The Saccharomyces cerevisiae Peroxisomal 2,4-Dienoyl-CoA Reductase Is Encoded by the Oleate-inducible Gene SPS19*

(Received for publication, January 31, 1997, and in revised form, June 3, 1997)

Aner Gurvitz‡§**, Hanspeter Rottensteiner‡§, Seppo H. Kilpeläinen‡§, Andreas Hartig§, J. Kalervo Hiltunen‡§, Maximilian Binder‡§, and Ian W. Dawes‡§, and Barbara Hamilton§

From the ‡School of Biochemistry and Molecular Genetics, University of New South Wales, Sydney NSW 2052, Australia, the §Institut für Biochemie und Molekulare Zellbiologie der Universität Wien and Ludwig Boltzmann-Forschungsstelle für Biochemie, Vienna Biocenter, Dr Bohrgasse 9, A-1030 Wien, Austria, the †Biocenter Oulu, Department of Biochemistry, University of Oulu, FIN-90570 Oulu, Finland, and the ¶Institut für Tumorbiologie-Krebsforschung der Universität Wien, Borschegasse 8a, A-1090 Wien, Austria

β-Oxidation is compartmentalized in mammals into both mitochondria and peroxisomes. Fatty acids with double bonds at even-numbered positions require for their degradation the auxiliary enzyme 2,4-dienoyl-CoA reductase, and at least three isoforms, two mitochondrial and one peroxisomal, exist in the rat. The Saccharomyces cerevisiae Sps19p is 34% similar to the human drial and one peroxisomal, exist in the rat. The Saccharomyces cerevisiae Sps19p is 34% similar to the human

The β-oxidation auxiliary enzyme 2,4-dienoyl-CoA reductase (EC 1.3.1.34) participates in the degradation of unsaturated fatty acids with double bonds at even-numbered (1, 2) and possibly also at odd-numbered positions (3, 4). It catalyzes an NADPH-dependent reduction of trans-2,cis/trans-4-dienoyl-CoA into trans-2-enoyl-CoA in eukaryotes (1) and into trans-2-enoyl-CoA in bacteria (5, 6). Reductases have previously been purified from Escherichia coli (7), from the yeasts Candida lipolytica (8) and Candida tropicalis (9), and from rat and bovine liver (10, 11). Mammals possess at least two mitochondrial reductases (molecular weights 120,000 and 60,000) and a third peroxisomal one (12, 13), and although the rat and human cDNAs for the M, 120,000 mitochondrial isoform have been cloned (14, 15), the peroxisomal isoform has remained uncharacterized at the molecular level. A reductase-deficient E. coli mutant unable to grow on petroselineate (cis-C18:1(9)) as the sole carbon source (16) further underscored the essential requirement for this enzyme, and a lethal inborn 2,4-dienoyl-CoA reductase deficiency in a human patient has been reported (17).

In Saccharomyces cerevisiae β-oxidation is solely confined to the peroxisomes (18). In cells grown on oleate, β-oxidation enzymes are induced and peroxisomes dramatically increase in number and size (19). The induction of a number of other S. cerevisiae genes encoding peroxisomal proteins such as catalase A (CTA1) and thiolase (FOX3) is mediated via a positive gene control sequence called the oleate response element (ORE1; 20, 21). OREs have recently been shown to act as the binding target for the products of PIP2 (20) and OAF1 (21), and strains deleted at either locus were unable to grow on oleate (cis-C18:1(9)) as the sole carbon source.

SPS19 was cloned together with the S. cerevisiae sporation-specific gene SPS18, that was in turn isolated from a genomic yeast DNA library fused to a promoterless E. coli lacZ gene following a screen for sporation-specific expression (22). Homozygous sps18/19Δ deletants demonstrated a 4-fold reduction in sporation, and the viable spores that did form failed to become resistant to ether and were more sensitive to lytic enzymes. Sequence analysis of the shared SPS18/19 promoter region showed that it contained potential OREs, and the deduced amino acid sequence of SPS19 revealed a carboxyl-terminal SKL peroxisome targeting signal (23, 24). A search of the data bases for similarities to the Sps19p sequence indicated that it was 34% similar to the human and rat mitochondrial 2,4-dienoyl-CoA reductase. Since the roles of β-oxidation and peroxisomes during eukaryotic development and particularly during yeast sporation are poorly understood, we were prompted to elucidate the properties and regulation of this gene product.

We show here that Sps19p is a peroxisomally localized 2,4-dienoyl-CoA reductase and that the sps19Δ deletant is unable to grow on petroselineate as the sole carbon source, although it remains viable on oleate. SPS19 is dispensable for ascosporo-
Characterization of the Yeast Gene SPS19

TABLE I

| Strain, plasmid, or oligonucleotide | Description | Source or Ref. |
|-----------------------------------|-------------|---------------|
| **S. cerevisiae**                 |             |               |
| 1) BJ1991                         | MATa leu2 ura3–52 trp1 pep4–3 pbrl-1122 | 60 N. Klecknera |
| 2) YK7057                         | MATa leu2 ura3–52 his44 lys HO-LYS |               |
| 3) YK303                          | MATa leu2–3,112 ura–3 his3–11 trp1 ade2–1 can1–100 | 61 R. Rothstein |
| 4) GAI–8C                        | MATa leu2 ura3–52 his5 trp1 ctt1–1 gal2 |               |
| MF24–6x4                         | URA3::CTA1–1841–198::CYC1-lacZ | 28            |
| 5) yAG1411                       | sps19A::LEU2 pAG129 digested with ScaI and StuI | This study |
| 6) yAG1466                       | Deleted as above | This study |
| 7) yAG1505                       | Deleted as above | This study |
| 8) yAG1516                       | Homozygous sps19A::LEU2 | This study |
| 9) yAG1628–22                    | Corresponding heterozygous wild type | This study |
| yAG1628–22                       | Homozygous sps19A::LEU2 | This study |
| 10) yAG3736–22                   | Corresponding heterozygous wild type | This study |
| yAG3735–22                       | Corresponding heterozygous wild type | This study |
| 11) yAG4561                      | pAG154 (SPS19-lacZ) | This study |
| 12) yAG551                      | pAG1534 (SPS18-lacZ) | This study |
| 13) yAG2951                      | pAG23 (ORE-mutated SPS19-lacZ) | This study |
| 14) yAG2295                      | pAG244 (SPS19 ORE::CYC1-lacZ) | This study |
| 15) yAG2571                      | pMF6 (UAS-less CYC1-lacZ vector) | This study |
| **Plasmid**                      |             | 28            |
| pIC18–2μ                         | Source of 1.8-kb KpnI-XbaI SPS18/19 for pAG113 | 22 |
| pJC18                            | SpI1p overexpression | 22 |
| pAG454                           | YIp357-19 containing 1.4-kb SphI-XbaI SPS18/19 | P. Yeoha |
| pAG223                           | YIp357-19M1 containing XhoI substituted ORE | P. Yeoha |
| pMF6                             | UAS-less CYC1-lacZ vector | 28 |
| pAG1113                          | This study |
| pAL113                           | BluscriptSKI (+) containing 1.8-kb KpnI-XbaI SPS18/19 | 28 |
| pAG129                           | pAG113 deleted at SPS19 (sps19A::LEU2) | This study |
| pAG534                           | YIp356R-18 containing 1.4-kb SphI-XbaI SPS18/19 | This study |
| pAG244                           | SPS19 ORE::CYC1-lacZ | This study |
| **Oligonucleotide**              |             | 28            |
| CYC1                             | SPS19ORE/SPS19          |                |
| 5′-AGTTGCTCGCCATCCACGCGC-3′      | This study |
| 5′-TCCGACATGTCAGGGAGTTTGATACCTTAAGCCCGTGAAGG-3′ | This study |
| SPS19ORE/SPS19-2                 | pIC18–2μ (22) was cloned into Bluscript® SK (+) (Stratagene, La Jolla CA) to yield pAG113. | This study |
| **Experimental Procedures**       |             |               |

### Strains and Plasmids

- The S. cerevisiae strains and plasmids used are listed in Table I. E. coli strain DH10B was used for all plasmid manipulations and isolations.

### Media and Growth Conditions

- For RNA isolations, logarithmetic cultures of the wild-type strain MF24-6x were shifted to YM medium (1% yeast extract, 2% peptone) containing the indicated carbon sources and grown for a further 16 h (20). For electron microscopy cells were propagated as described (25), and for β-galactosidase measurements cells were induced following a modified protocol (26).

### Stationary-phase haploid wild-type strains from overnight precultures consisting of YM medium and 5% glucose were transferred to 1-liter flasks; the extreme flocculence of the strain used obviated the requirement for absorbance determination prior to transfer into oleate medium.

### Plate Media

- Plates containing Tween 80 alone (Sps1), or Tween 80 with oleate (E. Merck AG, Darmstadt, Germany), or petroleosseine (Sigma) (0.67% yeast nitrogen base with amino acids, 0.1% yeast extract, 0.5% potassium P, at pH 6.0, and 2% agar, autoclaved with 0.5% Tween 80 alone or with 0.5% Tween 80 and either 0.125% oleate or 0.125% petroleosseine, respectively) were prepared by pouring a thin layer at a temperature below 55 °C. These plates were used to assess utilization of the fatty acids (clear-zone formation) and for sporulation (by direct microscopic examination of cells) after about 7-day incubation.

### Deletion of SPS19—A 1.8-kb KpnI-XbaI SPS18/19 fragment from pIC18–2μ (22) was cloned into Bluscript® SK (+) (Stratagene, La Jolla CA) to yield pAG113. The 189-base pair ClaI-SphI fragment within SPS19 was then replaced with a 2-kb Smal-SphI fragment containing the LEU2 gene from pAG250 (27), resulting in the disruption plasmid pAG129. A 2.8-kb fragment produced by digesting pAG129 with SacI and StuI was used for transformation (Fig. 18).

### SPS19 ORE::CYC1-lacZ Construct

- The Sps1-l delineated SPS19ORE/SPS19ORE/RE1 and ORE2 oligonucleotides (Table I) defining the ORE element within the shared SPS18/19 promoter region were annealed, and the double-stranded fragment was cloned into the XhoI site of the integrative vector pMF6 (28), a derivative of pLG669Z (29), to produce pAG244. Nucleotide sequencing revealed that the orientation of the ORE fragment with respect to the lacZ fusion boundary as being in the SPS18 direction, i.e. in the reverse orientation to SPS19, and integration of Sps1-linearized pMF6 and pAG244 into the ura3 locus of BJ1991 resulted in strains yAG257 and yAG259, respectively.

### Other Reporter Genes

- The plasmids YIp357-19 and YIp357-19M1 were kindly donated by P. Yeoh (University of New South Wales, Sydney, Australia). Briefly, a 1.4-kb XbaI-SphI fragment containing the intergenic region and part of the coding regions of both SPS18 and SPS19 was excised from pUC18-KCC (23) and inserted into the corresponding sites in YIp357 (30) to create YIp357-19 (pAG454; SPS19-lacZ) as well as into M13mp19 for the substitution of the S′ ORE half-site sequence 5′-ACCCCTTGAAG-3′ with a unique XhoI site using site-directed mutagenesis. The substituted DNA was verified by nucleotide sequencing and cloned into YIp357 to create YIp357-19M1 (pAG23). Integration of the Sps1-linearized plasmids containing the native and substituted DNA into the ura3 locus of BJ1991 resulted in strains yAG456 and yAG259, respectively. Cloning of the reverse orientation by inserting the 1.4-kb XbaI-SphI fragment isolated from pAG254 into the appropriate sites of YIp356R resulted in pAG354 (SPS18-lacZ). Similar integration of this plasmid yielded strain yAG561.

### Enzyme Assays—2,4-Dienoyl-CoA reductase was assayed spectrophotometrically at 23 °C as described (1). The assay mixture consisted of 0.1 M potassium P, (pH 7.4), 125 μM NADPH, and 60 μM 2,4-hexadienoyl-CoA (synthesized from trans-2,trans-4-hexadienoic acid via the
mixed anhydride system; (31)) as the substrate. Reductase activity was expressed as micromoles of substrate metabolized per min. β-Galactosidase activity was assayed in crude extracts prepared by breakage of cells with glass beads (32) and the values reported here, expressed as nanomoles of o-nitrophenol-β-galactopyranosidase hydrolyzed per min of protein, were the average of three experiments.

**Purification Procedure**—To purify Sps19p S. cerevisiae cells yAG162 harboring pJC18 (22) were grown in oleate medium for 3 days. The cells were collected by centrifugation, washed in 2 volumes of cold distilled water, and frozen at −70°C until required. All subsequent work was performed at 4°C. A cell pellet of 15 g wet weight was thawed, suspended in 150 ml of breakage buffer (50 mM potassium Pi, pH 7.0, 0.4 mM KCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol), and a crude extract of cells disrupted with glass beads was prepared (33). A volume of 100 ml of the 6 × 10^8 × g × min supernatant was diluted to 130 mM KCl, loaded onto a phosphocellulose P-11 column (2.5 × 15 cm; Whatman) in equilibrium with 20 mM potassium Pi, (pH 7.0). After washing the column with 55 ml of the same buffer, the reductase activity was eluted with 20 mM potassium Pi (pH 7.0) containing 0.5 mM NaCl. Following the adjustment of the salt concentration to 0.7 M NaCl, the eluted reductase-containing fraction was fractionated on a Matrex gel red A column (1.5 × 10 cm; Amicon Corp, Lexington, MA) in equilibrium with 20 mM potassium Pi (pH 7.0) containing 0.5 mM NaCl. The column was washed with 55 ml of 20 mM potassium Pi, (pH 7.0) containing 0.7 M NaCl, and the eluted reductase was eluted with 2.5 M NaCl in the same buffer. The reductase buffer exchange to 50 mM sodium Pi, (pH 7.6) containing 10% glycerol and 1 mM dithiothreitol was carried out with a HiTrap™ desalting column (Pharmacia Biotech Inc.). The purification of Sps19p was completed by applying the sample to a SMART™-linked Resource™ S column (Pharmacia Biotech Inc.) in equilibrium with the buffer mentioned above, and the bound proteins were eluted with a linear gradient (20 ml from 0–500 mM NaCl) in the equilibrium buffer.

**Demonstration of End Product Accumulation**—An aliquot containing 5 µg of Resource™ S-purified protein was incubated with 810 µl of a mixture that consisted of 0.01 mM potassium Pi, (pH 7.5), 125 µM NADPH, and 60 µM 2,4-hexadienoyl-CoA. After all the ester was metabolized, the reduced was isolated and purified by ultrafiltration and polyacrylamide gel electrophoresis, blotting and hybridization (27), and the reaction was monitored spectrophotometrically at A_{303} nm.

**Miscellaneous**—The following procedures were performed according to published methods: nucleic acid manipulations, formaldehyde gel electrophoresis, blotting and hybridization (30), DNA fragment isolation (37), yeast transformation (28), verification of single plasmid integration (39), yeast RNA preparation for Northern analysis (40), determination of protein concentration (41), sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (42), separation of organelles using homogenized spheroplasts generated from oleate-induced cells (43), and nucleotide sequencing of pAG244 using the CYC1 oligonucleotide (44).

**RESULTS**

**Sps19p Is Homologous to the Human and Rat 2,4-Dienoyl-CoA Reductases**—Analysis of the amino acid sequence of Sp19p revealed an overall 28% identity to the human mitochondrial 2,4-dienoyl-CoA reductase and 24% identity to the corresponding rat enzyme (Fig. 1A). Comparison of the NH_2-terminus residues showed that unlike its mitochondrially targeted mammalian homologues, Sp19p lacked the appropriate leader sequence and instead contained a carboxyl-terminal SKL peroxisomal targeting signal (24). This suggested that Sps19p may be an enzyme involved in β-oxidation (23), since in yeast this process is restricted to the peroxisomes (18). The known 2,4-dienoyl-CoA reductases are NADPH-dependent, and the NAD(P)^+ binding site includes a highly conserved β₃αβ₅ fold (48). The respective Sp19p sequence (Ala^30 to Gly^62) that came closest to this nucleotide-binding motif (Fig. 1A; Ref. 49) deviated from the consensus sequence both at the second and third glycine residues. Sp19p vaguely resembled a reductase, and to obtain a clearer indication for its physiological requirement for the breakdown of unsaturated fatty acids, an appropriately deleted yeast strain was constructed (Fig. 1B).

**Homologous sps19Δ Strains Do Not Grow or Sporulate on Petroselinolate**—To elucidate the potential participation of Sp19p in the metabolism of unsaturated lipids, wild-type and sps19Δ strains were grown on plates containing fatty acids with double bonds at odd-numbered (oleate) and even-numbered (petroselinolate) positions. In these solid media Tween 80 was added to help form the emulsion in the plate, but also to act as a relatively poor carbon source. Hence the strains could all grow on these plates (Fig. 2) but zones of clearing indicate utilization of the additional fatty acid substrate (oleate or petroselinolate).

We found that the deleted strain was unable to form clear zones and to sporulate at all on petroselinolate, but did so on oleate (Fig. 2). This indicated that Sp19p was involved in the removal of double bonds at even-numbered positions. Transformation of the sps19Δ strain with the multicopy plasmid pJC18 containing the intact SPS19 (22) complemented the mutant phenotype, since it restored its ability to utilize petroselinolate.

**Purification and Characterization of Sps19p**—Pilot-scale production of Sp19p using pJC18 transformed cells yielded crude extracts that contained a high 2,4-dienoyl-CoA reductase activity (55 nmol × min⁻¹ × mg⁻¹ protein). Since reductase activity in the extracts from similarly propagated parental strains (without pJC18) was below the detection limit of the used assay, Sps19p could be identified throughout the purification process by enzymatic monitoring. Reductase activity was chromatographically purified from homogenized oleate-induced cultures using phosphocellulose P-11 (cation exchanger), Matrex gel red A (general dye ligand), and Resource™ S (cation exchanger) columns (Fig. 3). In the final preparation, an overall purification of about 50-fold was obtained with an approximate 26% yield, and this preparation showed a specific activity of 1.79 µmol × min⁻¹ × mg⁻¹ protein for 2,4-hexadienoyl-CoA (Table I). SDS-polyacrylamide gel electrophoresis revealed a single protein band with an apparent molecular weight of 34,000, and size exclusion chromatography on a Superdex™ 200 HR 10/30 column (Pharmacia Biotech) yielded a native molecular weight of 69,000, indicating that the reductase was present as a homodimer. The monomer size so obtained agreed with the molecular weight of 31,400 calculated from the deduced amino acid sequence (23).

The amino-terminal residues of the protein were determined by microsequencing following reverse-phase chromatography and the resulting sequence (N-T-A-N-T-L-D-G-A-F-V-T-) compared to the deduced residues 5–15 of Sps19p. Analysis of the SPS19 nucleotide sequence had revealed two potential translational start sites, each of which was followed by the TTA motif for Asn (23). Comparison of the data base sequences for Sp19p had shown a number of differences, most notably at position 2, where Asn (in GenBank™ number M90351) is replaced by Asp (in GenBank™ number X78898; Fig. 1A). The
consensus for the N at position 5 is significant since asparagine is one of nine most abundant penultimate amino-terminal residues (4% of all Saccharomyces genes). Moreover, the finding that the Met at position 4 most likely acted as the signal for initiation of translation concurred with the consensus sequence: 5′-(A/Y)A(A/Y)A(A/Y)AUG-3′ with a strong preference for A at 2′ (50).

Demonstration of 3-Enoyl-CoA End Product Accumulation—In eukaryotes, the β-oxidation of fatty acids with double bonds at even-numbered positions will result in an unsaturated acyl-CoA intermediate with the double bonds at positions 2 and 4. This intermediate serves as a substrate for 2,4-dienoyl-CoA reductase, which introduces a double bond at position 3, resulting in 3-enoyl-CoA. This in turn is the substrate for Δ^9,Δ^2-enoyl-CoA isomerase (Fig. 4A). By generating a protein-free 2,4-hexadienoyl-CoA-depleted reductase reaction using purified Sps19p (Fig. 4B), and then adding the enzymes downstream in the β-oxidation pathway, we were able to identify the end product of the enzyme (Fig. 4C).

The addition of 2-enoyl-CoA hydratase I to the depleted reductase filtrate in the presence of NAD^+ and L-3-hydroxyacyl-CoA dehydrogenase failed to initiate the generation of 3-keto-6-hexanoyl-CoA. However, subsequent addition of Δ^3,Δ^2-enoyl-CoA isomerase to the mixture drove the reaction forward, clearly demonstrating that 3-enoyl-CoA and not 2-enoyl-CoA was the product of the previous reaction. With the enzymatic activity and the reaction end product defined, completion of the characterization of Sps19p required that its location within the cell be determined biochemically and immunohistochemically.

Sps19p Is Localized to the Peroxisomes—The protein A-Sepharose purified IgG fraction from a rabbit injected with the purified Sps19p as antigen was applied to immunoblots containing crude extracts of ethanol-propagated and oleate-induced wild-type and sps19Δ strains (Fig. 5A). A single band...
with the molecular weight of 34,000 was detected solely for the oleate-induced wild-type and not in the sps19Δ strain (for which no band was detected under any conditions), thereby confirming the antibodies' monospecificity and potential utility in studying the subcellular localization of Sps19p. Subsequent fractionation studies of homogenized yeast spheroplasts demonstrated that Sps19p was exclusive to the organellar pellet (Fig. 5B). We then studied the subcellular location of Sps19p using immunoelectron microscopy and found that anti-Sps19p antibodies applied to sections of wild-type cells grown on oleate medium clearly decorated the peroxisomal matrix (Fig. 5, C and D).

The SPS18/19 Promoter Region Is Unidirectionally Activated under Oleate Conditions—Oleate response elements, best described as palindromic CGG triplets spaced by 15–18 base pairs, mediate the transcriptional regulation of a number of peroxisomal protein-encoding genes, including POX1 (51), FOX3 (52), CTA1 (28), and PMP27 (53). The smallest element capable of relaying the fatty acid signal, a single ORE half-site (5'-CGGNNNTNA-3'), is sufficient for bi-directional induction (28). Previous nucleotide analysis of the shared SPS18/19 promoter region had identified potential ORE half-sites (Fig. 1C), two of which occurred as an appropriately spaced inverted repeat (23). We studied the ability of the intragenic region to initiate oleate induced bi-directional transcription by testing the expression of a lacZ reporter gene fused to either end of it (Fig. 6A). We noted that β-galactosidase expression by the SPS19-lacZ integrant under oleate conditions was 27-fold

![Fig. 2](image)

**Fig. 2.** SPS19 deleted strains fail to utilize petroselineate. The diploid deletant demonstrated clear zone formation (halo) and abundant sporulation on oleate, but not on petroselineate medium. No differences between the two strains were observed on Tween 80 (control) plates. The wild-type (WT) and sps19Δ strains used were yAG375 and yAG376, respectively.

![Fig. 3](image)

**Fig. 3.** Pure Sps19p was obtained following ion-exchange chromatography. A, Resource™ S chromatogram of purified protein from the pooled phosphocellulose P-11/Matrex gel red A fractions. B, Coomassie-stained SDS-polyacrylamide gel electrophoresis of the Resource™ S fractions. The lane numbers correspond to the fraction numbers in A. Numbers in the left margin refer to the migration of marker proteins: 14.4, lysozyme (14,400); 21.5, trypsin inhibitor (21,500); 31.0, carbonic anhydrase (31,000); 45.0, ovalbumin (45,000); 97.4, phosphorylase (97,400). The strain used for producing Sps19p was yAG162 transformed with pJC18.

**TABLE II**

| Step                     | Protein  | Total activity | Specific activity | Yield | Purification |
|--------------------------|----------|----------------|------------------|-------|--------------|
|                          | mg       | μmol × min⁻¹ | μmol × min⁻¹ × mg⁻¹ protein | %     | fold         |
| 100,000 × g supernatant  | 126.0    | 4.43           | 0.035            | 100   | 1            |
| P-11/Matrex gel red A    | 14.8     | 2.71           | 0.184            | 61    | 5.3          |
| Resource™ S              | 0.66     | 1.18           | 1.79             | 26.6  | 51.1         |

**Characterization of the Yeast Gene SPS19**

The purification protocol is described under “Experimental Procedures.”
Characterization of the Yeast Gene SPS19

FIG. 4. Sps19p is a 2,4-dienoyl-CoA reductase. A, the position of the enzyme in fatty acid breakdown (modified from Ref. 59). The $\Delta^2,\Delta^4$-dienoyl-CoA isomerase pathway (3, 4) is not included in the figure due to the observations described in this paper. The yeast genes coding for the designated enzymes are as follows: I, FOX1; II, SPS19; III, not identified; IV and V, FOX2 (Fox2p in yeast is a multifunctional enzyme catalyzing sequential 2-enoyl-CoA hydratase and d-specific 3-hydroxyacyl-CoA dehydrogenase reactions via $\Delta^3$-hydroxyacyl-CoA intermediates); VI, FOX3. The eukaryotic and bacterial reaction end products are indicated with a plain and dashed arrow, respectively. B, demonstration of reductase activity for Sps19p. Pure protein (arrow 1) was reacted with NADPH (arrow 2), and following the resetting of the spectrophotometer (*), 2,4-hexadienoyl-CoA substrate was added (arrow 3), and the oxidation of NADPH was monitored. C, the end product of the Sps19p reductase reaction is 3-enoyl-CoA. The previous protein-free, substrate-depleted reaction (arrow 4) was reacted with the NAD+ dependent, monofunctional mammalian mitochondrial enzyme 1-specific 3-hydroxyacyl-CoA dehydrogenase (arrow 5), the monofunctional mammalian mitochondrial enzyme hydrating 2-specific 3-hydroxyacyl-CoA to L-3-hydroxyacyl-CoA, 2-enoyl-CoA hydratase 1 (arrow 6), and finally with $\Delta^2,\Delta^4$-enoyl-CoA isomerase (arrow 7), and the generation of the Mg$^{2+}$-3-ketohexenoyl-CoA was monitored as described under "Experimental Procedures."

higher compared with that expressed by the strain carrying SPS18-lacZ. We also observed that haploids were able to express SPS19 in a sporulation-independent manner.

We investigated the transcription of the two genes under different carbon source conditions (Fig. 6B) and showed that while the profile of SPS19 was similar to that of oleate-inducible FOX3, no signal was detected on the Northern filter when radiolabeled SPS18 was applied. Hence, SPS19 transcription was likely to be mediated by an orientation-governed ORE, and identification of the responsive element, so as to demonstrate its sufficiency for oleate induction, would be facilitated using a reporter gene in which it was mutated.

The SPS19 ORE Is Sufficient for Oleate-dependent Initiation of Transcription—Of the three potential ORE half-sites present within the promoter, we chose to test the one that fully conformed to the 5’-CGGNNNTNA-3’ consensus (Fig. 1C). A derivative of the SPS19-lacZ reporter gene with the distal end of the palindromic ORE replaced by an XhoI site was tested against the parent construct (Fig. 6C). The loss of all $\beta$-galactosidase activity in the strain carrying the mutated reporter clearly indicated that this was the sequence responsible for the oleate inducibility of SPS19. We then monitored the expression of a UAS-less CYC1-lacZ reporter gene into which the palindromic SPS19 ORE had been inserted in the direction of SPS18. The single integrant of the recombinant construct demonstrated a 22-fold increase in $\beta$-galactosidase expression com-
The deduced Sps19p amino acid residues deviate from the conserved NADPH-binding site in reductases, serving to broaden the consensus for this moiety (49). The E. coli 2,4-dienoyl-CoA reductase consists of a single polypeptide chain with a molecular weight of 70,000 (6), and the Candida lipolytica enzyme (33,000 monomer) has been reported to consist of 10–12 identical subunits (8). The mammalian reductases (molecular weight 120,000) act as homotetramers, whereas Sps19p (molecular weight 69,000) is a homodimer. The significant homology of the yeast protein to the mammalian homologues, together with the demonstration of its reductase activity and peroxisomal localization, places Sps19p as a closely related enzyme to the one proposed to exist in humans. Work is currently underway to test whether the phenotype of the yeast mutant is complemented by the appropriately targeted human mitochondrial reductase.

The end products of mammalian 2,4-dienoyl-CoA reductases are always trans-3-enoyl-CoA esters (Fig. 4A), although they accept both trans-2,cis-4-, and trans-2,trans-4-dienoyl-CoA as substrates (6, 10). The inability of sps19A strains to grow on petroselineate plates demonstrated that the S. cerevisiae reductase is physiologically indispensable for the degradation of the trans-2,cis-4-dienoyl-CoA intermediate that arises from the degradation of this fatty acid (cis-6-octadecenoic acid). In our end product accumulation assays we had used trans-2,trans-4-hexadienoyl-CoA. Although the formation of the 3-enoyl-CoA product for the S. cerevisiae reductase had clearly been demonstrated, there exists some uncertainty regarding the ester’s chirality, and we reason that it is likely to be the trans isomer as it is in mammals.

We also provide here physiological data on the phenotype of the first organism devoid of a 2,4-dienoyl-CoA reductase. Although a reductase-deficient E. coli strain had been described previously, it retained 12% of the wild-type enzymatic activity and expressed this defective protein to apparently normal levels (16). Our observation that the deleted yeast strain was unable to utilize petroselineate as the sole carbon source, and that it remained viable on oleate, agrees with the data presented for the bacterial reductase mutant.

If the Δ^2,4-dienoyl-CoA isomerase pathway as demonstrated in rats is taken into account, then reductases are also required for the efficient breakdown of fatty acids with double bonds at odd-numbered positions (3, 4). However, the likelihood that the observed normal growth of sps19A cells on oleate may have been due to the action of a second, antigenically related 2,4-dienoyl-CoA reductase isomerase seems low. Anti-Sps19p antibodies capable of cross-reacting with the rat homologue from livers of clofibrate-treated animals (data not shown) did not react with crude extracts from the oleate-induced deletant. In addition, no isoforms are known to exist for other β-oxidation enzymes such as Pox1p (55), Fox2p (56), and Fox3p (57), where antibodies have been due to the action of a second, antigenically related 2,4-dienoyl-CoA reductase isomerase. In the sps19A cells to grow on oleate suggested that dienoyl-CoA isomerase is not obligatory for the breakdown of unsaturated fatty acids with double bonds at odd-numbered positions.

The unidirectional transcription of SPS19 is mediated by an ORE within the SPS18/19 intergenic region. We identified the site responsible for oleate-induced expression of the reductase gene and demonstrated its sufficiency for oleate-mediated transcription. The shared SPS18/19 promoter region is capable of versatile regulation, where in diploids under sporulation conditions both genes are activated during meiosis, but SPS18 is induced to a 4-fold greater extent than SPS19. In contrast, under oleate conditions SPS19 is exclusively transcribed, and this induction is independent of ploidy.
Curiously, Sp19p is dispensable for the breakdown of oleate, although it is nonetheless induced by the presence of this fatty acid. We speculate that under physiological conditions yeast cells are unlikely to be confronted with a single lipid species as the sole carbon source, and hence when sensing the fatty acid signal cells will metabolically prime themselves to oxidize carbon double bonds at both odd- as well as at even-numbered positions. A deeper insight into how fatty acids induce Sp19p is required.

The yeast locus for the reductase had previously been isolated in a screen for sporulation genes, and the acetate medium (data not shown). Although functionally, the deletant’s incapacity for sporulation on this nonfermentable lineate medium, on which cells transferred to acetate medium (data not shown), although functionally, the deletant’s incapacity for sporulation on this nonfermentable lineate medium, on which cells transferred to acetate medium (data not shown).

Acknowledgments—We thank Hannelore Wrb, Tanja Kokko, Leila Wabnegger, and Walter Stadler for excellent technical assistance and Arnoud J. Kal for his insights from the inception of this work. We especially thank Geoff Kornfeld for facilitating the liaison between the laboratories where this work was conducted. Aner Gurvitz thanks Professor Helmut Ruis for making his stay in Vienna possible.

Characterization of the Yeast Gene SPS19

10. Kimura, C., Kondo, A., Koeda, N., Yamanaka, H., and Mizugaki, M. (1984) J. Biol. Chem. 259, 16489–16495
11. Roe, C. R., Millington, D. S., Norwood, D. L., Kodo, N., Sprecher, H., Mohammed, B. S., Nada, N., Shultz, H., and McVie, R. (1990) J. Clin. Invest. 85, 1703–1707
12. Kunau, W. H., Bühne, S., Moreno de la Garza, M., Kionka, C., Matebholis, M., Schultz-Borchard, U., and Thieringer, R. (1988) Biochem. Soc. Trans. 16, 418–420
13. Veelhuis, M., Matebholis, M., Kunau, W.-H., and Harder, W. (1987) Yeast 3, 77–84
14. Yoon, S.-Y., Coolsy, S., and Schulz, H. (1989) J. Biol. Chem. 264, 16489–16495
15. Coe, J. G., Murray, L. E., and Dawes, I. W. (1992) Mol. Microbiol. 6, 75–81
16. Coe, J. G., Murray, L. E., and Dawes, I. W. (1994) Mol. Gen. Genet. 244, 661–672
17. Gould, S. J., Keller, G.-A., Hosken, N., Wilkinson, J., and Subramani, S. (1989) J. Cell Biol. 108, 1657–1664
18. Krüger, F., Langeder, A., Raupachova, J., Binder, M., and Hartig, A. (1993) J. Cell Biol. 120, 665–673
19. Simon, M. M., Pavlik, P., Hartig, A., Binder, M., Ruis, H., Cook, W. J., Denis, C. L., and Schanz, B. (1995) Mol. Gen. Genet. 249, 289–296
20. Jones, J. S., and Prakash, L. (1990) Yeast 6, 363–366
21. Filipits, M., Simon, M. M., Rapatz, W., Hamilton, B., and Ruis, H. (1993) Gene (Amst.) 132, 49–55
22. Guarente, L., and Ptashne, M. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 2199–2203
23. Myers, A. M., Tragaloff, A., Kinney, D. M., and Lusty, C. J. (1986) Gene (Amst.) 45, 299–310
24. Goldman, P., and Vagepols, P. R. (1961) J. Biol. Chem. 236, 2620–2623
25. Miller, J. H. (1972) Experiments in Molecular Genetics, pp. 352–355, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
26. Moreno de la Garza, M., Shultz-Borchard, U., Crab, J. W., and Kunau, W.-H. (1985) Eur. J. Biochem. 148, 285–291
27. Zeelen, J. P., Paap, R. A., Wierenga, R. K., Kunau, W.-H., and Hiltunen, J. K. (1992) J. Mol. Biol. 224, 273–275
28. Palosaari, P. M., Kilponen, J. M., Sormunen, R. T., Hassinen, I. E., and Kunau, W.-H. (1990) J. Biol. Chem. 265, 3347–3353
29. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
30. Boyle, J. S., and Lew, A. M. (1995) Trends Genet. 11, 8
31. Chen, D.-C., Yang, B.-C., and Kuo, T.-T. (1992) J. Biol. Chem. 267, 3353–3357
32. Miller, J. H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.