.Pep   ETLIKTALGK DATKAFSGGV YRHSNAAQNV LADMRVAVIK ..ESKNSAIR
    Consensus   EEVL-E--G- DATE-F-EDV -GHSTDARE- LK-Y-IGEI- ------P-DR

                101                                                149
      Rat.Pep   SKIAKPSETL ITTVESNSSW WTNWVIPAIS ALVVALMYRL YMAED*...
   Rabbit.Pep   SKLSKPMETL ITTVDSNSSW WTNWVIPAIS ALIVALMYRL YMADD*...
    Human.Pep   PKLNKPPETL ITTIDSSSSW WTNWVIPAIS AVAVALMYRL YMAED*...
   Bovine.Pep   SKITKPSESI ITTIDSNPSW WTNWLIPAIS ALFVALIYHL YTSEN*...
     Rice.Pep   TKYVPPKQPH YNQDKTPEFI IKILQFLVPL AILGLAVAIR IYTKSESA*
 Tobacco.Pep    TKYTPPNQPH YNQDKTSEFV VKLLQFLVPL IILGVAFGIR FYTKQSSA*
Brassica.Pep    T.YVAPVQPA YNQDKTPEFM IKILQFLVPI LILGLALVVR QYTKKE*..
    Yeast.Pep   ...VSTSE.. .NQSKGSGTL VVILAIL... .MLGVAY... .YLLNE*..
     Ole1.Pep   M......... ..ASKRGEIY ETGKFF*... .......... .........
    Consensus   -K---P-E-- ----K----W -T---I---- A-L---M--- Y--------
```

FIG. 7. **Pileup diagram of animal, plant, and *Saccharomyces* cytochrome $b_5$ protein coding sequences compared with the Ole1p cytochrome $b_5$-like domain (*OLE1.pep*).** *Shaded* regions indicate the position of conserved amino acids that correspond to a heme-binding pocket common to cytochrome $b_5$ peptides.

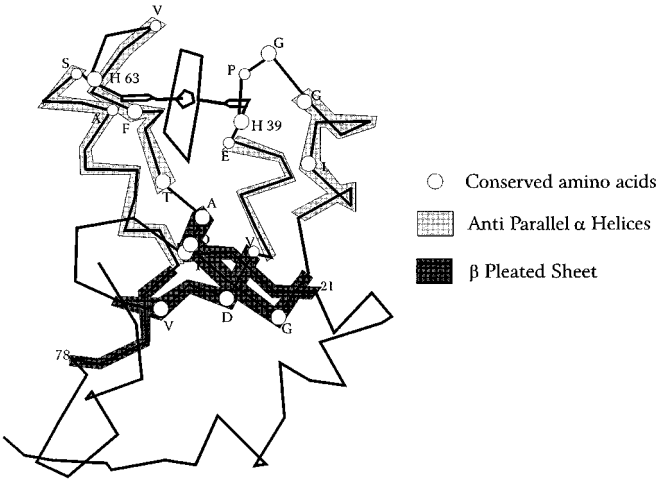


FIG. 8. **Three-dimensional diagram of bovine cytochrome *b*5 redrawn from Mathews *et al.* (23) showing positions of conserved amino acids with the cytochrome $b_5$-like domain of Ole1p, antiparallel helices associated with the heme crevice, and *β*-pleated sheet structures that form the floor of the heme pocket.**

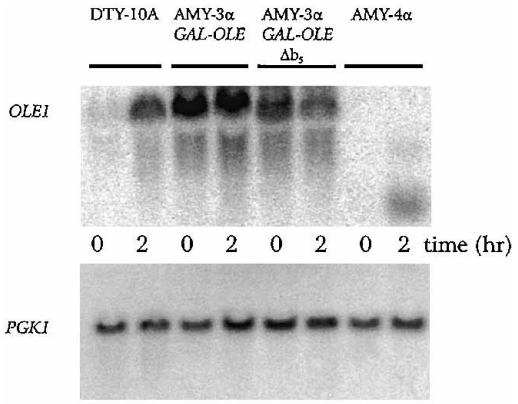


FIG. 9. **Northern blot of wild type (DTY-10a), AMY-3*α* + *YCp-GAL-OLE*, AMY-3*α* + *YCpGAL-OLE*Δb$_5$, and AMY-4*α* total RNA under repressed (unsaturated fatty acid-supplemented) and derepressed (no fatty acid supplement) conditions.** Cultures were grown to a density of $2 \times 10^7$ cells/ml in synthetic galactose medium and unsaturated fatty acids as described under "Results." At times 0 aliquots were taken for mRNA isolation. The remaining cells were washed and resuspended in nonsupplemented synthetic galactose medium and grown for 2 h to derepress transcription of genes under control of the *OLE1* promoter prior to isolation of total RNA. Blots of the fractionated RNAs were probed with radiolabeled *OLE1* and *PGK1* gene fragments.

terminal ends of the protein have not been successful.[3]

Given that deletion of residues within this domain blocks the function of the yeast desaturase, these experiments suggest that the cytochrome $b_5$-like domain functions as a primary electron donor to the desaturase domain of Ole1p. We cannot rule out the possibility, however, that the residual carboxyl-terminal peptide sequences in the truncated forms of Ole1p may block the ability of the diffusible cytochrome $b_5$ to participate in electron transfer that normally occurs in wild type cells. With certain substrates, cytochrome $b_5$ is known to provide a second electron to cytochrome P450 mixed function oxidases (26). The approximately 2-fold increase in *OLE1* mRNA

[3] Immunoprecipitations using these antibodies with [$^{35}$S]methionine-labeled cells as well as monoclonal antibodies against functional epitope-tagged forms of Ole1p suggest that the protein has an extremely short half-life (<2 min), which may account for this inability to detect the native protein by either immunoprecipitation or Western blotting methods (S. Galuska, unpublished data).

levels in cytochrome $b_5$ gene-disrupted cells may represent a compensation mechanism for decreased catalytic efficiency in the absence of the diffusible cytochrome $b_5$. Neither can we rule out the possibility that a truncated form of Ole1p similar to that of the homologous rat desaturase (with an appropriate combination of COOH-terminal residues) can accept electrons from the diffusible membrane-bound cytochrome $b_5$.

The chimeric yeast desaturase gene appears to have evolved through an event in which the $NH_2$-terminal protein coding regions of an ancestral cytochrome $b_5$ gene were fused to the carboxyl terminus of coding sequences within an independent desaturase gene. There may be some selective advantage for this type of enzyme system, given that cytochrome $b_5$ in liver fatty acid desaturation appears to diffuse laterally across the membrane surface among NADH cytochrome $b_5$ reductase, its electron donor, and the desaturase (27, 28). Tethering the cytochrome $b_5$ to the desaturase could potentially speed up the electron transfer by presenting a correctly oriented heme group with respect to the dioxo-iron cluster, eliminating the need for diffusion and reorientation of the reduced cytochrome $b_5$. The linkage of the heme domain with the desaturase domain of Ole1p raises questions, however, concerning the docking sites for electron transfer among cytochrome $b_5$, its electron donor, and electron acceptor. Fusion of the cytochrome $b_5$ domain to the desaturase probably results in a relatively fixed orientation of heme group with respect to the dioxo-iron center. If the membrane-bound form of NADH reductase or a similar membrane-tethered reductase acts as the electron donor to the Ole1p heme group, the docking site of the electron donor may reside in a different location than the site of electron transfer between the heme moiety and the desaturase iron cluster.

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# A Novel Cytochrome $b_5$-like Domain Is Linked to the Carboxyl Terminus of the *Saccharomyces cerevisiae* Δ-9 Fatty Acid Desaturase

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