Fah1p, a *Saccharomyces cerevisiae* Cytochrome $b_5$ Fusion Protein, and Its *Arabidopsis thaliana* Homolog That Lacks the Cytochrome $b_5$ Domain Both Function in the α-Hydroxylation of Sphingolipid-associated Very Long Chain Fatty Acids*

(Received for publication, January 24, 1997, and in revised form, August 25, 1997)

Andrew G. Mitchell and Charles E. Martin‡

From the Department of Biological Sciences and the Bureau of Biological Research, Rutgers University, Nelson Laboratories, P.O. Box 1059, Piscataway, New Jersey 08855-1059

A search of the *Saccharomyces cerevisiae* genome data base for cytochrome $b_5$-like sequences identified a 1.152-kilobase pair open reading frame, located on chromosome XIII at locus YMR272C (FAH1). That gene encodes a putative 384-amino-acid protein with an amino-terminal cytochrome $b_5$ domain. The $b_5$ core domain shows a 52% identity and 70% similarity to that of the yeast microsomal cytochrome $b_5$ and a 35% identity and 54% similarity to the $b_5$ core domain of *OLE1*, the *S. cerevisiae* Δ-9 fatty acid desaturase. Expression of the *S. cerevisiae* FAH1 cytochrome $b_5$ domain in *Escherichia coli* produces a soluble protein that exhibits the typical oxidized versus reduced differential absorbance spectra of cytochrome $b_5$.

Sequence analysis of Fah1p reveals other similarities to Ole1p. Both proteins are predicted to have two hydrophobic domains, each capable of spanning the membrane twice, and both have the IXX2-29(X)H motifs that are characteristic of membrane-bound fatty acid desaturases. These similarities to Ole1p suggested that Fah1p played a role in the biosynthesis or modification of fatty acids.

Disruption of the *FAH1* gene in *S. cerevisiae* did not give any visible phenotype, and there was no observable difference in content or distribution of the most abundant long chain saturated and unsaturated 14–18-carbon fatty acid species. Northern blot analysis, however, showed that this gene is expressed at much lower levels (−150-fold) than the *OLE1* gene, suggesting that it might act on a smaller subset of fatty acids. Analysis of sphingolipid-derived very long chain fatty acids revealed an approximately 40-fold reduction of α-HO 26:0 and a complementary increase in 26:0 in the gene-disrupted fahΔ strain. GAL1 expression of the *S. cerevisiae* FAH1 genes in the fahΔ strain restores α-HO 26:0 fatty acids to wild type levels. Also identified are a number of homologs to this gene in other species. Expression of an *Arabidopsis thaliana* FAHI gene, which does not contain the cytochrome $b_5$ domain, in the fahΔ strain produced an approximately 25-fold increase in α-HO 26:0 and reduced the levels of its 26-carbon precursor, suggesting that it functions in very long chain fatty acid hydroxylation using an alternate electron transfer mechanism.

Cytochrome $b_5$ is a ubiquitous eukaryotic membrane protein that is an essential component of a number of endoplasmic reticulum (ER)1-linked redox enzyme systems. One of its primary roles in animal cells is in the formation of unsaturated fatty acids. In the process of double bond formation, the membrane-bound cytochrome $b_5$ transfers electrons by lateral diffusion from NADH cytochrome $b_5$ reductase to the Δ-9 fatty acid desaturase (1, 2). Cytochrome $b_5$, however, appears to function in other fatty acid-modifying reactions. At least one step in the formation of very long chain fatty acids (20–26 carbons) is thought to be cytochrome $b_5$-dependent (3). These membrane-bound fatty acid elongation systems act independent of the soluble fatty acid synthase complex (4). Their very long chain products, which in yeast are predominantly 26 carbons in length (26:0),2 are minor but physiologically important fatty acids that are incorporated into sphingolipids. In these lipid, the hydrophobic ceramide portion is composed of the long chain base phytosphingosine, which is amide-linked to the very long chain fatty acid, the majority of which is hydroxylated at the α-position (5, 6). It is suggested that cytochrome $b_5$ is also the intermediate electron donor for α-hydroxylation (7) and that this reaction appears to involve the direct hydroxylation of a sphingolipid-bound fatty acid (8, 9). The oleate 12-hydroxylase (*FAH12*) activity of *Ricinus communis* is also shown to be dependent upon cytochrome $b_5$ (10).

The *Saccharomyces cerevisiae* genome contains a number of sequences related to cytochrome $b_5$. A gene encoding a microsomal cytochrome $b_5$ analogous to the mammalian enzyme, was cloned by Truan et al. (11). It is a small 120-amino-acid polypeptide that contains the typical carboxyl-terminal hydrophobic membrane-anchoring sequence that is necessary for its binding to the ER (12). We also recently identified a cytochrome $b_5$-like sequence as a carboxyl-terminal extension to the Δ-9 fatty acid desaturase (*OLE1*). This gene appears to have originated from a fusion of the ancestral cytochrome $b_5$ and fatty acid desaturase genes. The cytochrome $b_5$ domain of *OLE1* was shown to be essential for desaturase activity (13).

The *S. cerevisiae* Ole1p is not the only occurrence of a cytochrome $b_5$ gene fusion in eukaryotes. Our analysis of the *Histoplasma capsulatum* Δ-9 fatty acid desaturase reported by Gargano et al. (14) indicates that it also possesses a cytochrome $b_5$ carboxyl-terminal extension. An amino-terminal cytochrome

---

1 The abbreviations used are: ER, endoplasmic reticulum; OLE1p, *S. cerevisiae* Δ-9 fatty acid desaturase protein; ORF, open reading frame; GC, gas chromatography; Fah1p, *S. cerevisiae* fatty acid hydroxylase protein; PCR, polymerase chain reaction; EST, expressed sequence tag.

2 Fatty acids are denoted by a standard designation that indicates the number of carbons, followed by the number of double bonds (*e.g.* 26:0, a 26-carbon fatty acid with no double bonds; 16:1, a 16-carbon fatty acid with one double bond).

---

*The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Tel.: 732-445-4081; Fax: 732-445-5870; E-mail: martin@biology.rutgers.edu.


b5 fusion to a protein similar to an acyl lipid desaturase was found in Arabidopsis (15), and recently an amino-terminal cytochrome b5 to a Δ-6 fatty acid desaturase was identified in Borago officinalis (16).

A search of the S. cerevisiae genome revealed a presumptively cytochrome b5-containing gene, YMR272C, which also had the characteristics of a fatty acid desaturase. These enzymes have been shown to contain the conserved general motif HX$_2$-a-XH, where the histidine residues act to coordinate a μ-oxo-bridged diiron cluster (Fe-O-Fe) that functions as part of the reaction center (18, 19). These motifs are also found in the bacterial alkane hydroxylase (20) and xylene monooxygenase (21), in the plant oleate 12-hydroxylase (FAH12) (10), and in the yeast and human methyl sterol oxidase (Etg25) (22). The YMR272C ORF contains five putative HX$_2$-a-XH motifs that are conserved in its homologs.

On the basis of this gene that might encode a fatty acid-modifying enzyme, GC fatty acid methyl ester profiles of wild type and YMR272C gene-disrupted strains were compared. While no changes in fatty acid desaturation were observed, a peak fying enzyme, GC fatty acid methyl ester profiles of wild type strain. This laboratory

### MATERIALS AND METHODS

**PCR Cloning, Sequencing, and Disruption of the YMR272C ORF—** Standard molecular biological techniques were used for all cloning procedures (25, 26). Vent DNA polymerase (New England Biolabs) was used for the amplification reactions, and the resulting PCR products were subcloned into the pCRscript SK+ vector (Stratagene). Three independent amplifications were confirmed by Sequenase sequencing (U.S. Biochemical Corp.). The PCR primers AGM039 and AGM040 (Life Technologies, Inc.) (Table I) were designed to allow amplification of the YMR272C ORF (chromosome XIII base pair coordinates 810777–809623) from DTY-11A genomic DNA (Table II). This PCR product was subcloned to create pAM109. To prepare a gene disruption, the HindIII site of pAM110's multiple cloning site was first destroyed by Klenow fill in and religation (pAM111). This plasmid was then cut as BamHI/BstXI site of pAM109's multiple cloning site was first destroyed by Klenow fill in and religation (pAM111). This plasmid was then cut as BamHI and used for transformation of the DTY-10A and 11A strains. The YEASTMAKER yeast transformation system (CLONTECH) was used for these transformations. The resulting transformants were confirmed by PCR and Northern blot analysis, as described previously (13).

**PCR Cloning of an Arabidopsis thaliana Homolog—** A search of the Arabidopsis EST database revealed a number of overlapping sequences with homology to the S. cerevisiae YMR272C ORF (Z32613, R65395, S0777–809623) from DTY-11A genomic DNA (Table II). This PCR product was subcloned to create pAM109. To prepare a gene disruption, the HindIII site of pAM109's multiple cloning site was first destroyed by Klenow fill in and religation (pAM111). This plasmid was then cut as BamHI and used for transformation of the DTY-10A and 11A strain. The YEASTMAKER yeast transformation system (CLONTECH) was used for these transformations. The resulting transformants were confirmed by PCR and Northern blot analysis, as described previously (13).

**GAL1 Expression—** For overexpression of the YMR272C and A. thaliana ORF's in yeast, blunt SacI/BamHI fragments of pAM109 and pAM129 were independently ligated into a blunt HindIII/BamHI-cut YcpGAL plasmid (containing the galactose-inducible (GAL1) promoter) to create pAM132 and pAM133, respectively. These plasmids were then transformed into the YMR272C gene disruption strain (fah1Δ) (Table II).

**Expression of the Cytochrome b5 Domain in Escherichia coli—** The cytochrome b5 domain was excised from pAM109 using BamHI, which cuts within YMR272C and at the BamHI site of the polycloner. This 471-base pair fragment was subcloned into the BamHI site of pET11d (Novagen) and orientation establishing (pAM151). To remove the remaining polynucleotide and to restore the reading frame, pAM151 was cut as NotI and SalI, blunt ended with Klenow, and religated (pAM154). The pET11d and pAM154 plasmids were transformed into the BL21(DE3) pLysE E. coli expression strain. To induce expression of the recombinant protein, 2 ml of an overnight culture were used to inoculate 100 ml of LB (200 μg/ml ampicillin). The cultures were then grown with shaking (250 rpm) at 37 °C for 2 h. Thirty minutes before induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside, a 75 μg/liter concentration of the heme precursor 5-aminolevulinic acid was added (27), and the cells were then grown for 20 h at the reduced temperature of 30 °C (28). The cells were harvested by centrifugation at 5000 × g for 5 min and washed once with 100 mM Tris-HCl, pH 8, 1 mM phenylmethylsulfonyl fluoride. Cells were then lysed in the same buffer by French press, and the extract was centrifuged at 30,000 × g for 30 min. The soluble fractions were analyzed using a Perkin-Elmer Lambda 12 spectrometer. Redox absorbance spectra (400–600 nm) were obtained by comparison of air-oxidized against sodium dithionite-reduced samples. Cytochrome b5 content was determined from the a-band absorbance maxima (558 nm) of the reduced cytochrome b5 using an absorption coefficient of 26.5 mM$^{-1}$ cm$^{-1}$ (29).

**Fatty Acid Analysis and Sphingolipid Extraction—** Fatty acid methyl esters were prepared by HCl methanolysis as described previously (24). Gas chromatography was performed on a Varian 3400CX GC using a Supelcowax10 30 m × 0.32 mm column (Supelco) run at 240 °C. Data were collected and analyzed using the Class-VP Chromatography Data System version 4.1 (Shimadzu Scientific Instruments) software. Gas chromatographic electron-ionizing mass spectroscopy was performed on a Varian 3400 gas chromatograph connected to a Finnigan MAT 8230 mass spectrometer, using the same column under similar conditions. a-Hydroxyhexacosanoic acid (a-hydroxy 26:0) was obtained from Sigma.

Sphingolipid and glycerolipid fatty acids were fractionated from log-arithmetic phase cells as described by Pinto et al. (30). 5% trichloroacetic acid-washed cell pellets were subjected to mild alkaline hydrolysis. The methanolic-KOH extract, which contains saponifiable fatty acids, was then acidified and removed from the cell pellet by centrifugation. Sphingolipids were then extracted from the saponified cell pellets with an ethanolic/water/diethylether/pyridine/NH$_4$OH (15:15:5:1:0.018) solvent. After drying under nitrogen, sphingolipid fatty acid methyl esters were prepared by HCl methanolysis.

**RESULTS AND DISCUSSION**

**Identification of S. cerevisiae Cytochrome b5-like Sequences—** Cytochrome b5 is an essential component of a number of ER-linked redox enzyme systems. It functions in the modification of xenobiotic substances by P450 enzymes (35) as well as in a number of fatty acid-modifying enzyme systems, such as desaturation (1, 2), elongation (3), and hydroxylation (10, 36). A number of cytochrome b5 fusion proteins have recently been reported. These are the OLE1 gene, where the cytochrome b5 is a carboxyl-terminal extension to the desaturase domain, and two plant genes that include an Arabidopsis amino-terminal cytochrome b5 fusion to a polypeptide similar to the plant acyl lipid desaturases (15) and a Δ-6 fatty acid desaturase identified in B. officinalis (16). The animal Δ-5 fatty acid desaturases do

---

**Table I**

| Strain   | Genotype       | Source             |
|----------|----------------|--------------------|
| DTY-10A  | MATα, CYTbα, OLE1, FAH1, leu-3, leu-112, TRP1, can1-100, ura3-1, ade2-1, his3-11, his3-15 | This laboratory |
| DTY-11A  | MATα, CYTbα, OLE1, FAH1, leu-3, leu-112, trp1-1, can1-100, ura3-1, ade2-1, HIS3 | This laboratory |
| fah1Δ    | MATα, CYTbα, OLE1, fah1Δ::LEU2, trp1-1, can1-100, ura3-1, ade2-1, HIS3 | This laboratory |

---

**Table II**

| Strain | Genotype used in this study |
|--------|----------------------------|

---

3 Available on the World Wide Web at http://genome-www.stanford.edu/saccharomyces.
not possess this cytochrome $b_2$ extension and are dependent on the diffusible microsomal cytochrome $b_5$ protein, which provides electrons to the mammalian enzyme. The $b_5$ fold is also found in a number of other heme-binding proteins such as the yeast cytochrome $b_2$, sulfite oxidases, and nitrate reductases (37).

In the x-ray crystal structure of the bovine cytochrome $b_5$, residues 21–78 define a heme-containing crevice, the walls of which are formed by two roughly antiparallel $\alpha$-helices, with a floor of $\beta$-pleated sheets (31). Histidines 36 and 63 of this region are responsible for binding the heme iron group. This region is highly conserved among eukaryotic cytochromes $b_5$ and is referred to as the cytochrome $b_5$ "fold." The homologous cytochrome $b_5$ fold amino acid sequence of the yeast microsomal protein (32) was used for the database search. This search identified a number of cytochrome $b_5$-like sequences (Table III and Fig. 1), including the microsomal cytochrome $b_5$, the $b_5$ domain of OLE1, cytochrome $b_5$, and a previously undescribed sequence that has 52% identity and 70% similarity to the yeast microsomal cytochrome $b_5$ heme binding domain. This predicted 1.152-kilobase pair open reading frame is located on chromosome XIII at locus YMR272C and encodes a 384-amino acid protein that contains an approximately 100-amino acid amino-terminal cytochrome $b_5$ domain (Fig. 2).

A characteristic of the microsomal cytochrome $b_5$ is the abundance of acidic residues (Glu and Asp) located within the region that includes the heme binding pocket. These residues appear to be part of a highly dynamic surface that is able to adapt its conformation to interact with numerous substrates (33). Fig. 1 shows that this region of the microsomal cytochrome $b_5$ has 13 such residues. YMR272C is comparable in having 11, while Ole1p and cytochrome $b_2$ each have only 4 of these residues. It was previously proposed (13) that the lower number of charged residues in the $b_5$ domain of Ole1p results from the reduced need to form many charge-pair interactions with the desaturase domain because this domain is directly linked to its electron-accepting substrate. The closer homology of the $b_5$ domain of YMR272C to the native cytochrome $b_5$ may reflect a more recent evolutionary fusion event than the $b_5$ fusion to the $D$-9 fatty acid desaturase. The different positions of their $b_5$ domains also suggest that these two fusions occurred as independent evolutionary events rather than the divergence of a common $b_5$ fused ancestral gene.

**PCR Cloning and Disruption of the YMR272C Open Reading Frame**—The YMR272C ORF was PCR-amplified from wild type DTY-11A genomic DNA, and this sequence was disrupted with the LEU2 gene as described under "Materials and Methods." Linear DNA disruption of this gene in either DTY-10A or 11A did not give any visible phenotype. Wild type levels of growth were observed on media containing either glucose or the nonfermentable carbon source glycerol.

Present in the GenBank™ data base are a number of homologs to YMR272C (Z49260). Table IV shows these homologies, and Fig. 3 shows the peptide sequence alignment of these ORFs. Based on the presence of the conserved histidine residues

---

**TABLE III**

Results of a search of the S. cerevisiae genome database for the microsomal cytochrome $b_5$ fold motif

| Gene       | Chromosome | Locus | Identity | Similarity |
|------------|------------|-------|----------|------------|
| Cytochrome $b_5$ | XIV       | YNL111C | 100      | 100        |
| Identified as FAH1 | XIII    | YMR272C | 52.2     | 69.6       |
| OLE1      | VII       | YGL055W | 43.5     | 60.9       |
| Cytochrome $b_2$ | XIII     | YML054C | 35.3     | 60.0       |

**Fig. 1. Alignment of S. cerevisiae cytochrome $b_5$ domains.** The cytochrome $b_5$-like domains identified in Table III were aligned using the GCG Pileup program. Homologies between these sequences were highlighted using the BOXSHADE program (available on the World Wide Web at http://ulrec3.unil.ch/software/BOX_form.html).

**Fig. 2. Nucleotide and amino acid sequence of the YMR272C ORF.** Marked are the cytochrome $b_5$ core domain (underlined), histidine motifs (boxed), and the transmembrane domains (shaded).
dues of the $b_5$ fold, the Caenorhabditis elegans (Z81038) sequence appears to have a cytochrome $b_5$-like region; however, the Schizosaccharomyces pombe (Z97209) and a reconstructed A. thaliana sequence (Fig. 4) do not.

E. coli Expression of the YMR272C Cytochrome $b_5$ Domain—To demonstrate that the N-terminal extension of YMR272C coded for a cytochrome $b_5$ domain, a 471-base pair fragment was cut from the pAM109 plasmid, and subcloned into the pET11d E. coli expression vector, as described under “Materials and Methods.” This fragment encodes 150 amino acids of a soluble cytochrome $b_5$. As reported for the expression of other cytochromes $b_5$ (16, 34), after 20 h of induction at the lower temperature of 30 °C (28), cells containing the cytochrome $b_5$ sequence appear reddish in color, as compared with pET11d control cell inductions. Spectral analysis of a soluble extract from these cells demonstrates the oxidized versus reduced absorbance differential typical of a cytochrome $b_5$. Absorbance maxima are observed at 558 and 426 nm (Fig. 5). Using the absorption coefficient of 26.5 mM$^{-1}$ cm$^{-1}$ for the $a$-band (558 nm) (29), the cytochrome $b_5$ content was determined to be 7.6 μg/mg of soluble protein extract. This low expression level compares with that reported by Sperling et al. (15) for the expression of the Arabidopsis acyl desaturase cytochrome $b_5$ domain. Similarly, the recombinant protein was not detected by SDS-PAGE in initial soluble cell lysate (data not shown).

PCR Cloning of the A. thaliana FAH1 Open Reading Frame—A number of A. thaliana ESTs that have homology to the S. cerevisiae FAH1 gene are present in the GenBank™ database. This sequence information, which covers almost the entire coding sequence from a cDNA library (as described under “Materials and Methods”), allowed us to design PCR primers to amplify the entire coding sequence from a cDNA library (as described under “Materials and Methods”). The sequence of the resulting PCR was found to be consistent with the overlapping EST data, except for a single base discrepancy (C to A at base pair 565; Fig. 4), which converts a threonine to a lysine residue. The lysine residue appears to be more consistent with the multiple sequence alignment data shown in Fig. 3. There is a region of this ORF that is not covered by an EST. The sequence and ORF presented for this region (lowercase letters in Fig. 4) comes from sequenced PCR products (GenBank™, accession number AF021804). This A. thaliana Fah1p shows 42.6% identity and 62.1% similarity to the S. cerevisiae Fah1p. From the EST data there does not appear to be any cytochrome $b_5$-like sequences in any reading frame 5′ to the predicted start methionine.

Sequence Analysis—Examination of the non-$b_5$ domain of YMR272C revealed a number of motifs that are found in lipogenic enzymes. Sequence comparisons between known membrane-bound desaturases, including OLE1 and the Arabidopsis sequence (15), revealed the conserved general motif HX$_{2–3}$(XH)H. These are a characteristic of membrane-bound desaturases (19) but are also found in the bacterial alkane hydroxylase (20) and xylene monooxygenase (21), in the plant oleate 12-hydroxylase (10), and in the yeast and human methyl sterol oxidase (22). The histidine residues act to coordinate a μ-oxo diiron cluster (Fe-O-Fe) that functions as part of the reaction center. Removal of any one of these histidines from the rat Δ9 fatty acid desaturase was shown to disrupt desaturase function (19). Ole1p contains three of these motifs, two HX$_2$H and one HX$_3$H. Examination of the S. cerevisiae, S. pombe, C. elegans, and A. thaliana amino acid sequences shows five conserved histidine-rich motifs (two HX$_2$H, one HX$_3$H, a HX$_4$H, and a (D/H)$_2$H). Figs. 2–4 show the sequence and positioning of these motifs.

Hydrophobic Analysis—Stukey et al. (38) proposed a model for Ole1p in which there are two long hydrophobic domains, each capable of spanning the membrane twice (Fig. 6). This allows the three histidine-containing motifs to lie on the cytoplasmic face of the ER membrane. Similarly, transmembrane sequence analysis of the A. thaliana Fah1p revealed a number of motifs that are found in lipogenic enzymes. Sequence comparisons between known membrane-bound desaturases, including OLE1 and the Arabidopsis sequence (15), revealed the conserved general motif HX$_{2–3}$(XH)H. These are a characteristic of membrane-bound desaturases (19) but are also found in the bacterial alkane hydroxylase (20) and xylene monooxygenase (21), in the plant oleate 12-hydroxylase (10), and in the yeast and human methyl sterol oxidase (22). The histidine residues act to coordinate a μ-oxo diiron cluster (Fe-O-Fe) that functions as part of the reaction center. Removal of any one of these histidines from the rat Δ9 fatty acid desaturase was shown to disrupt desaturase function (19). Ole1p contains three of these motifs, two HX$_2$H and one HX$_3$H. Examination of the S. cerevisiae, S. pombe, C. elegans, and A. thaliana amino acid sequences shows five conserved histidine-rich motifs (two HX$_2$H, one HX$_3$H, a HX$_4$H, and a (D/H)$_2$H). Figs. 2–4 show the sequence and positioning of these motifs.

Hydrophobic Analysis—Stukey et al. (38) proposed a model for Ole1p in which there are two long hydrophobic domains, each capable of spanning the membrane twice (Fig. 6). This allows the three histidine-containing motifs to lie on the cytoplasmic face of the ER membrane. Similarly, transmembrane sequence analysis of the A. thaliana Fah1p revealed a number of motifs that are found in lipogenic enzymes. Sequence comparisons between known membrane-bound desaturases, including OLE1 and the Arabidopsis sequence (15), revealed the conserved general motif HX$_{2–3}$(XH)H. These are a characteristic of membrane-bound desaturases (19) but are also found in the bacterial alkane hydroxylase (20) and xylene monooxygenase (21), in the plant oleate 12-hydroxylase (10), and in the yeast and human methyl sterol oxidase (22). The histidine residues act to coordinate a μ-oxo diiron cluster (Fe-O-Fe) that functions as part of the reaction center. Removal of any one of these histidines from the rat Δ9 fatty acid desaturase was shown to disrupt desaturase function (19). Ole1p contains three of these motifs, two HX$_2$H and one HX$_3$H. Examination of the S. cerevisiae, S. pombe, C. elegans, and A. thaliana amino acid sequences shows five conserved histidine-rich motifs (two HX$_2$H, one HX$_3$H, a HX$_4$H, and a (D/H)$_2$H). Figs. 2–4 show the sequence and positioning of these motifs.
α-Hydroxylation of Sphingolipid Very Long Chain Fatty Acids

28285

1 GTGTTTTTTTGGATTATGCTATCGTTGTGACGCGCTATGATGGGCTCGCACTGCACTC
60 CACATTACGGCTGATGTGCTACTTCTACATGCTATACGCGCTGGTGTCTTGTTC
120 ATGTGCTCTGCGCGCCACGACGACGACTTCATGCGACGGCGGAGCTTTCTCAC
180 GCTGGGCTTTCTTTTCTTCTGCTGATGGGCTCTTGCGTGTGCTGCCTCC
240 AGCTGCTCTGCGCGCCACGACGACGACTTCATGCGACGGCGGAGCTTTCTCAC
300 CCGGAGAGTAGTGGGATGGGTGCTCCACACTTATGTCAGGCGCTGGTCTCC
360 LQEDYE SVGRQPIATKEGPR

FIG. 4. Nucleotide and amino acid sequence of the A. thaliana FAH1 ORF. Marked are the histidine motifs (boxed), and the transmembrane domains (shaded). Lowercase nucleotide sequence indicates data not covered by any ESTs.

predictions⁴ for YMR273C and its homologs show the two hydrophobic domains, each of which is sufficient for two passes of the membrane bilayer (Fig. 6). As expected, there is a striking conservation of size and hydrophobicity of these transmembrane domains among the four Fah1p homologs. These data, taken together with the presence of the histidine motifs, suggest a similar topology to that proposed for OLE1. The relative positioning of the histidine motifs to the transmembrane domains are also consistent with the findings of Shanklin and co-workers that these are usually located close to, but not within, the membrane-spanning sections (19, 39) (Figs. 2 and 4). These properties suggest that the active site is assembled within, the membrane-spanning sections (19, 39) (Figs. 2 and 4). These properties suggest that the active site is assembled within, the membrane-spanning sections (19, 39) (Figs. 2 and 4). These properties suggest that the active site is assembled within, the membrane-spanning sections (19, 39) (Figs. 2 and 4). These properties suggest that the active site is assembled within, the membrane-spanning sections (19, 39) (Figs. 2 and 4). These properties suggest that the active site is assembled within, the membrane-spanning sections (19, 39) (Figs. 2 and 4). These properties suggest that the active site is assembled within, the membrane-spanning sections (19, 39) (Figs. 2 and 4). These properties suggest that the active site is assembled within, the membrane-spanning sections (19, 39) (Figs. 2 and 4). These properties suggest that the active site is assembled within, the membrane-spanning sections (19, 39) (Figs. 2 and 4). These properties suggest that the active site is assembled within, the membrane-spanning sections (19, 39) (Figs. 2 and 4). These properties suggest that the active site is assembled within, the membrane-spanning sections (19, 39) (Figs. 2 and 4). These properties suggest that the active site is assembled within, the membrane-spanning sections (19, 39) (Figs. 2 and 4). These properties suggest that the active site is assembled within, the membrane-spanning sections (19, 39) (Figs. 2 and 4). These properties suggest that the active site is assembled within, the membrane-spanning sections (19, 39) (Figs. 2 and 4). These properties suggest that the active site is assembled within, the membrane-spanning sections (19, 39) (Figs. 2 and 4). These properties suggest that the active site is assembled within, the membrane-spanning sections (19, 39) (Figs. 2 and 4). These properties suggest that the active site is assembled within, the membrane-spanning sections (19, 39) (Figs. 2 and 4). These properties suggest that the active site is assembled within, the membrane-spanning sections (19, 39) (Figs. 2 and 4). These properties suggest that the active site is assembled within, the membrane-spanning sections (19, 39) (Figs. 2 and 4). These properties suggest that the active site is assembled within, the membrane-spanning sections (19, 39) (Figs. 2 and 4). These properties suggest that the active site is assembled within, the membrane-spanning sections (19, 39) (Figs. 2 and 4). These properties suggest that the active site is assembled within, the membrane-spanning sections (19, 39) (Figs. 2 and 4). These properties suggest that the active site is assembled within, the membrane-spanning sections (19, 39) (Figs. 2 and 4). These properties suggest that the active site is assembled within, the membrane-spanning sections (19, 39) (Figs. 2 and 4).

Northern Blot Analysis—A Northern blot of wild type DTY-11A RNA was probed with OLE1, cytochrome b₅, and the YMR272C sequences and quantified by phosphor imaging. The remaining lipids from the cell pellet were then extracted as described by Pinto et al. (30), a procedure used to quantitatively solubilize sphingolipids. The sphingolipid frac-

⁴ Available on the World Wide Web at http://ubrec3.unil.ch/software/TMPRED_form.html.
tion extracted by that procedure was then subjected to acid methanolysis to determine the acyl content of those lipids. Fig. 9, a and b, compares the GC profiles of total fatty acid methyl esters of trichloroacetic acid-washed wild type DTY-11A and the fah1Δ cells. As previously shown (Fig. 8), the gene-disrupted strain shows the loss of the α-HO 26:0 peak and an increase in the 26:0 peak. Fig. 9, c–f, shows fatty acid methyl esters derived from the extraction procedure. Traces c and d are derived from the sphingolipid fraction. These fractions contain very low levels of 14–18-carbon species, which is typical of sphingolipids. As expected, Fig. 9c (wild type) shows that the very long chain fatty acids are predominantly the 26-carbon species of which 80% is hydroxylated. Fig. 9d, however, shows that the primary sphingolipid species in fah1Δ is 26:0 and that the α-HO 26:0 peak represents less that 0.5% of that fraction. The residual HO 26:0 appears to be an intermediate product of the very long chain fatty acid elongation cycle rather than a product of an independent hydroxylase.

Fig. 9, e and f, shows fatty acid methyl esters from the alkali-labile lipids removed in the extraction procedure. The low amount of α-HO 26:0 in the wild type alkali-labile fraction in trace e (~0.1%) demonstrates, first, that virtually all sphingolipids are retained in the cell pellet by this method and, second, that there is a pool of 26:0 fatty acid that is not sphingolipid-bound. This is consistent with earlier reports that some 26:0 species are found in triglycerides (6). The absence of α-HO 26:0 in this pool also demonstrates the specificity of the hydroxylating enzyme for sphingolipid acyl groups. This observation is further supported by reports of mutant strains of S. cerevisiae that do not make sphingolipids when cultured without the sphingolipid long chain base phytosphingosine (9). These mutants are auxotrophic for long chain bases, and when grown with phytosphingosine they make sphingolipids in normal amounts. Suppressor mutants of these strains bypass the need to synthesize this long chain base by making novel inositol glycerophospholipids that structurally mimic sphingolipids and therefore compensate for some sphingolipid function(s) necessary for growth. These phosphatidylinositol-containing lipids contain one molecule of 26:0 fatty acid (5) but apparently do not contain hydroxylated 26:0, suggesting that these O-acyl glycerolipids are not substrates for hydroxylation.

GAL1 Expression of the S. cerevisiae and A. thaliana ORFs—To demonstrate that the PCR-cloned genes were able to repair the disruption strain phenotype, the YCpGAL, pAM132, and pAM133 plasmids were independently transformed into the fah1Δ strain. Cells were grown in galactose-containing...
media, sphingolipids were extracted, and fatty acid methyl esters were prepared as described under "Materials and Methods." Under those conditions, 14–18-carbon fatty acid methyl esters have retention times of between 6 and 8 min. The 26:0 and hydroxy-26:0 species have retention times of 17.5 and 38.5 min, respectively.

Analysis of sphingolipid-derived very long chain fatty acids revealed an approximately 40-fold reduction of $\alpha$-HO 26:0 and a complementary increase in 26:0 in the gene-disrupted fah1 strain as compared with wild type (Fig. 10). Expression of the S. cerevisiae FAH1 gene from the GAL1 promoter (pAM132), restored the $\alpha$-HO 26:0 fatty acid to wild type levels in the fah1 strain. Expression of the A. thaliana gene (pAM133), which does not contain the cytochrome $b_5$ domain, in the fah1 strain produced an approximately 25-fold increase in $\alpha$-HO 26:0 and reduced levels of its 26-carbon precursor, indicating that it plays a similar role in very long chain fatty acid hydroxylation, apparently using an alternate electron donor. This activity of the A. thaliana homolog, which lacks a cytochrome $b_5$ domain, in the fah1 strain, parallels the observation of Stukey et al. (38) that the rat $\Delta 9$ desaturase, which also lacks a cytochrome $b_5$ domain, functions in an ole1 strain.

**Conclusions**—We believe this to be the first report of the identification of a gene that acts in the hydroxylation of very long chain fatty acids. Extraction of sphingolipids demonstrated the specificity of this hydroxylase activity for the very long chain fatty acids associated with these lipids. Presumably, it encodes for the hydroxylating enzyme or one of its components, although conclusive proof will require expression and demonstration of its activity in a heterologous system. The ability of the homologous A. thaliana gene to substantially repair the hydroxylation defect also reinforces the idea that the gene encodes a component of the hydroxylation system.

As would be expected from the similarity of the reaction mechanisms for desaturases and hydroxylases (19), this S.

**FIG. 8.** GC analysis of fatty acid methyl esters of wild type and fah1 strains. Fatty acid methyl esters were prepared and analyzed by gas chromatography as described under "Materials and Methods." Under those conditions, 14–18-carbon fatty acid methyl esters have retention times of between 6 and 8 min. The 26:0 and hydroxy-26:0 species have retention times of 17.5 and 38.5 min, respectively.

**FIG. 9.** fah1 cells are defective in the $\alpha$-hydroxylation of sphingolipid-bound 26:0 fatty acid. The fatty acyl composition of sphingolipids was compared with that of the glycerolipid fraction and total cellular fatty acids. Percentages indicated refer to the wild type percentage of total fatty acids. Total cellular fatty acids were extracted from trichloroacetic acid-washed wild type DTY-11A (a) and YMR272C (fah1A)-disrupted (b) cells by HCl methanolysis. Sphingolipids were extracted from whole cells as described by Pinto et al. (30). After drying under nitrogen, these were then subjected to HCl methanolysis. The sphingolipid-associated fatty acids are shown for wild type DTY-11A (c) and the fah1A (d) strain. The alkaline-labile fraction (see "Materials and Methods") was also dried and further extracted by HCl methanolysis. Non-sphingolipid-associated fatty acids are similarly shown for wild type DTY-11A (e) and the fah1A strain (f).

**FIG. 10.** GAL1 expression of the FAH1 gene. Sphingolipids were extracted and fatty methyl esters derived from galactose-grown cells of wild type DTY-11A and the fah1A strain carrying the YCpGAL, pAM132, and pAM133 plasmids. The percentages refer to wild type percentage of 26 carbon fatty acid.
*α*-Hydroxylation of Sphingolipid Very Long Chain Fatty Acids

cerevisiae α-hydroxylase gene, FAH1, has a similar topology to OLE1. Both possess two hydrophobic domains, each capable of spanning the membrane twice, and contain the histidine-rich motifs associated with coordination of the μ-oxo-bridged diiron cluster. Surprisingly, both *S. cerevisiae* genes (OLE1 and FAH1) have fused cytochrome b5 domains. Because the positioning of these b5 domains differ, it would appear that the two fusions occurred as independent evolutionary events rather than the divergence of a common b5 fused ancestral gene. The closer homology of the b5 domain of FAH1 to the native cytochrome b5 may reflect a more recent evolutionary fusion event than that which selected for the b5 fusion to the Δ9 fatty acid desaturase. It is still not clear why *S. cerevisiae* has evolved these two cytochrome b5 fusion proteins, but one can speculate that their presence confers some evolutionary selectable advantage.

REFERENCES
1. Dailey, H. A., and Strittmatter, P. (1978) *J. Biol. Chem.* 253, 8203–8209
2. Dailey, H. A., and Strittmatter, P. (1980) *J. Biol. Chem.* 255, 5184–5189
3. Cinti, D. L., Cook, L., Nagi, M. N., and Suneja, S. K. (1991) *Eur. J. Biochem.* 199, 1–51
4. Bessoule, J., Lessire, R., Rigoulet, M., Guein, B., and Casagne, C. (1988) *Eur. J. Biochem.* 177, 207–211
5. Lester, R. L., Wells, G. B., Oxford, G., and Dickson, R. C. (1990) *J. Biol. Chem.* 265, 8543–8553
6. Nurminen, T., and Suomalainen, H. (1971) *Biochem. J.* 125, 963–969
7. Shigematsu, H., and Kishimoto, Y. (1987) *Int. J. Biochem.* 207–211
8. Kaya, K., Ramesha, C. S., and Thompson, G. A., Jr. (1984) *J. Biol. Chem.* 259, 3543–3553
9. Dickson, R. C., Wells, G. B., Schmidt, A., and Lester, R. L. (1990) *Mol. Cell. Biol.* 10, 2176–2181
10. Smith, M. A., Jonsson, L., Stymne, S., and Stobart, K. (1992) *Biochem. J.* 287, 141–144
11. Truan, G., Epinat, J.-C., Rouguerelle, C., Cullin, C., and Pompon, D. (1994) *Gene* (Amst.) 149, 123–127
12. Vergeres, G., and Waskell, J. (1992) *J. Biol. Chem.* 267, 12583–12591
13. Mitchell, A. G., and Martin, C. E. (1995) *J. Biol. Chem.* 270, 29766–29772
14. Gargano, S., Di Lallo, G., Kobayashi, G. S., and Maresca, B. (1995) *Lipids* 30, 899–906
15. Sperling, P., Schmidt, H., and Heinz, E. (1995) *Eur. J. Biochem.* 232, 798–805
16. Sayanova, O., Smith, M. A., Lapinskas, P., Stobart, A. K., Debon, G., Christie, W. W., Shewry, P. R., and Napier, J. A. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 4211–4216
17. Elledge, S. J., Mulligan, J. T., Ramer, S. W., and Davis, R. W. (1990) *Proc. Natl. Acad. Sci. U. S. A.* 87, 1731–1735
18. Shanklin, J., Whittle, E., and Fox, B. G. (1994) *Biochemistry* 33, 12787–12794
19. Fox, B. G., Shanklin, J., Somerville, C. R., and Munck, E. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 2486–2490
20. Kok, M., Oldenburs, R. v., van der Linden, M. P. G., Raatjes, P., Kingma, J., van Lelyveld, P. H., and Wiholt, B. (1989) *J. Biol. Chem.* 264, 5435–5441
21. Suzuki, M., Hayakawa, T., Shaw, J. P., Reik, M., and Harayama, S. (1991) *J. Bacteriol.* 173, 1690–1695
22. Liangtao, L., and Kaplan, J. (1996) *J. Biol. Chem.* 271, 16927–16933
23. Sherman, F., Fink, G. R., and Lawrence, C. W. (1982) *Methods in Yeast Genetics: A Course Manual*, Cold Spring Harbor Press, Cold Spring Harbor, NY
24. Stukey, J. E., McDonough, V. M., and Martin, C. E. (1989) *J. Biol. Chem.* 264, 16537–16544
25. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1998) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Press, Cold Spring Harbor, NY
26. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds.) 1995 *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc., New York
27. Nishimoto, M., Clark, J. E., and Siler Masters, B. S. (1989) *Biochemistry* 28, 8660–8670
28. Schein, C. H., and Noteborn, M. H. M. (1989) *Bio/Technology* 7, 1141–1148
29. Ozol, J. (1974) *Biochemistry* 13, 426–434
30. Pinto, W. J., Srinivasan, B., Shepherd, S., Schmidt, A., Dickson, R. C., and Lester, R. L. (1992) *J. Bacteriol.* 174, 2565–2574
31. Mathews, F. S., Levine, M., and Argos, P. (1976) *J. Mol. Biol.* 120, 449–464
32. Lederer, F. (1979) *Biochemistry* 18, 674–692
33. Stoch, E. M., and Daggett, V. (1995) *Biochemistry* 34, 9682–9693
34. Smith, M. A., Napier, S. A., Stymne, S., Tatham, A. S., Shewry, P. R., and Stobart, A. K. (1994) *Biochem. J.* 303, 73–79
35. Guengerich, F. P. (1991) *J. Biol. Chem.* 266, 10019–10022
36. Van De Loo, F. J., Broun, P., Turner, S., and Somerville, C. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 6743–6747
37. Guiard, B., and Lederer, F. (1989) *J. Mol. Biol.* 217, 674–692
38. Stukey, J. E., McDonough, V. M., and Martin, C. E. (1990) *J. Biol. Chem.* 265, 10019–10022
39. Fox, B. G., Shanklin, J., Ai, J., Loehr, T. M., and Sanders-Loehr, J. (1994) *Biochemistry* 33, 12776–12786