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A *Saccharomyces cerevisiae* Gene Required for Heterologous Fatty Acid Elongase Activity Encodes a Microsomal β-Keto-reductase*

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A number of *Saccharomyces cerevisiae* membrane-bound oxidoreductases were examined for potential roles in microsomal fatty acid elongation, by assaying heterologous elongating activities in individual deletion mutants. One yeast gene, YBR159w, was identified as being required for activity of both the *Caenorhabditis elegans* elongase PEA1 (F56H11.4) and the *Arabidopsis thaliana* elongase FAE1. Ybr159p shows some limited homology to human steroid dehydrogenases and is a member of the short-chain alcohol dehydrogenase superfamily. Disruption of YBR159w is not lethal, in contrast to previous reports, although the mutants are slow growing and display high temperature sensitivity. Both Ybr159p and an *Arabidopsis* homologue were shown to restore heterologous elongase activities when expressed in *ybr159*Δ mutants. Biochemical characterization of microsomal preparations from *ybr159*Δ cells revealed a primary perturbation in β-ketoacyl reduction, confirming the assignment of YBR159w as encoding a component of the microsomal elongase.

Unsaturated fatty acids are essential cellular constituents, serving not only as structural components of membranes but also as bioactive metabolites. One important class of these lipids is collectively known as polyunsaturated fatty acids (PUFAs)[1]; these are defined as fatty acids of 18 carbons or more (C_{18+}), which contain two or more double bonds (1). In mammals, 20 carbon (C_{20}) PUFAs have been shown to be the biological precursors of a group of molecules called the eicosanoids, which includes the prostaglandins, leukotrienes, and thromboxanes (2). The eicosanoids have roles in inflammation responses as well as cardiac and reproductive function. This obvious importance of PUFAs has resulted in considerable interest in the characterization of their biosynthetic pathway (3), and we have identified a new class of fatty acid desaturases required for PUFA biosynthesis, which contain an N-terminally fused cytochrome b_5 domain (4).

More recently, we have also identified a component of the PUFA fatty acyl-chain elongation system, which when heterologously expressed in yeast, directs the C_2 elongation of the C_{18} PUFA γ-linolenic acid (18:3(*n*-6); GLA) to the C_{20} PUFA di-homo-γ-linolenic acid (20:3(*n*-6)) (5). This component (*Caenorhabditis elegans* ORF F56H11.4, designated PEA1 for polyunsaturated fatty acid elongating activity (5, 6)) shows some limited homology to the yeast *ELO* gene family, which have been shown genetically to be required for the synthesis of saturated medium and very long-chain fatty acids (7, 8). Although the precise biochemical function of the *C. elegans* PUFA-elongating activity PEA1 and the polypeptides encoded by the *ELO* genes remain to be elucidated, it is generally considered that they serve as condensing enzymes (5, 6). The steps in the biosynthetic pathway of C_{20} PUFAs are indicated in a generalized scheme (Fig. 1), with the key step of C_2 elongation of C_{18} substrates highlighted.

The biosynthesis of C_{20+} fatty acids has also been observed in higher plants, although the elongated fatty acids are quite distinct to those observed in mammals. Although C_{18} PUFAs such as α-linolenic acid and linoleic acid accumulate to high levels in plants, the presence of C_{20} PUFAs has not been observed (1). However, some higher plants, most notably members of the Brassicas such as oil seed rape and *Arabidopsis*, synthesize C_{20} and C_{22} monounsaturated fatty acids (MUFAs) (9). These C_{20+} fatty acids are the product of (several cycles of) C_2 fatty acyl elongation of a monounsaturated C_{18} substrate, oleic acid ($18:1\Delta^9$). The biosynthesis of higher plant $C_{20/22}$ MUFAs has been shown to require a single gene, FAE1 (fatty acid elongation), which encodes a putative condensing enzyme (10). The Fae1p condensing enzyme displays specificity only for saturated and monounsaturated fatty acids (9, 10). Importantly, although Fae1p shows some limited homology to analogous condensing enzymes such as chalcone and stilbene synthases, it displays no similarity to the Elo protein family; conversely no homologues of Fae1p are present in yeast.

Fatty acid elongation is carried out by a microsomal "elongase," which consists of four distinct enzymatic reactions (11). In sequential order, these are a condensation reaction between a CoA-esterified fatty acyl substrate and malonyl-CoA, β-keto reduction, dehydration, and a final enoyl reduction. It has been hypothesized that the specificity of any particular elongation reaction is conferred through the selectivity of the first condensation step. Conversely it is believed that the three other components (two reductases and a dehydratase) are common to all microsomal fatty acyl elongases and have no particular substrate specificity (11). This is given credence by the observation that heterologous expression in yeast of the plant Fae1p condensing enzyme successfully reconstitutes a functional C_{20+} MUFA-specific elongase (9). Similarly, we have also observed effective reconstitution of the PUFA-specific elongase (via by

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[1] The abbreviations used are: PUFA, polyunsaturated fatty acid; GLA, γ-linolenic acid; MUFA, monounsaturated fatty acid; ORF, open reading frame; PEA1, polyunsaturated fatty acid elongating activity; FAE1, fatty acid elongation; ts, temperature-sensitive; ER, endoplasmic reticulum; YPD, yeast peptone dextrose media; GC-MS, gas chromatography-mass spectrometry; WT, wild type.

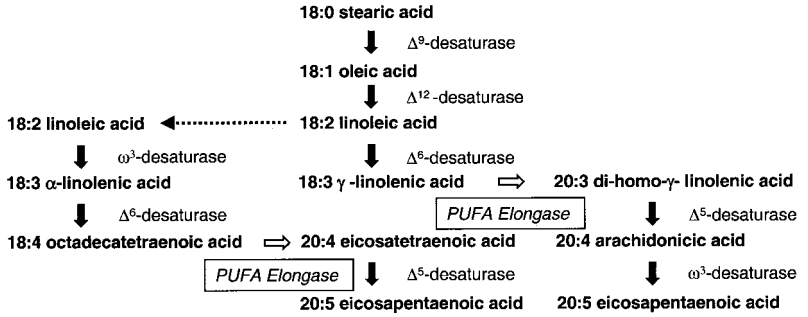

FIG. 1. **Generalized pathway for the biosynthesis of polyunsaturated fatty acids.** A generalized scheme of the enzymes responsible for aerobic desaturation and elongation of fatty acids is shown. The reaction directed by the polyunsaturated fatty acid elongase PEA1, resulting in the production of C_{20} PUFAs, is *boxed*. The yeast *S. cerevisiae* does not synthesize PUFAs, serving as a convenient host for "gain-of-function" screening.

heterologous expression of the *C. elegans* Pea1p in yeast) (5), even though yeast has no endogenous capacity for either of these particular biosynthetic reactions.

We have hypothesized that these heterologous elongating activities function by "hi-jacking" endogenous microsomal elongases, which in the case of yeast, are primarily synthesizing the C_{20-26} saturated fatty acid components of sphingolipids (5). This results in the presumptive redirection of the three endogenous components (reductases and dehydratase) toward nonnative substrates. Until very recently, little was known about the identity of these other three enzyme activities and their precise contribution(s) to the activity and specificity of microsomal fatty acid elongases. Kohlwein *et al.* (12) characterized the *TSC13* gene, which is one of a number of genes identified in a screen for temperature-sensitive (ts) mutants with defects in sphingolipid synthesis (13). Mutants with ts alleles of *TSC13* displayed phenotypes similar to the *elo2*Δ and *elo3*Δ mutants, including the accumulation of high levels of long-chain bases, the accumulation of ceramides with chain lengths of less than 26 carbons, and a deficiency in very long chain fatty acid synthesis (12). Microsomal fatty acid elongation assays revealed that the *tsc13* mutation caused the accumulation of *trans*-2,3- and 3-hydroxy-acyl intermediates; these observations, taken together with the homology between Tsc13p and steroid-5-α-reductase, are consistent with the *TSC13* gene encoding the enoyl-reductase component of the microsomal elongase. Furthermore, epitope-tagged Tsc13p was shown to coimmunoprecipitate with the presumptive condensing enzymes Elo2p and Elo3p (12). Interestingly, a precise ER location for Tsc13p, enriched at sites of vacuole-nuclear envelope interaction, was observed. *TSC13* is essential, as is expected for a gene encoding a non-redundant fatty acid elongating activity (12).

As part of our studies on microsomal fatty acid elongation, we have sought to identify other enzymatic components of the fatty acid elongase. To that end, we screened yeast knockout mutants for loss-of-function of our heterologous PUFA elongating activity PEA1 (5). Using this approach we identified a previously functionally uncharacterized gene required for the reconstitution of heterologous elongase activity.

MATERIALS AND METHODS

Cloning of Putative β-Ketoacyl Reductases in a Yeast Expression Vector—The *Saccharomyces cerevisiae* ORF encoded by YBR159w was cloned by PCR into the pESC-TRP vector (Stratagene). A crude DNA extract was prepared using the reference strain W303-1A. 1 ml of an overnight culture in rich medium (YPD) was centrifuged at 3000 × *g* for 5 min at room temperature, and the cells were resuspended in 0.5 ml of TE buffer (10 mM Tris, pH 8.0, mM EDTA). This suspension was boiled for 10 min, and the extract was centrifuged at 10,000 × *g* for 5 min. 1 μl of the supernatant was used for PCR amplification using primers YBR159.for (5′-GCGGGATCCACCATGACTTTTATGCAACAGC-3′) and YBR159.rev (5′-GCGGGTACCCTATTCCTTTTTTAACCGTCT TGC-3′). The amplified sequence was then restricted using *Bam*HI and *Kpn*I (underlined in the forward and reverse primers, respectively), purified using the Qiagen PCR purification kit, and ligated into *Bam*HI/*Kpn*I-cut pESC-TRP plasmid vector (Stratagene). An *Arabidopsis thaliana* expressed sequence tag (GenBank™ accession number: AA10E08; kindly provided by Prof. H. J. Bohnert, University of Arizona) derived from gene F12A21.31 was used as template DNA for PCR amplification using primers At159.for (5′-GCGGGATCCACCATGGAGATCTGCACT-TACTTC-3′) and At159.rev (5′-GCGCTCGAGTCATTCTTTCTTCATG-GAGTC-3′). The amplified sequence was then restricted using *Bam*HI and *Xho*I (underlined in the forward and reverse primers, respectively) and cloned into *Bam*HI/*Xho*I-cut pESC-TRP as described above.

Functional Characterization in Yeast—ORFs encoding putative β-ketoacyl-reductase activities and elongase or desaturase constructs were introduced in *Saccharomyces cerevisiae* using a lithium acetate-based method (14). Expression of the transgenes was induced by addition of 2% (w/v) galactose in the presence or absence of exogenously supplied fatty acid substrates as described previously (15). The mutant strain used in this study was CEN.RO16; Mat a/α; ura3–52/ura3–52; his3Δ1/his3Δ1; leu2–3_112/leu2–3_112; trp1–289/trp1–289; *ybr159*Δ::HIS3/YBR159w, obtained from European *Saccharomyces cerevisiae* Archive for Functional Analysis (EUROSCARF) (available at www.uni-frankfurt.de/fb15/mikro/euroscarf/index.html). This strain was transformed with a pYES2 construct containing the *C. elegans* PUFA elongating activity PEA1 (F56H11.4) and sporulated in SPM liquid media as described previously (16). After sporulation, asci were digested with β-glucuronidase (Sigma Chemical Co.), and the tetrads were dissected using light microscopy and a micromanipulator as described (17). Separated ascospores were grown on YPD for up to 2 weeks at 22 °C, and the mating type of each haploid colony tested using two yeast strains, a *sst1* and α *sst2* (18). "Wild type" (Mat a or α; ura3–52; his3Δ1; leu2–3_112; trp1–289) and mutant spores (Mat a or α; ura3–52; his3Δ1; leu2–3_112; trp1–289; *ybr159*Δ::HIS3) were identified by replica plating on synthetic dextrose medium lacking histidine. This was also confirmed by PCR using primers 159_prom.for (5′-CGGATT-TGGAAGTCCTTTATAG-3′) and 5′_his3.rev (5′-CGCTTTACTAGGGC-TTTCTGC-3′). Wild type and mutant spore colonies containing the PUFAs-elongase construct in pYES2 were selected by replica plating on synthetic dextrose medium lacking uracil.

Fatty Acid Analysis—Total fatty acids extracted from yeast cultures were analyzed by gas chromatography (GC) of methyl ester derivatives. Lipids were extracted and transmethylated with methanolic HCl. Fatty acid methyl esters were analyzed as described before (19).

GC-MS Analysis—Induced peaks were characterized using GC-MS (Kratos Analytical Instruments MS80RFA) operating at an ionization voltage of 70 eV with a scan range 40–500 Da and as described before (19).

Elongase Activity Assays—Microsomes were prepared from the wild type or *ybr159*Δ mutant cells as has been previously described (20). Total elongase activity was measured in a volume of 200 μl containing 50 mM Tris, pH 7.5, 1 mM $MgCl_2$, 150 μM Triton X-100, 1 mM NADPH, 1 mM NADH, 10 mM β-mercaptoethanol, 40 μM palmitoyl-CoA, 60 μM 2[^{14}C]malonyl-CoA (0.05 μCi/ml) at 37 °C. The reaction was initiated by the addition of 0.4 mg of microsomal protein. Protein concentrations were determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories). For assays of only the condensing activity, the NADPH and NADH were omitted. At various times (0.2, 1, or 5 min) the reaction was terminated by adding 200 μl of 5 M KOH/10% MeOH and heating at 80 °C for 1 h. Following addition of 200 μl of 10 N H_2SO_4, fatty acids were recovered by two 1.5-ml extractions into hexane. The extracted fatty acids were resolved by silica gel TLC using hexane:diethyl ether: acetic acid (30:70:1) as the developing solvent and detected and quantified using a PhosphorImager SI (Molecular Dynamics, Inc.).

RESULTS

In an attempt to identify genes or genes that encode the β-keto-reductase of the microsomal elongase, we searched the complete genome sequence of the yeast *S. cerevisiae* (predicted

TABLE I

Yeast oxidoreductases identified as candidate components of the microsomal elongase

YBR159w was previously reported as being essential, although results obtained in this study indicate that *ybr159*Δ mutant cells are actually viable. The activity of the heterologous elongating activity PEA1 in deletion mutant strains is indicated.

Gene name/synonyms	Function/similarity	Null mutant phenotype	PUFA elongation
YBR159w	17β-Hydroxysteroid dehydrogenase[a]	Lethal/reduced viability	No
YBR265w/TSC10	3-Ketosphinganine reductase	Lethal in the absence of phytosphinganine	Yes
YMR226c	Short-chain alcohol dehydrogenase[a]	Viable	Yes
YIR036c	7α-Hydroxysteroid dehydrogenase[a]	Viable	Yes
YIR035c	11β-Corticosteroid dehydrogenase[a]	Viable	Yes
YIL124w/AYR1	1-Acyl-dihydroxyacetone phosphate reductase	Viable	Yes
YDL114w	Short-chain alcohol dehydrogenase[a]	Viable	Yes
YLR426w	3-Oxoacyl-ACP reductase/alcohol dehydrogenase[a]	Viable	Yes
YML131w	NAD-dependent oxidoreductase[a]	Viable	Yes
YBR046c/ZTA1	Quinone oxidoreductase	Viable	Yes

[a] ORFs with unassigned biochemical function but displaying significant similarity to functionally characterized activities.

to encode between 5000 and 6000 polypeptides) (21) for protein sequences likely to encode oxidoreductases. This was carried out either on a homology basis to known oxidoreductases or via the presence of the diagnostic NADH binding motif (22). Because this generated over 200 candidates, the search was refined to exclude proteins already clearly functionally characterized, as well as cytosolic oxidoreductases. By excluding predicted ORFs, which lacked a canonical dilysine ER retention motif, the search was further refined to identify predicted oxidoreductases likely to have a transmembrane topology and to be located in the ER. This approach allowed us to consider a small number (~10) of genes for functional characterization as potential microsomal fatty acyl elongase components (listed in Table I). A number of these selected oxidoreductases ORFs had previously been shown by high throughput deletion analysis to be non-essential for viability. Yeast mutants in which the non-essential candidate genes were disrupted were assayed for any loss of ability to carry out heterologous PUFA elongation. Thus, a galactose-inducible, URA3-marked plasmid carrying the *C. elegans* PEA1 PUFA elongating activity (5) was transformed into these mutant strains, and the transformed yeast cells were assayed for their ability to direct the C_2 elongation of GLA, forming the basis of a "loss-of-function" screen.

Characterization for loss-of-function in haploid knockout mutants of the eight non-essential oxidoreductases (Table I) revealed no alteration in their ability to reconstitute the heterologous PUFA elongase and redirect the elongation of C_{18} PUFAs. Although the synthesis of very long-chain fatty acids is essential for yeast cell viability (8), previous directed ethyl methanesulfonate (EMS) mutant screens for defects in fatty acid elongation only identified the *ELO1* gene as a potential elongase component, implying that other elongase genes are either essential or display functional redundancy (7, 8, 23). To address the possibility that the (annotated as) essential gene YBR159w encoded an oxidoreductase component of the microsomal elongase, a diploid yeast strain heterozygous for disruption of YBR159w was transformed with a plasmid containing the PEA1 ORF. Following sporulation and tetrad dissection, the spores were allowed to germinate on YPD media. Using this approach, we observed that the *ybr159*Δ haploid deletion was in fact viable, although PCR confirmed the insertional disruption of this oxidoreductase ORF in mutant haploid cells (data not shown). When compared with wild type spores isolated from the same tetrad, spores containing the disrupted gene showed a very reduced growth rate, with small colonies appearing usually only after 10 days of incubation at 22 °C.

An initial study described deletion of YBR159w (in strain ENY.MR17) as resulting in poor growth (only at low temperatures) with a pseudo-hyphal phenotype (24), although in a subsequent study it was reported that YBR159w was an essential gene in the CEN.PK2 background (25). In our current study, we used the same parental diploid heterozygote as used in the latter study (CEN.PK2 strain background), although after sporulation the haploid *ybr159*Δ mutants were viable even at 30 °C. Indeed, although it took about 10 days for mutant spores to germinate and to form a colony after meiosis (see above), *ybr159*Δ cells are able to grow at 30 °C in rich medium (in the absence of fatty acid supplements) but at a slower rate than wild type (Fig. 2). Interestingly, cultivating *ybr159*Δ cells in a media supplemented with medium and long-chain fatty acids did not improve the growth rate (data not shown). However, we observed that the *ybr159*Δ mutant displayed temperature sensitivity when transferred to 37 °C. We also examined the phenotypic appearance of the CEN.PK strain *ybr159*Δ mutant cells and observed the previously reported (for strain ENY.MR17) pseudo-hyphal growth (24). However, genetic analysis indicates that this phenotype is unlinked to the *ybr159*Δ mutation.[2]

The Oxidoreductase Ybr159p Is Specifically Required for Heterologous Elongation Activity in S. cerevisiae—The ability of the heterologous elongating activity Pea1p to function in the *ybr159*Δ deletion was tested by galactose induction as described above. Wild type haploid colonies displayed PUFA elongating activity resulting in the conversion of 18:3(*n*-6) (GLA) into 20:3(*n*-6) (di-homo-γ-linolenic acid), whereas deletion mutant haploids completely failed to elongate heterologous C_{18} PUFAs (Fig. 3, *top panel*). We tested the galactose inducibility of other pYES2-directed enzyme activities in this haploid knockout strain to assess the possibility of pleiotropic effects on either galactose uptake or lipid metabolism. However, when two other (heterologous) enzymes of the PUFA biosynthetic pathway (borage Δ^6-desaturase (19), *C. elegans* ω^3-desaturase (26); see also Fig. 1) were tested in this mutant background, they displayed unaltered activities when compared with wild type yeast (Table II). Northern blot analysis of mutant cells expressing the heterologous elongase revealed no alteration in transcript abundance when compared with wild type (data not shown). Thus, deletion of YBR159w had a specific effect on the activity of the heterologous PUFA elongase reconstituted by expression of PEA1. This was given additional weight by our observation that YBR159w was also required for the activity of the *Arabidopsis* microsomal elongase condensing enzyme FAE1 (10); expression of Fae1p in *ybr159*Δ haploid strains failed to accumulate C_{20+} MUFAs (Fig. 3, *bottom panel*).

Several ORFs Related to YBR159w Are Present in S. cerevisiae and in Other Yeast, Plant, and Animal Genomes—Exam-

[2] T. Dunn, unpublished observations.

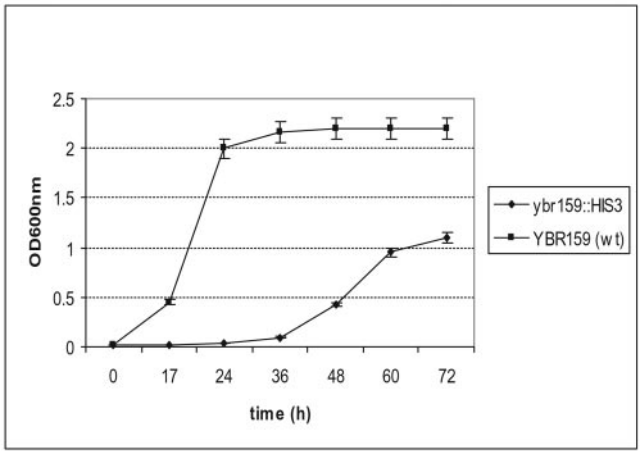

FIG. 2. **Wild type and ybr159Δ mutant cells display different growth rates in YPD at 30 °C.** Liquid cultures in YPD were inoculated with wild type and mutant cells, picked from YPD plates conserved at 4 °C, and incubated for up to 72 h at 30 °C. Cell growth was monitored by measuring the optical density of the cultures at 600 nm.

ination of the deduced amino acid sequence encoded by YBR159w indicated that the predicted polypeptide (of 347 amino acids) showed some similarity to human estradiol-17β-hydroxysteroid dehydrogenase (32% similarity). For this reason, it has recently been suggested that the enzyme encoded by YBR159w could be responsible for steroid dehydrogenase activities observed *in vitro* in yeast extracts (27). However, no sequences homologous to YBR159w could be detected by PCR and Northern hybridization in the mesophilic yeasts *Candida tropicalis* and *Cryptococcus tsukubaensis* even though steroid dehydrogenase activity was observed. These observations have led the authors to speculate that the Ybr159p oxidoreductase does not function as a steroid dehydrogenase (27); this is in agreement with our present study. Presumptive orthologues of Ybr159p are also present in fission yeast (43% similarity), *Drosophila melanogaster* (34% similarity), *C. elegans* (30% similarity), and *Arabidopsis* (30% similarity) (Fig. 4*A*). Not only do all these sequences contain the diagnostic NADH binding motif (22), they also share a number of conserved residues, in particular the catalytically essential (for estradiol-17β-hydroxysteroid dehydrogenase) motif Y-X_3-K (28). There are also canonical dilysine ER retention motifs present in both Ybr159p and the *Arabidopsis* orthologue, consistent with these proteins' predicted multiple (presumptively endoplasmic reticulum) membrane-spanning topology. When the polypeptide sequence of Ybr159p was used to search the complete yeast genome sequence, several related, but distinct, ORFs were detected. These included Ymr226p (which shows some homology to insect short-chain alcohol dehydrogenase; 33% similar), Ayr1p (1-acyl-dihydroxyacetone-phosphate reductase (29); 24% similar), and Yir036p (which shows homology to 7α-hydroxysteroid dehydrogenase; 23%) (Fig. 4*B*). Individual deletion analysis of these three ORFs had previously indicated that none of these genes encode essential proteins. More importantly, individual deletion of any of these ORFs (YMR226c, AYR1/YIL124w, YIR036c) did not alter the activity of the heterologous PUFA elongase PEA1 as determined by our loss-of-function assay (data not shown; see also Table I for summary).

ybr159Δ Mutants Can Be Complemented by a Homologous ORF from A. thaliana—Further confirmation of the role of Ybr159p in heterologous microsomal elongation activity was obtained by rescue of either Pea1p or Fae1p (PUFA or MUFA, respectively) elongating activities by episomal co-expression of either the wild type YBR159w ORF from *S. cerevisiae* or a presumptive *A. thaliana* homologue ORF (F12A21.31). In *ybr159Δ* cells, the galactose-induced co-expression of YBR159w with PEA1 resulted in restoration of 72% of C_{18} PUFA elongating activity, respectively, when compared with the activity of the same enzymes in wild type cells (Table III). When the presumptive *Arabidopsis* orthologue F12A21.31 was co-expressed instead of YBR159w, this resulted in a very similar restoration of PUFA elongation (Table III); for that reason we hereafter refer to F12A21.31 as At-YBR159. Similarly, co-expression of YBR159w with FAE1 in *ybr159Δ* cells resulted in a 52% restoration of C_{18} MUFA elongation, whereas co-expression with At-YBR159 resulted in restoration of 42% of FAE1 activity, compared with wild type cells (Table IV).

Interestingly, episomal galactose-induced overexpression of YBR159w in wild type spore colonies (derived from the same tetrads that had yielded the *ybr159Δ* haploids) resulted in moderate increases in both PEA1 (PUFA) and FAE1 (MUFA) heterologous elongation activities, compared with the expression of these elongating activities in wild type cells (Tables III and IV). This suggests that, in contrast with native elongase systems, the condensation reaction may not be the only rate-limiting activity for heterologous elongation in yeast. Co-expression of the same elongating activities with At-YBR159 in wild type yeast resulted in almost equivalent results, again demonstrating the functional equivalence of the *Arabidopsis* protein.

ybr159Δ Mutant Cells Have Reduced Endogenous Fatty Acid Elongation Activity—ybr159p clearly plays a role in the fatty acid elongation mediated by the heterologously expressed Fae1p and Pea1p activities in yeast. Based on its homology to the oxidoreductases, it was considered a good candidate for a β-keto-reductase, reducing the 3-keto intermediate formed by the condensing activity during each cycle of fatty acid elongation. To address how elongation is affected in the *ybr159Δ* mutant cells, microsomes were prepared and assayed for elongase activity *in vitro*. The elongation cycle initiates with the condensation of malonyl-CoA with an acyl-CoA (*e.g.* palmitoyl-CoA) to form a 3-ketoacyl-CoA intermediate. Omitting pyridine nucleotide from the assay mix prevents the reduction of the 3-ketoacyl-CoA intermediate and thereby allows the first step of elongation to be measured (12). The condensation activity measured over a time course of 5 min was very similar whether wild type or *ybr159Δ* microsomes were used for the assay (Fig. 5, compare *lanes 4–6* and *10–12*). However, when the time course of the overall elongation reaction (with NADH/NADPH included) was conducted and the products were analyzed by TLC using conditions that resolved the four intermediates of fatty acid elongation, differences between the wild type and *ybr159Δ* mutant were apparent. The only intermediate seen in the elongation catalyzed by the wild type microsomes was a small amount of 3-hydroxy intermediate in the 5-min time point (Fig. 5, *lane 3*) under conditions where a large amount of fully elongated product accumulated. In contrast, a large amount of the 3-keto intermediate accumulated and the formation of the fully elongated product were greatly delayed during the elongation catalyzed by the *ybr159Δ* mutant microsomes (Fig. 5, *lanes 8* and *9*). Based on the homology of Ybr159p to oxidoreductases, it may directly catalyze the reduction of the 3-keto intermediate that is formed in each cycle of elongation. However, in addition to the 3-keto intermediate, elevated amounts of the 3-hydroxy intermediate also accumulated (compare the difference in the ratio of this intermediate to the fully elongated product in the mutant to that in the wild type, Fig. 5, *lane 3 versus lane 9*). Although the accumulation of the 3-keto intermediate of elongation would be consistent with Ybr159p functioning as a β-keto-reductase, it is not clear why the 3-hy-

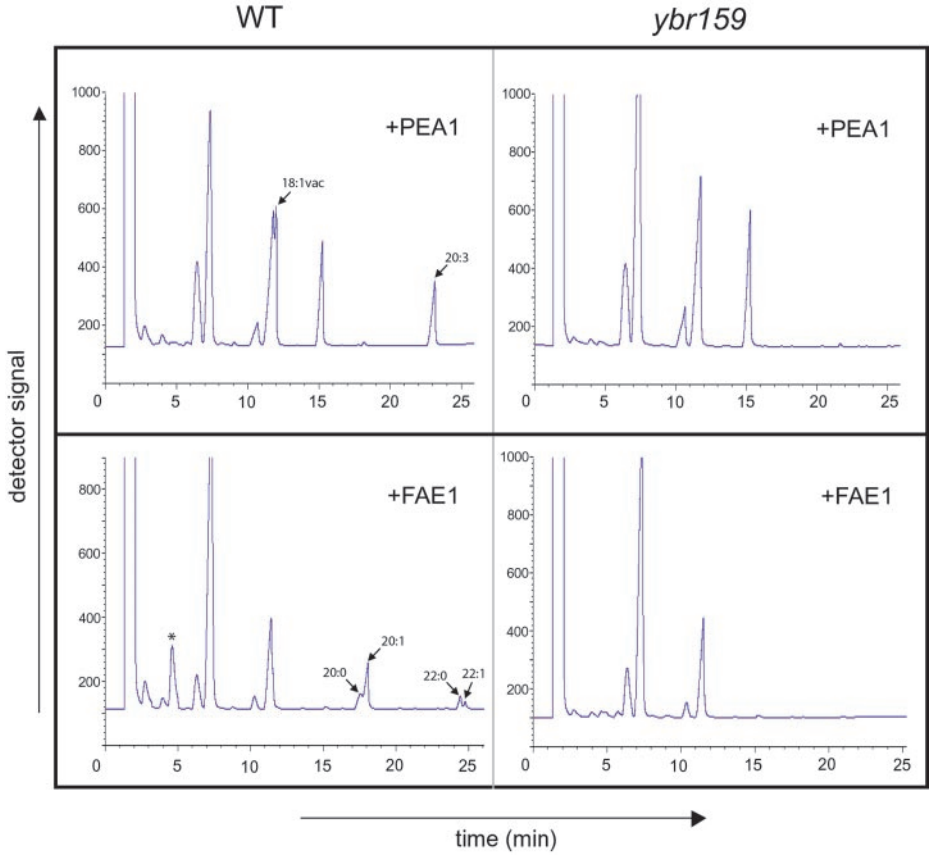

FIG. 3. **Analysis of fatty acid elongation activity in ybr159Δ yeast.** Chromatograms of fatty acid methyl esters prepared form wild type (*WT*) and mutant (*ybr159*Δ) yeast expressing either *C. elegans* PUFA elongating activity PEA1 (*top panel*) or *A. thaliana* MUFA elongating activity FAE1 (*bottom panel*). γ-Linolenic acid (18:3 $\Delta^{6,9,12}$) was supplied as an exogenous substrate to cells expressing PEA1. Fatty acid elongation was detected by flame ionization, with peak identification by subsequent GC-MS. In wild type cells the expression of PEA1 or FAE1 resulted in the production of 18-, 20-, and 22-carbon elongation products (peaks indicated by *arrows*). In *ybr159*Δ cells elongation activity was completely absent. *Asterisk*, the expression of FAE1 in wild type yeast cells also resulted in the accumulation of 14-carbon-saturated and monounsaturated fatty acids.

TABLE II
Expression of desaturase genes in ybr159Δ yeast

Fatty acid composition of *ybr159*Δ or wild type yeast transformed with either the borage Δ^6-fatty acid desaturase (BorΔ6) or the *C. elegans* ω^3-fatty acid desaturase (EAT1) cloned into the pYES2 vector (Invitrogen) under the control of the GAL1 promoter. Linoleic acid (C18:2$\Delta^{9,12}$) was added to the cultures before induction with galactose. Induced cultures were incubated at 22 °C for 48 h, and the fatty acid composition was analysed as described under "Materials and Methods." All values are expressed as mol% of total fatty acids. Standard deviations are also given. The percent desaturation of substrates was calculated using the following formula: Product/(substrate + product) × 100, with substrate and product being the mol% of LA and ALA or GLA, respectively.

Episomal gene Haploid genotype	BorΔ6 (+LA)		FAT1 (+LA)	
	*ybr159*Δ	WT	*ybr159*Δ	WT
		mol%		
Fatty acid				
16:0	14.5 ± 0.9	15.6 ± 1.2	14.1 ± 0.6	16.2 ± 0.7
16:1Δ^9	43.5 ± 3.7	38.8 ± 2.6	48.2 ± 2.4	43.8 ± 2.3
16:2$\Delta^{6,12}$	2.9 ± 1.2	3.1 ± 1.1		
18:0	4.2 ± 1.0	4.2 ± 0.7	3.1 ± 0.4	4.0 ± 0.3
18:1Δ^9	15.8 ± 1.9	13.5 ± 1.3	17.1 ± 1.2	14.1 ± 0.7
18:2$\Delta^{9,12}$ (LA)	13.9 ± 2.5	17.4 ± 2.6	16.1 ± 1.5	19.7 ± 0.9
α18:3$\Delta^{9,12,15}$ (ALA)			1.4 ± 0.5	2.2 ± 0.7
γ18:3$\Delta^{6,9,12}$ (GLA)	5.2 ± 2.2	7.4 ± 1.7		
		%		
% Desaturation				
16:1Δ^9	6	7.5		
18:2$\Delta^{9,12}$ (LA)	27	29.8	8.0	10.0

droxy intermediate would also accumulate in the mutant (see "Discussion" for possible explanations). In addition, although Ybr159p is not essential for viability, very long chain fatty acids are. Therefore, if Ybr159p is a β-keto-reductase activity that functions in elongation, there must be functionally redundant activity. This is consistent with the non-lethality of the disruption of this gene.

DISCUSSION

In this study, we report the identification of a yeast gene required for the reconstitution of a heterologous microsomal fatty acid elongase. This gene, identified as YBR159w is predicted to encode an integral membrane protein, most likely located in the ER. The encoded protein shows some limited homology to mammalian steroid dehydrogenases, most notably estradiol-17β-hydroxysteroid dehydrogenase, and is a member of the short-chain dehydrogenase superfamily (Sanger Protein Family Data base ID: Pfam00106). Interestingly, the *Arabidopsis* homologue of Ybr159p functionally characterized by us in this study was previous annotated by Xu *et al.* (30) as being an orthologue of the maize Glossy8 gene. Transposon-induced mutations in this maize gene result in defects in the synthesis of the cuticular waxes deposited on the outer epidermis of the

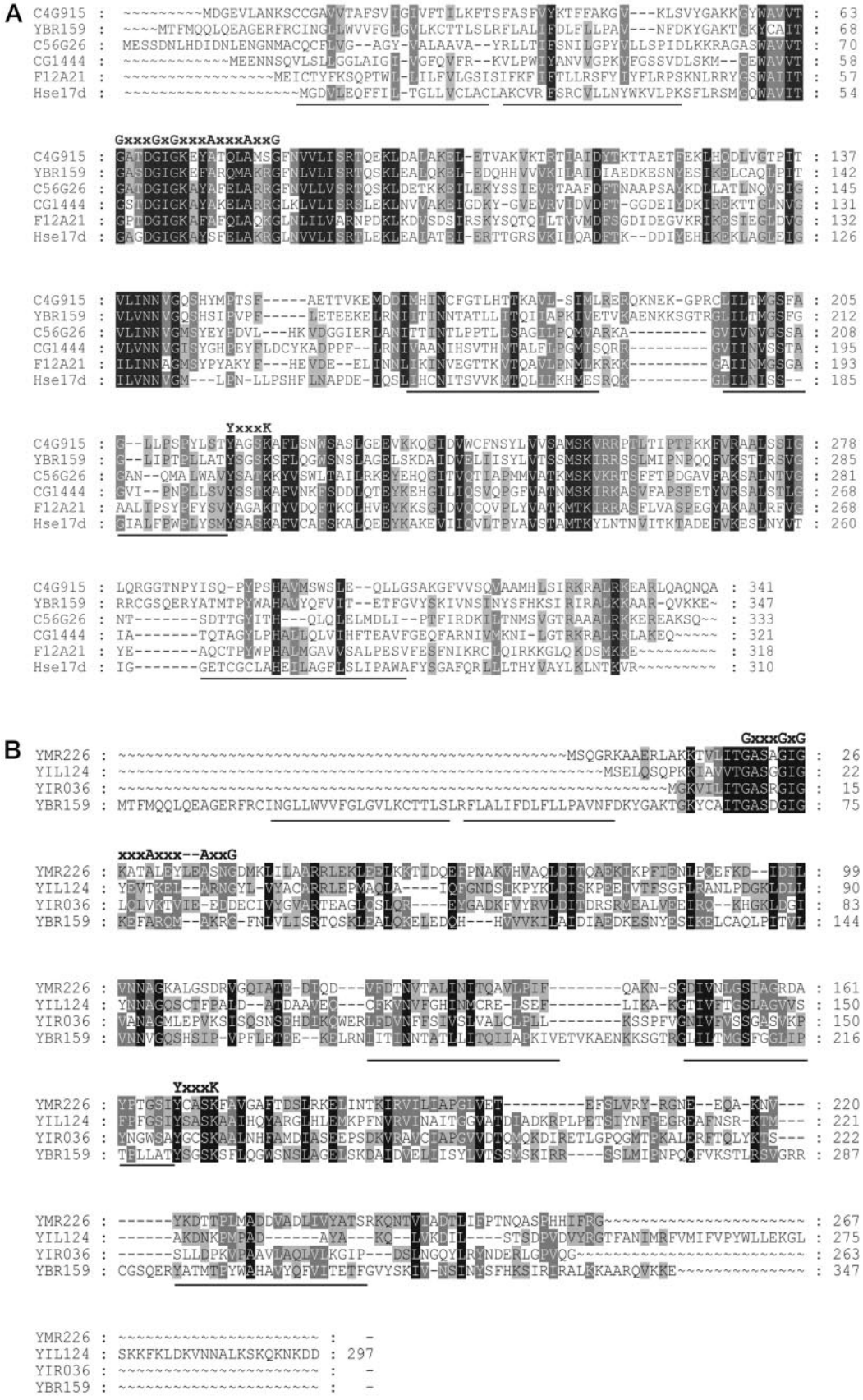

FIG. 4. *A*, sequence comparisons of *S. cerevisiae* gene product Ybr159p and homologues from other species. SPAC4G9.15: putative short-chain alcohol dehydrogenase from *S. pombe* (CAA93565); C56G2.6: possible estradiol-17β-hydroxysteroid dehydrogenase from *C. elegans* (AAA64333); CG1444: putative oxidoreductase from *D. melanogaster* (BI370369); F12A21.31: putative β-ketoacyl-reductase from *A. thaliana* (AF361844); HSD17B3: 17β-hydroxysteroid dehydrogenase from *Homo sapiens* (NP_000188). *B*, sequence comparisons of Ybr159p and related sequences from *S. cerevisiae*: Ymr226p (CAA90197); Yil124p (CAA86868); Yir036p (CAA86196). Potential membrane-spanning regions were identified using the TopPred2 topology prediction server (available at bioweb.pasteur.fr/seqanal/interfaces/toppred.html) and are indicated by *black lines*. The putative NADH binding motif $(G(X)_3GXG(X)_3A(X)_3A(X)_2G)$ and the essential catalytic motif $(Y(X)_3K)$ are also indicated.

TABLE III

Rescue of PUFA elongation activity in ybr159Δ yeast

Fatty acid composition of mutant (*ybr159Δ*) or wild type (WT) yeast coexpressing PEA1 (cloned into pYES2) and either *S. cerevisiae* ORF YBR159w or AtYBR159, an homologue ORF from *A. thaliana* (both cloned into pESC-TRP). All episomal gene constructs were under the control of the GAL1 promoter. γ-Linolenic acid (18:3 $\Delta^{6,9,12}$) was supplied as exogenous substrate and the fatty acid composition of induced yeast was analyzed as described in Table 2. The percent elongation of substrates was calculated using the following formula: Product /(substrate + product) × 100, with substrate and product being the mol% of 16:1Δ^{9} and 18:1Δ^{11} or of GLA and DHGLA, respectively.

Episomal gene 1	PEA1 (+GLA)					
Episomal gene 2	–		+ YBR159		+ AtYBR159	
Haploid genotype	*ybr159Δ*	WT	*ybr159Δ*	WT	*ybr159Δ*	WT
	mol%					
Fatty acid						
16:0	14.2 ± 1.0	15.4 ± 3.2	11.3 ± 0.6	14.3 ± 0.1	12.5 ± 1.5	16.3 ± 4.4
16:1Δ^{9}	50.3 ± 5.2	39.5 ± 5.1	43.9 ± 2.1	26.7 ± 0.6	49.3 ± 1.0	35.6 ± 3.4
18:0	3.1 ± 0.6	3.3 ± 0.8	3.7 ± 0.3	3.8 ± 0.1	2.6 ± 0.2	3.5 ± 0.8
18:1Δ^{9}	27.1 ± 3.5	27.8 ± 6.3	26.4 ± 4.2	21.2 ± 0.1	23.8 ± 2.1	23.0 ± 4.3
18:1Δ^{11} (Vac)		6.2 ± 2.5	5.8 ± 1.1	5.9 ± 0.2	6.0 ± 0.4	7.5 ± 3.5
γ18:3$\Delta^{6,9,12}$ (GLA)	5.3 ± 1.2	3.5 ± 2.0	5.4 ± 0.5	9.8 ± 0.2	3.6 ± 0.6	7.6 ± 3.0
20:3$\Delta^{8,11,14}$ (DHGLA)		4.3 ± 1.8	3.5 ± 0.5	17.9 ± 0.5	2.2 ± 1.0	6.5 ± 2.5
			%			
% Elongation						
16:1Δ^{9}		13.5	11.7	18	11	17.4
γ18:3$\Delta^{6,9,12}$ (GLA)		55	40	65	38	46

TABLE IV

Rescue of MUFA elongation activity in ybr159Δ yeast

Fatty acid composition of mutant (*ybr159Δ*) or wild type (WT) yeast coexpressing FAE1 and either *S. cerevisiae* ORF YBR159w or *A. thaliana* ORF AtYBR159. Fatty acid analysis was performed as described in Table 2. The percent elongation of substrates was calculated using either the formula: Product /(substrate + product) × 100, with substrate and product being the mol% of 20:0 and 22:0 or of 20:1Δ^{11} and 22:1Δ^{13}, respectively; or the formula: (Product1 + product2) /(substrate + product1 + product2) × 100, with substrate product1 and product2 being the mol% percentage of 18:0, 20:0, and 22:0 or of 18:1Δ^{9}, 20:1Δ^{11}, and 22:1Δ^{13}, respectively.

Episomal gene 1	FAE1					
Episomal gene 2	–		+ YBR159		+ AtYBR159	
Haploid genotype	*ybr159Δ*	WT	*ybr159Δ*	WT	*ybr159Δ*	WT
	mol%					
Fatty acid						
16:0	13.4 ± 0.5	8.5 ± 0.9	12.2 ± 0.7	7.7 ± 0.4	11.0 ± 0.7	9.6 ± 0.6
16:1Δ^{9}	56.9 ± 4.8	62.5 ± 1.3	69.5 ± 2.1	65.3 ± 0.5	70.2 ± 2.4	63.9 ± 0.8
18:0	3.3 ± 0.2	2.5 ± 0.4	2.4 ± 0.6	2.2 ± 0.1	2.4 ± 0.1	2.6 ± 0.1
18:1Δ^{9}	26.4 ± 4.1	15.4 ± 1.3	13.0 ± 1.1	14.8 ± 0.7	14.4 ± 0.9	14.7 ± 0.2
20:0		2.5 ± 0.5	0.7 ± 0.1	2.4 ± 0.1	0.6 ± 0.1	2.0 ± 0.1
20:1Δ^{11}		5.7 ± 0.7	1.6 ± 0.2	5.7 ± 0.1	1.1 ± 0.2	5.7 ± 0.5
22:0		1.4 ± 0.2	0.4 ± 0.1	1.3 ± 0.2	0.2 ± 0.1	1.0 ± 0.1
22:1Δ^{13}		1.1 ± 0.2	0.2 ± 0.1	0.6 ± 0.1	0.1 ± 0.0	0.5 ± 0.1
			%			
% elongation						
18:0		60.0	31.4	62.7	25.0	53.6
18:1Δ^{9}		30.6	12.2	29.9	7.7	29.7
20:0		35.8	36.4	35.1	25.0	33.0
20:1Δ^{11}		16.2	11.1	10.0	8.3	8.0

plant's cells, giving the mutant plant a characteristic "glossy" appearance (30). The maize *glossy8* locus was cloned via transposon inactivation tagging and, although not characterized biochemically, was hypothesized (on the basis of homology to other dehydrogenases) to encode a β-ketoacyl-reductase (30). Homozygous *glossy8* maize mutants display decreased levels of C_{24+} components of wax esters but are viable, indicating either gene redundancy or the presence of distinct elongases for fatty acid and wax synthesis. A partial-length cDNA clone from *Arabidopsis* (GenBank™ accession number U89512) was also identified in that study as being a likely orthologue of maize Glossy8. That *Arabidopsis* transcript is derived from F12A21.31, the gene identified by us as encoding a functional homologue of the yeast microsomal β-keto-reductase Ybr159p. Based on the data presented in this paper, we believe it is now possible to assign the function of β-keto-reductase to the maize Glossy8 locus.

Previous assessments of the requirement of YBR159w for yeast viability yielded slightly contradictory data, with the gene being described as essential in the CEN.PK2 background but viable at reduced temperatures in the ENY.MR17 background. One explanation for the discrepancy in previous viability data may be explained by our observation of a long "lag" period in the initial growth of newly isolated haploid mutants. This initial slow growth may be related to some form of adaptive response to the loss of this microsomal elongase component. After this "adaptation" the mutant spore colonies formed are viable in the absence of fatty acid supplement, although growing at a much slower rate than wild type cells (Fig. 2). In that respect it may be analogous the adaptation of *srp54Δ* null mutants, in which the loss of a component of the ER protein targeting machinery is bypassed through an adaptive mechanism (31).

Mutagenic approaches to the identification of genes involved in fatty acid elongation have previously only identified one component, Elo1p, that is believed to be a condensing enzyme.

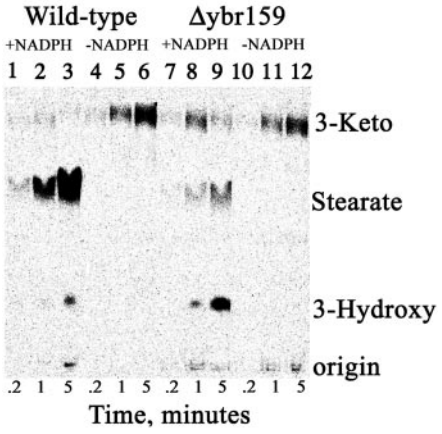

FIG. 5. **ybr159Δ cells are deficient in fatty acid-chain elongation.** Fatty acid elongation activity in wild type (*lanes 1–6*) and *ybr159*Δ mutant (*lanes 7–12*) microsomes was compared using palmitoyl-CoA and radiolabeled malonyl-CoA as substrates. The assays were performed in the absence of NADH/NADPH (*lanes 4–6* and *10–12*) to measure condensation activity and in the presence of NADH/NADPH (*lanes 1–3* and *7–9*) to measure overall elongation activity. The reactions were stopped at the indicated times, and the fatty acids were extracted and separated by TLC. The positions where the 3-ketostearate (*3-Keto*), 3-hydroxystearate (*3-Hydroxy*), and stearate standards (included on the TLC and visualized by charring after exposure to the PhosphorImager screens) migrated are indicated.

The failure to recover mutants in the other enzymatic reactions that comprise the elongase system have been interpreted as indicating either functional redundancy (as exemplified in the case of the *ELO2/3* genes) or lethality upon disruption. The essential nature of the activity of the microsomal elongase for yeast cell viability is clear from the synthetic lethality of *elo2elo3* mutants, as well as the requirement for a functional copy of the *TSC13* gene, which encodes the enoyl-reductase. Our observation that YBR159w, a non-essential gene, is required for microsomal elongase activity is intriguing, especially because of the lack of (functionally redundant) candidate homologues in the yeast genome. It is also clear that the lethality (or not) on the loss of YBR159w is strain-dependent; our current studies in the CEN.PK2 background indicate the viability of *ybr159*Δ mutants. Although there are no obvious homologues of Ybr159p, it is possible that related, ER co-located oxidoreductases (*i.e.* paralogues of Ybr159p) are capable of (inefficiently) metabolizing 3-ketoacyl-CoA intermediates. This could explain the viability of the *ybr159*Δ mutant as well as the observed pronounced reduction (but not ablation) in elongase activity.

The experiments presented here suggest that Ybr159p is the β-keto-reductase activity of the microsomal fatty acid elongating system. Consistent with this proposal, the *in vitro* elongation assays reveal an accumulation of the 3-keto-fatty acyl elongation intermediate. The accumulation of 3-hydroxacyl-CoA intermediates in the flux through the elongase of *ybr159*Δ is equally intriguing. This could be due to a perturbation of the physical interactions between the various distinct enzymatic components as a result of the loss of Ybr159p. As such, this would give further credence to the hypothesis that the microsomal elongase is likely to function as a large multimeric protein complex. In that respect, proximal physical interactions between other microsomal elongase components such as Tsc13p and Elo2p/Elo3p have previously been demonstrated (12).

Conclusions—In this study we have used a "loss-of-heterologous-function" screen to genetically identify a component of the microsomal fatty acid elongase. Biochemical characterization confirms the identification of the encoded polypeptide as the β-keto-reductase. As such, this is the first report of the identification of a gene encoding this microsomal enzyme. Moreover, overexpression of the β-keto-reductase can result in enhanced activity of (heterologous) elongases, challenging the concept that the condensing enzyme is the rate-limiting step in fatty acyl elongation.

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