Regulatory Elements That Control Transcription Activation and Unsaturated Fatty Acid-mediated Repression of the Saccharomyces cerevisiae OLE1 Gene*

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In Saccharomyces cerevisiae, unsaturated fatty acids are formed from saturated acyl-CoA precursors by Ole1p, a Δ-9 fatty acid desaturase. OLE1 mRNA levels are differentially regulated by the addition of saturated or unsaturated fatty acids to the growth medium. One component of this regulation system involves the control of OLE1 transcription. Saturated fatty acids induce a 1.6-fold increase in transcription activity, whereas a large family of unsaturated fatty acids repress OLE1 transcription as much as 60-fold. A deletion analysis of OLE1 promoter::lacZ fusion reporter genes identified a 111-base pair (bp) fatty acid-regulated (FAR) region approximately 580 bp upstream of the start codon that is essential for transcription activation and unsaturated fatty acid repression. Deletion of an 88-bp sequence within that region resulted in a complete loss in transcription activation and unsaturated fatty acid regulation. The 111-bp FAR element strongly activates transcription and confers unsaturated fatty acid regulation on a heterologous CYC1 promoter test plasmid. Essential elements required for unsaturated fatty acid repression of OLE1 were found in the 5' and 3' region of the 111-bp sequence. The FAR element-mediated activation and fatty acid repression of transcription was found to be closely tied to fatty acyl-CoA metabolism. Two fatty acid activation genes, FAA1 and FAA4, were found to be essential for unsaturated fatty acid repression of OLE1 through the FAR sequences. Disruption of either gene results in reduced levels of unsaturated fatty acid repression; disruption of both genes completely blocks the regulatory response. Acyl-CoA binding protein (ACBP) plays a role in determining the level of FAR element activated transcription. Disruption of the ACBP gene causes a >5-fold activation of OLE1 transcription and a similar increase in OLE1 mRNA levels. Unsaturated fatty acid repression of OLE1 transcription, however, is not affected by the disrupted ACBP gene. These studies show that promoter elements responsible for unsaturated fatty acid-mediated transcription repression are tightly linked to OLE1 activation sequences and that OLE1 transcription levels are closely tied to acyl-CoA metabolism.

Nutrient fatty acids can exert strong regulatory effects on a number of lipogenic enzymes in fungi. Medium and long chain fatty acids are readily internalized by fungi and incorporated into membrane and storage lipids. Saturated and unsaturated acids appear to differentially regulate the expression of a number of lipid biosynthetic genes, including those encoding acetyl-CoA carboxylase (2–4), fatty-acid synthase (5, 6), and fatty acid desaturase (1). The mechanisms by which cells sense fatty acids, discriminate among molecular species, and modulate gene activity in different parts of the lipid metabolic web is unclear, although it appears that multiple systems have evolved to regulate different lipogenic functions.

To identify the mechanisms that control lipid synthesis in response to extracellular acids, we examined the regulation of the Δ-9 fatty acid desaturase, an enzyme involved in the formation of unsaturated fatty acids. The OLE1 gene, which encodes that enzyme in Saccharomyces, is strongly regulated in response to extracellular fatty acids (1). This can be seen as a rapid reduction in OLE1 mRNA levels when unsaturated acids are added to the growth medium and an increase in enzyme activity when cells are exposed to saturated fatty acids. The exogenous fatty acids that trigger these responses are rapidly incorporated into membrane lipids; thus it is reasonable to expect that the sensors and signal transducers that regulate OLE1 in response to nutrient fatty acids may also be a part of broader controls that regulate membrane lipid composition in response to other stimuli.

Our recent studies suggest that fatty acid regulation of OLE1 is under at least two forms of control (7). One component acts to repress transcription of the OLE1 gene; a second acts by post-transcriptional mechanisms to further modify OLE1 mRNA levels (7). In this paper, we examine the promoter of the desaturase gene to identify transcriptional controlling elements that respond to unsaturated fatty acids and to assess their contribution to OLE1 expression. An essential transcription activation region, designated the FAR element, is identified that also contains the elements required for unsaturated fatty

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The abbreviations used are: FAR, fatty acid-regulated; UDrT, uracil drop-out synthetic medium with tertigol; bp, base pair(s); PCR, polymerase chain reaction; kb, kilobase(s); ACBP, acyl-CoA binding protein; FAA, S. cerevisiae fatty acid activation gene (acyl-CoA synthetase); ER, endoplasmic reticulum.
acid-mediated repression of transcription activity. There appears to be a close connection between cellular acyl-CoA metabolism and regulators that act on that region of the OLE1 promoter.

MATERIALS AND METHODS

Strains, Growth Medium, and DNA Manipulations—Strains used in this study are shown in Table I. Yeast cells containing lacZ fusion plasmids were grown at 30 °C on uracil dropout synthetic dextrose (8) medium supplemented with 1% tergitol (UDt) and 1 mM appropriate fatty acid (obtained from Sigma or Nu-Chek Prep). All unsaturated fatty acids used in this paper contained double bonds in the cis configuration. All recombinant DNA manipulations were performed according to standard methods (8, 9). Plasmid amplifications and bacterial transformations were performed using Escherichia coli strains HB101, DH5α (Life Technologies, Inc.), or XL1-Blue (Stratagene). Yeast transformations were performed by the method of Ito et al. (10) or by electroporation (Life Technologies, Inc.) according to the manufacturer’s protocols.

Construction of OLE1 Promoter-lacZ Fusion Deletion Series—Plasmids used in this study are shown in Table II. Plasmid p62 was constructed by exchanging a HindIII/Stul fragment from plasmid YCp50 (11) with a DNA fragment from plasmid YEP356R (7), which contains a multiple cloning sequence fused to bases 3582 through 3614 of the E. coli lacZ gene. Plasmid p62:–934 was constructed by the insertion of an OLE1 1015-bp HindIII/Sall fragment into the p62 multiple cloning site. That fragment extends from –934 base pairs upstream of the start codon through codon 27 of the OLE1 protein region to form an in-frame fusion with the E. coli lacZ sequence. A nested series of OLE1 promoter deletions were derived from that vector; these cover the region from nucleotides –934 to –142 relative to the first in-frame ATG codon. They were generated by a combination of restriction sites and Bal31 digestions. All Bal31-generated end points were determined by DNA sequencing.

Plasmid p62:–934 was constructed by isolating a fragment consisting of OLE1 nucleotides –934 to –576 and fusing it to a fragment containing bases –489 to +81. The resulting fragment was ligated into the parent p62 plasmid, yielding an OLE1 insert with the same ends as p62:–934 but lacking 88 bases between –576 and –489. All fragments in plasmid p62 series had a 3′ end point at nucleotide +81. The promoter deletion constructs were used to transform the phenotypically wild-type OLE1 strain, L8–25A, to uracil prototrophy.

Construction of pCt1 Vectors Containing OLE1 Promoter Sequences—Plasmids derived from vector pCT2 are shown in Table II. Promoter test vector pCTΔ was obtained from Kornberg (12, 20). It contains a multiple cloning site fused to bases –248 through +5 of the Saccharomyces CYC1 promoter region, which is fused in-frame to E. coli lacZ. The CYC1 fragment includes two functional TATA elements, but it does not contain the CYC1 upstream activating sequences. During the course of this study, a sequence contained in the KpnI-Smal restriction sites in the multiple cloning region of the vector was found to be a transcription-repressing element that acted independent of the inserted test sequences. Vectors in which that site was removed by restriction enzyme digestion of flanking sequences were designated as pCTm (pCT modified) vectors.

Vectors pCT714 and pCTm714 were constructed by inserting a 714-bp HindII/HpaI fragment that extended between bases –934 and –221 of the OLE1 promoter into the pCTΔ multiple cloning region. Vectors pCTm111 and pCTm111 were similarly constructed by inserting 111 bp of the OLE1 promoter region that extends from bases –576 to –466. Vector pCTm111E10 was made by inserting an EcoRI linker (sequence CCGAATTCCG) into the Smal restriction site at the 5′ end of the 111-bp fragment of pCTm111. pCTm100 was derived from pCTm111E10 by EcoRI digestion to remove the 11 upstream base pairs of the OLE1 promoter sequence.

Vectors pCTm67 and pCTm40 were constructed using synthetic paired oligonucleotides. Vector pCTm67 contains bases –582 to –516, and plasmid pCTm40 includes bases –582 and –543 of the OLE1 promoter sequences.

Vector pCTm11 was constructed using a 91-bp fragment encompassing bases –556 to –466 of the OLE1 promoter (derived by PCR amplification of OLE1 promoter sequences). The base sequence of the PCR fragment was determined by DNA sequencing. Plasmid pCTm114 contains bases –579 to –466 of the OLE1 promoter. Vector pCTm25 was prepared by digestion of vector pCTm114 at the Apal restriction site within the 114-bp OLE1 sequence and the vector Xhol site.

Integrating vectors pCTm714 and pCTm111 were constructed by removing the ARS and CEN sequences from their respective pCTm parents by HindIII digestion and religation.

β-Galactosidase Assays—Assays of cells containing plasmids derived from OLE1 promoter-lacZ fusion plasmids were performed as described previously (8). Cell densities for those assays were determined either by measurement at A550 or by hemocytometer counts. Corrections were made for light scattering by A420 absorption. Assays of extracts from cells containing pCT plasmids were assayed by the procedure of Buchman et al. (12). The latter assays were correlated with total protein in cell extracts by the method of Bradford (13) using the Bio-Rad assay kit. Two or three independent yeast transformants were assayed for each of the plasmid constructs given in Table II. β-Galactosidase activities reported here are the results of at least three independent experiments. Each experimental assay was performed in triplicate.

Fatty Acid Repression/Derepression Studies—Cells containing an OLE1-lacZ reporter gene were tested for unsaturated fatty acid regulation according to protocols that measure repression of activity from a derepressed state. Cells were grown overnight in uracil dropout synthetic dextrose medium supplemented with 1% UDt. These cultures were used to inoculate (at one-tenth total volume) fresh UDt medium or
Deletions of the OLE1 promoter::lacZ fusion constructs and their activity in the phenotypically wild-type strain L8–25A.

The upper left scale represents DNA sequences upstream of the OLE1 protein coding region. The relative locations of two proposed TATA boxes at positions −160 (TATAA) and −30 (TATAAAA), and the start codon are indicated. Restriction sites shown on the scale that were used for constructions have the following designations: H, HindIII; S, SmaI; P, PstI. Deletion constructs are shown in the column below the scale. The narrow line represents OLE1 promoter sequences, the solid black bar represents the amino terminal 27 amino acids of the OLE1 coding sequence fused to E. coli lacZ (hatched bar). The number above each line indicates the position of the deletion end point with respect to the ATG start codon of the wild-type base sequence with the A of the codon designated as +1. Deletion end points were verified by sequencing as described under "Materials and Methods." The p62::934Δ88 construct represented by the bottom line contains an 88-bp deletion at the indicated position in the promoter region. Bars to the right of each diagram illustrate the β-galactosidase activity in Miller units (30) produced by each construct in cells grown without unsaturated fatty acids (hatched bars) and with unsaturated fatty acids (solid bars). Units of activity indicated are the average of at least three independent experiments performed on two separate transformants. Standard deviations of these values were less than 20% of the mean.

UDT medium supplemented with 0.5 mM of each of the unsaturated fatty acids (16:1 and 18:1) or 1 mM of 18:2. These cultures were incubated at 30 °C with rotary shaking for 8–10 h followed by harvesting by centrifugation for analysis of β-galactosidase activity.

RNA Isolation and Northern Blot Analysis—Total yeast RNA was isolated essentially as described previously (14, 15). Equal amounts (10 μg) of total RNA from each time point of an experiment were analyzed by Northern blots according to standard procedures for separation of RNA using 1% formaldehyde gels (8). RNA from the gels was transferred to the Zeta Probe membrane (Bio-Rad) in 10× SSC for 90 min at 5 inches of mercury using a Vacuum Blotter (model 785) from Mini Oven MK II was used as described in the instruction manual. A hybridization oven (Hybaid Mini Oven MK II) was used as described in the instruction manual supplied by the manufacturer. The membrane was prehybridized at 65 °C for 10–20 min in 1× SSC, 0.25 M Na2HPO4, pH 7.2, and 7% SDS. The membrane was hybridized at 65 °C overnight with 2.5 × 104 cpm/ml. Following incubation the membrane was washed twice (30 min/wash) at 65 °C with 1× SSPE, 40 mM Na2HPO4, pH 7.2, and 1% SDS. Northern blots were quantitated using a phosphorimaging analyzer (Molecular Dynamics).

Preparation of Radiolabeled Probes—For the detection of OLE1 mRNA, a radiolabeled DNA probe was made using a 0.5-kb EcoRI fragment from the OLE1 protein coding region. For an internal control of cellular mRNA levels, a 1-kb HindIII-KpnI fragment of the Saccharomyces phosphoglycerate kinase gene (PGK1) was isolated from plasmid pRP1PGK from S. Pelz (Robert Wood Johnson Medical School). All DNA fragments were separated by agarose gel electrophoresis in 1× TAE and purified by Gene Clean II (Bio 101) according to manufacturer’s recommendations. The purified DNA fragments were labeled to high specific activity with [α-32P]dATP (DuPont NEN) by the random primer extension method (16) using a PROBE-EZE reaction kit (5 Prime→3 Prime). Unincorporated nucleotides were removed from the sample using a Sephadex G-50 spin column (5 Prime→3 Prime). Specific activity of labeled probes was determined by liquid scintillation counting.

Fatty Acid Extraction and Analysis—Total cellular fatty acids were obtained by HCl methanlysis of extensively washed cell pellets according to the modified procedure of Brouse et al. (17) as described previously (7). Lipid fractionation and preparation of fatty acid methyl esters were performed as described previously (7). Fatty acid methyl esters were separated on a Superoxawax 10 30 m × 0.032 mm capillary column using helium as a carrier gas in a Varian 3400CX gas chromatograph.

Cloning and Disruption of ACBP—A 1.6-kb DNA fragment encoding the S. cerevisiae acyl-CoA binding protein (ACBP) gene was cloned by PCR amplification of genomic DNA. PCR primers used for the amplification were 5′-TTCCACATGTGAATTACATTGGTG-3′ (bases −853 to −831 of ACBP gene, where +1 is the A in the ATG start codon) and 5′-GTGTAATGACATACATTG-3′ (positions +728 to +749). This fragment was cloned into PCR-script SK (+) (Stratagene). A 700-bp fragment containing the ACBP protein coding region and flanking vector 5′ and 3′ sequences were removed by BglII/BclI digestion. This was replaced with a 2-kb fragment consisting of the Saccharomyces LEU2 gene. A 2.8-kb linear DNA fragment encompassing the LEU2 gene and flanking ACBP gene sequences was isolated from the plasmid and used to perform a one-step gene disruption (18, 19) of the chromosomal ACBP gene in strain DTY-10A. The disruption of ACBP was confirmed by leucine prototrophy and PCR analysis of genomic DNA.

RESULTS

Deletion Analysis of OLE1 Promoter::lacZ Fusion Vectors—DNA fragments from the OLE1 promoter consisting of a nested series of 5′ deletions (starting from base −934 relative to the start codon) were placed in frame with β-galactosidase coding sequences in the single copy CEN plasmid (p62) and transformed into a phenotypically wild-type (OLE1+) strain L8–25A for analysis. β-Galactosidase activities for the reporter genes are shown in Fig. 1.

Deletion of bases −934 to −576 caused an approximate 3-fold loss of reporter gene activity. Removing bases −576 through −488 caused a further 20-fold drop in reporter gene activity, indicating the presence of an activating sequence in the region near base −576. Deletions producing 5′ ends between −396 and −255 exhibited small (2-fold) increases in expression corresponding to approximately one-thirtieth of the activity seen with the entire 934-bp promoter fragment. Deletions beyond base −255 produced reporter gene activities near the basal level exhibited by the plasmid that contains no OLE1 promoter sequences. An 88-bp deletion in the putative activation region between bases −576 through −489 (Fig. 1) showed a 27-fold reduction in β-galactosidase activity, indicating that
Regulation of Saccharomyces OLE1 Transcription

Fatty Acid Regulation of the Promoter Deletion Vectors—

Previous studies of a gene fusion containing the entire 934-bp promoter region fused to lacZ, indicated that reporter gene activity was strongly repressed by a set of mono- and polyunsaturated fatty acids (7). To identify the position of potential unsaturated fatty acid regulation regions within the promoter, parallel cultures grown in the presence or absence of a mixture of 16:1 and 18:1 were assayed for parallel cultures grown in the presence or absence of a mixture of 16:1 and 18:1. These experiments were performed using standardized growth conditions that cause an 8–10-fold repression of transcription activity from the plasmid that contains the original promoter fragment in construct p62::−934, with OLE1 fragments inserted into heterologous constructions employing yeast CYC1:lacZ gene fusion plasmids. Plasmid pCT (20) contains a polylinker upstream of the S. cerevisiae CYC1 promoter that lacks the CYC1 UAS sequence. Fragments of the OLE1 promoter were inserted into the polylinker region to test for the ability to activate transcription and confer fatty acid regulation on this test plasmid, as described under “Materials and Methods.” Plasmid pCT714 contains a 714-bp fragment extending from bases −934 to a HpaI site at position −221, which lies upstream of the OLE1 TATA sequence. OLE1 promoter sequences are indicated by the black line. The left-most box placed in the OLE1 promoter sequences indicates the position of the 111-bp FAR region shown to be essential for transcription activation and unsaturated fatty acid repression. Black bars represent CYC1 promoter sequences; hatched boxes indicate lacZ coding regions. Activities of heterologous promoter test plasmids are shown to the right of the corresponding figure and are expressed in nmol of o-nitrophenyl-β-D-galactopyranoside (ONPG) hydrolyzed/min/mg protein. Activities indicated are the averages of at least three independent experiments ± S.D. for each vector. Hatched bars represent activities in cells grown without added fatty acids (NFA), shaded bars represent activities of cells grown in the presence of 0.5 mM 16:1 and 0.5 mM 18:1 (UFA).

Activity (nmols ONPG / min / mg Protein)

Fig. 2. Expression of reporter gene activity from heterologous pCT vectors containing OLE1 promoter sequences. Diagrams on the left compare the OLE1 promoter fragment in construct p62::−934, with OLE1 fragments inserted into heterologous constructions employing yeast CYC1:lacZ gene fusion plasmids. Plasmid pCT (20) contains a polylinker upstream of the S. cerevisiae CYC1 promoter that lacks the CYC1 UAS sequence. Fragments of the OLE1 promoter were inserted into the polylinker region to test for the ability to activate transcription and confer fatty acid regulation on this test plasmid, as described under “Materials and Methods.” Plasmid pCT714 contains a 714-bp fragment extending from bases −934 to a HpaI site at position −221, which lies upstream of the OLE1 TATA sequence. OLE1 promoter sequences are indicated by the black line. The left-most box placed in the OLE1 promoter sequences indicates the position of the 111-bp FAR region shown to be essential for transcription activation and unsaturated fatty acid repression. Black bars represent CYC1 promoter sequences; hatched boxes indicate lacZ coding regions. Activities of heterologous promoter test plasmids are shown to the right of the corresponding figure and are expressed in nmol of β-nitrophenyl-β-D-galactopyranoside (ONPG) hydrolyzed/min/mg protein. Activities indicated are the averages of at least three independent experiments ± S.D. for each vector. Hatched bars represent activities in cells grown without added fatty acids (NFA), shaded bars represent activities of cells grown in the presence of 0.5 mM 16:1 and 0.5 mM 18:1 (UFA).

essential transcription activation elements were located in that region.

Fatty Acid Regulation of the Promoter Deletion Vectors—Previous studies of a gene fusion containing the entire 934-bp promoter region fused to lacZ, indicated that reporter gene activity was strongly repressed by a set of mono- and polyunsaturated fatty acids (7). To identify the position of potential unsaturated fatty acid regulation regions within the promoter, parallel cultures grown in the presence or absence of a mixture of 16:1 and 18:1 were assayed for β-galactosidase activity (Fig. 1). These experiments were performed using standardized growth conditions that cause an 8–10-fold repression of reporter gene activity from the plasmid that contains the original 934-bp upstream OLE1 promoter region. Higher levels of repression (up to 60-fold reductions in activity) can be produced from the same construct by continuous feeding of unsaturated fatty acids.2 Reporter plasmids containing deletions from −934 to −576 showed the full range of repression normally seen under these experimental conditions. Deletions downstream from base −576, however, lost the ability to be repressed concomitantly with the loss of transcription activation.

The activity of the reporter containing the 88 bp deletion within the 934-bp fragment was reduced 2-fold in response to unsaturated fatty acids.

Tests performed on the effects of saturated fatty acids on strains containing the promoter deletion series produced a different pattern of regulation (data not shown). Supplementation of media with 0.5 mM 14:0 produced a 1.2–1.5-fold increase in activities of cells that contained plasmids p62::−934 and p62::−792. By contrast, reporter activity of the plasmid p62::−576 was decreased 5-fold by the addition of 14:0. Activities of plasmids containing more extensive 5′ deletions in cells incubated with 14:0 did not differ significantly from those grown in media with no fatty acid supplements.

A 111-bp FAR Element Contains the Primary Transcription Activation and Unsaturated Fatty Acid Regulatory Elements of OLE1—Because sequence elements between −576 and −489 were found to be required for transcription activation and unsaturated fatty acid regulation, a series of OLE1 promoter fragments was tested for the ability to activate and confer unsaturated fatty acid repression on an unrelated gene. A CEN vector (designated vector pCT) that contains the basal promoter elements of the Saccharomyces CYC1 gene fused to lacZ (20) (Fig. 2) was used for these tests. This vector lacks the CYC1 UAS sequences and produces low levels of reporter activity when activating sequences are absent from a multiple cloning site upstream of the basal promoter region. Vector pCT contains two TATA elements of the CYC1 gene; these have an arrangement similar to that found in elements in the OLE1 gene.

Plasmid pCT714 contains a 714-bp OLE1 promoter fragment that extends between bases −934 and −221 (Fig. 2). Insertion of that fragment upstream of the CYC1 TATA region produced 66-fold higher levels of reporter gene activity, compared with the parent vector that contains no insert. Plasmid pCT714Δ88 contains the same 88-bp deletion as that in p62::−934Δ88. That vector showed only a 2-fold increase in activity above the basal levels produced by the parent plasmid.

Reporter gene activity was also strongly activated in vector pCT111, which contains a 111-bp fragment derived from bases −576 to −466. The 5′-end of that fragment coincides with the 5′-end of the 88 bases deleted from the pCT714Δ88 vector that were found to be essential for transcription activation. Plasmid pCT111 exhibited a 28-fold increase in activity over the control plasmids that contained no OLE1 promoter sequences.

Vectors that included either the 111-bp sequence or the intact 714-bp OLE1 promoter sequence were repressed by the addition of unsaturated fatty acids to the growth medium (Fig.

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2 S. Hwang and C. Martin, unpublished observations.
Nucleotide sequences associated with the FAR element responsible for transcription activation and unsaturated fatty acid regulation. Underlined sequences represent symmetric GC-rich sequences found within the 114-bp fragment of plasmid pCTm114. Bases in lower case and boldface represent the FAR region sequence required for unsaturated fatty acid-mediated repression of OLE1 transcription in plasmid pCTm100.

Vector pCT111 showed an approximate 2-fold repression of activity under conditions where the reporter that contained the entire promoter was repressed 7-fold. The vector containing the 88-bp deletion within the 714-bp promoter sequence was not repressed by unsaturated fatty acid regulation. These data suggest that primary elements essential for unsaturated fatty acid regulation are contained within the 111-bp promoter sequence. A parallel set of results was also obtained in tests of these fragments in the related pCZ plasmid (20). That vector differs from the plasmid pCTΔ in that it contains a shorter part of the CYC1 basal promoter region that includes only one TATA sequence (data not shown). Given the ability of the 111-bp DNA fragment to strongly activate transcription and confer fatty acid-specific repression on heterologous vectors, it was designated as the OLE1 FAR sequence. The nucleotide sequence of this fragment and flanking sequences is shown in Fig. 3.

Symmetric GC-rich Sequences Associated with the FAR Region—There are three symmetric base sequences located at the distal end of the FAR region. Two identical sequences (CCCGGG) are separated by five base pairs. Another in the reverse order (GGGCCC) is located six bases downstream. During the course of this study we also determined that the pCT vector contained another CCCGGG element within the multiple cloning site; this element was situated between the inserted promoter fragments and the CYC1 basal promoter elements. That part of the vector (derived from the pUC19 vector multiple cloning site) has been shown to be a fortuitous binding site for the general transcription factor REB1(12). Because the vector sequence might provide a target for an interfering transcription factor or compete with OLE1 regulators, it was removed by restriction digest of flanking sequences. The modified vectors expressed 3-4 fold greater activity than the original vectors that contain the additional CCCGGG site (Table III). Given those results, all further tests of the FAR region were carried out using the modified pCT vector (designated as pCTm) in which that site and flanking sequences were deleted.

Fatty Acid Regulation of Heterologous Vectors Containing Sequences Derived from the FAR Elements—Activities of pCTm vectors containing fragments associated with the FAR region were tested to determine the minimum size required for transcription activation and unsaturated fatty acid regulation (Fig. 4). In this series of experiments, linoleic acid (18:2) was used to repress transcription. We have previously shown that it is an efficient repressor of OLE1 expression and has the advantage that its incorporation in yeast can be accurately monitored by gas chromatography (7).

Because the previously identified 111-bp FAR region fragment contains only the GGG bases of the upstream CCCGGG sequence, vector pCTm114 was constructed to include both members of the symmetric pair. Compared with pCTm111, the activity of pCTm114 was slightly reduced, but there were no significant changes in the level of unsaturated fatty acid repression. Two additional vectors were constructed to test the effects of disrupting both CCCGGG sequences. A 10-bp linker was inserted into the remaining CCCGGG sequence of pCTm111 to produce pCTm111E10. That vector exhibited an approximate 50% decrease in reporter activity (compared with pCTm111) with no significant changes in the level of unsaturated fatty acid repression. Vector pCTm100 was constructed to contain only the GGG of the downstream CCCGGG site. It showed activities and level of fatty acid repression nearly identical to vector pCTm111E10. Deletion of an additional nine base pairs (pCTm91), however, resulted in a 50% reduction of derepressed activity and complete loss of fatty acid repression.

Plasmids were transformed into strain L8–25A and tested for β-galactosidase activity after growth in UDT medium with no fatty acids (NFA) or in the presence of 1 mM 18:2 (UFA). Unsaturated fatty acid-mediated repression is expressed as the ratio of activities from the plasmid in cells grown under depressed (NFA medium) and repressed (UFA; unsaturated fatty acid-supplemented) conditions. NFA/UFA ratios ≥1 indicate a loss of the regulatory response. β-Galactosidase activity was measured relative to soluble protein by the method of Buchman et al. (12) as described under “Materials and Methods.” Data represent average of three or more independent experiments ± S.D.

| Vector      | Supplement | Activity | NFA/UFA |
|-------------|------------|----------|---------|
| pCT714      | NFA        | 13.3 ± 0.3 | 8.3     |
| pC714       | UFA        | 1.6 ± 0.6  |         |
| pCTm111     | NFA        | 36.9 ± 3.0 | 7.7     |
| pCT714      | UFA        | 4.8 ± 0.7  |         |
| pCT111      | NFA        | 5.6 ± 1.2  | 2.3     |
| pCTm111     | UFA        | 2.4 ± 0.6  |         |
| pCT714      | UFA        | 20.2 ± 3.2 | 1.9     |
| pCTm110     | NFA        | 0.2 ± 0.0  | 0.25    |
| pCT714      | UFA        | 0.8 ± 0.3  |         |
| pCTm111     | NFA        | 0.6 ± 0.1  | 0.35    |
| pCT714      | UFA        | 1.7 ± 0.0  |         |
While that observation indicates that the 9-bp fragment contains sequences essential for fatty acid-mediated repression, deletion of a 50-bp fragment from the distal end of the FAR region (pCTm67) indicates that those sequences are also essential for activation and repression. Shorter test fragments containing 40- and 25-bp sequences encompassing the distal part of the FAR region showed a complete loss of activation and repression. They exhibit levels of activity similar to that of the parent vector with no inserted promoter sequences. Vectors containing fragments shorter than the 100-bp fragment in pCTm100 also exhibit slightly increased levels of activation in the presence of unsaturated fatty acids, similar to that observed with the vector alone.Taken together, these data indicate that both the 5’- and 3’-ends of the pCT111FAR region are required for maximal activation and unsaturated fatty acid-mediated repression.

To determine their response to saturated fatty acids, pCT plasmid-bearing strains were tested by supplementing cells with 14:0 (data not shown). Similar results were obtained to plasmid-bearing strains were tested by supplementing cells containing the 714-base promoter region exhibited a 1.6-fold increase in activity in cells grown in the presence of 0.5 mM 14:0. Vectors containing elements of the FAR region, including pCTm114, pCTm111, pCTm91, and pCTm67, did not show any significant increase in activity in cells incubated with the saturated fatty acid.

Integration of pCTm111 and pCTm174 into a Chromosomal Locus Results in Increased Levels of Derepressed Activity and Unsaturated Fatty Acid-mediated Repression—To determine if the gene activation and unsaturated fatty acid repression effects seen with the reporter genes were plasmid-specific, plasmids pCTm111 and pCTm174 were converted to integrating vectors by cutting with HindIII to remove the ARS and CEN sequences. These were integrated into the URA3 locus of strain L8-25A. Activity of the integrating vector (pITm) was increased approximately 5-fold over that of the autonomous pCTm plasmid in cells grown in unsupplemented medium (Table IV). Its activity was increased approximately 2-fold in cells grown in the presence of unsaturated fatty acids. Activity of the integrated pITm111 and pITm174 reporters was increased approximately 3-fold in unsupplemented medium, compared with their plasmid counterparts. The pITm111 reporter activity was repressed approximately 3-fold and pITm174 more than 10-fold by exposure of cells to 18:2. All three integrated reporters exhibited parallel behavior to that of their plasmid counterparts with respect to relative levels of activity and response to unsaturated fatty acid supplements.

Mutants Defective in Fatty Acid Activation Fail to Regulate OLE1 Transcription—Exogenous fatty acids must be converted to acyl-CoA species to be incorporated into membrane and storage lipids. S. cerevisiae contains at least five genes that activate fatty acids to form acyl-CoAs. Four unlinked acyl-CoA synthetase genes designated FAA1-4 have been recently identified and cloned (27). To test the effects of fatty acid activation on the expression of OLE1, combinations of null mutations in these four loci were analyzed using OLE1 promoter::lacZ plasmids (Table V). Tests of pCTm174 reporter activity showed that strains containing either the faa1Δ or faa4Δ disrupted genes significantly reduced the level of unsaturated fatty acid repression, compared with the wild-type parent. In the wild type, 18:2 repressed levels of OLE1 average 12% of that in cells not exposed to the fatty acid. By comparison, 18:2 repressed reporter activities in single mutants faa1 and faa4 were 40-42% that of unsupplemented cells. Repression was completely blocked in strain YB525, which is a double faa1Δ,faa4Δ mutant. Mutants faa2Δ and faa3Δ showed levels of 18:2 repression that were close to those observed in the wild-type parent. Repressed levels of reporter activity in faa2Δ and faa3Δ strains were approximately 17% of that of their corresponding derepressed levels. Reporter activity in strain YB526, which contains disruptions in all four FAA genes, also failed to be repressed, showing no difference in levels between 18:2 fed and nonfed cells. To test whether the effects of the faa1Δ and faa4Δ mutants act through the FAR region, plasmid pCTm111 was tested in strains containing the double faa1Δ,faa4Δ disruptions and the quadruple faa1,2,3,4Δ disruptions. Reporter activities failed to be repressed in both disrupted strains under conditions where activity in the wild-type parent was repressed 1.9-fold.

The presence of doubly disrupted faa1Δ and faa4Δ genes also

| Integrated sequence | Supplement | Activity | NFA/UFA |
|---------------------|------------|----------|---------|
| NMol/mg protein/min ± S.D. |
| pCTm174 | NFA | 92.0 ± 0.5 | 18.4 |
| | UFA | 50.0 ± 2.8 |
| pCTm111 | NFA | 638 ± 11.1 |
| | UFA | 20.0 ± 5.9 |
| pCTm | NFA | 32 ± 0.3 |
| | UFA | 5.2 ± 1.6 |

| Strain | Relevant genotype | Plasmid pCTm174 (714-bp promoter region) | Plasmid pCTm111 (111-bp FAR region) |
|--------|------------------|--------------------------------------|--------------------------------------|
| | | NFA activity | NFA/UFA | NFA activity | NFA/UFA |
| | | nmol/min/mg protein ± S.D. | ± S.D. | nmol/min/mg protein ± S.D. | ± S.D. |
| YB322 | Wild type | 29.4 ± 6.74 | 8.6 ± 0.53 | 20.7 ± 1.4 | 1.9 ± 0.4 |
| YB513 | faa1Δ | 20.3 ± 0.25 | 32.6 ± 0.81 | 7.5 ± 0.28 |
| YB489 | faa2Δ | 17.9 ± 0.75 | 32.6 ± 0.81 | 7.5 ± 0.28 |
| YB492 | faa3Δ | 20.3 ± 2.3 | 5.9 ± 0.004 |  |
| YB524 | faa4Δ | 40.0 ± 6.3 | 25.0 ± 0.8 |  |
| YB525 | faa1Δ | 63.0 ± 11.6 | 11 ± 0.3 | 76.3 ± 6.4 | 0.9 ± 0.02 |
| YB526 | faa1,2,3,4Δ | 57.3 ± 8.9 | 1.0 ± 0.03 | 96.1 ± 10.6 | 0.7 ± 0.05 |
appears to have a strong effect on the activation levels of the reporter genes. Derepressed activities of both pCTm714 and pCTm111 in the double disruptant were increased 2-3-fold over the wild-type parent.

**Acyl-CoA Binding Protein Affects the Level of OLE1 Transcription and Cellular Fatty Acyl Composition—**The connection between acyl-CoA synthase activity and unsaturated fatty acid repression suggested that the ACBP could be a component of the molecular sensor that acts in the fatty acid-mediated regulation of OLE1. Almost all of the cell’s fatty acyl-CoA species are bound to this ubiquitous 10-kDa protein that binds medium and long chain acyl-CoA species with high specificity and high affinity. Furthermore, ACBP does not bind CoA, non-esterified fatty acids, or acyl carnitines (26).

To assess the effects of this protein on OLE1 regulation, the gene was cloned by PCR and disrupted by replacing sequences that flank the coding region of the protein with the Saccharomyces LEU2 gene. Surprisingly, no visible phenotype other than a slight retardation in growth was observed in the disruptant strains. The effect of the disruption, however, was to produce an approximate 3-4-fold increase in transcription activity of reporter plasmids that contain the entire OLE1 upstream region (p62:::LEU2) and an approximate 5-fold increase in transcription activity with the pCTm111 vector containing the 111-bp FAR region (Table VI).

The effect of the ACBP gene disruption on transcription had a concomitant effect on OLE1 mRNA levels (Fig. 5). Quantitative phosphor image analysis of mRNA from the Saccharomyces PGK1 gene as an internal standard, revealed that OLE1 mRNA levels in the disruptant strain were 5.5-fold higher than in its wild-type parent grown in fatty acid-deficient medium. Surprisingly, disruption of the ACBP gene had no effect on relative levels of unsaturated fatty acid repressed OLE1 transcription (Fig. 5). ACBP disruption also appears to have profound effects on cellular fatty acid composition. The increased levels of OLE1 transcript seemed to result in a net increase in desaturase activity relative to saturated fatty acid biosynthesis. Analysis of total cellular fatty acids revealed a striking increase in 14-16 carbon species and an increase in the ratio of unsaturated to saturated fatty acids. Due to increased desaturation of 14:0 and 16:0, 14:1 levels increased to 5% wild type from less than 1% and 16:1 levels increased from 41.7-59.2% in the acbp:::LEU2 disruptant. Levels of the 18 carbon species were reduced in the disruptant, primarily as a function of the reduction in 18:1-21% (down from 29%) of the total fatty acid mass (Table VII).

Participation of Hap1p in FAR Region Activated Transcription—Endoplasmic reticulum desaturases are thought to require heme-dependent electron transfer as part of their reaction mechanism. We have recently found that Ole1p has a cytochrome b_5-like heme binding domain at its carboxyl terminus. Given that the Hap1p and Hap2/3/4p transcription factors are components of regulatory systems that govern synthesis of heme proteins in Saccharomyces, we used the OLE1 reporter genes to test whether these transcription factors were essential for activation and unsaturated fatty acid mediated regulation of OLE1 (25). Plasmids p62:::LEU2 and pCTm111 tested in the hap1::LEU2 gene disrupted strain and its wild-type parent (Table VIII). β-Galactosidase activities from the 934- and 111-bp promoter sequences were reduced by 40 and 30%, respectively, in the hap1 disrupted strain, compared with wild type. By contrast, a hap2/3::LEU2 disruptant showed no reduction in activity over the congenic wild-type strain.

The absence of the Hap1p transcription factor appeared to have no significant effect on fatty acid regulation of the reporter gene. Under the standard assay conditions, activity in the hap1::LEU2 disruptant strain grown in the presence of unsaturated fatty acids was reduced to approximately 20% of that found in cells grown in fatty acid-deficient medium (data not shown). Given the effects of increased OLE1 transcription on fatty acyl composition seen in the acbp:::LEU2 disruptant strain, it was thought that complementary changes might be produced by the reduced level of OLE1 transcription in the hap1::LEU2 disrupted strain. Analysis of the disruptant and its parent strain revealed no significant differences in either the fatty acyl composition or the total cellular fatty acid content of the two strains (data not shown).

**DISCUSSION**

Saccharomyces, like other eukaryotes, maintains a balanced ratio of unsaturated and saturated fatty acids in its membrane lipids under a wide range of physiological conditions. This requires the coordinated regulation of fatty acid synthesis, which produces saturated fatty acids, and fatty acid desaturation, which converts most of the saturated acids to unsaturated species. The maintenance of fatty acyl composition appears to be important in controlling the properties of both cellular membranes and storage lipids. The Δ-9 fatty acid desaturase is a critical component of this system and is a highly regulated activity that responds to both nutrient and physiological controls (7). A major question concerns how cells monitor the availability of fatty acid precursors and the acyl composition of glycerolipids to regulate the activities of the desaturase and

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3 Mitchell and Martin, submitted for publication.
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**TABLE VIII**

Comparison of reporter gene activities in isogenic HAP1, hap1::LEU2, and hap2::LEU2 strains

| Strain/plasmid/construct | β-Galactosidase activity | HAP1 activity |
|--------------------------|--------------------------|---------------|
| HAP1/p62                 | 16.7 ± 2.5               | 100           |
| hap1/p62                 | 6.6 ± 2.9                | 39.8          |
| hap2/p62                 | 16.3 ± 3.2               | 97.7          |
| HAP1/pCT111              | 4.6 ± 0.15               | 100           |
| hap1/pCT111              | 1.4 ± 0.25               | 30.4          |

Other lipogenic enzymes. Recent evidence from this laboratory suggests that the regulation of unsaturated fatty acid formation involves a diverse array of controls.

This analysis of the OLE1 promoter indicates that the formation of unsaturated fatty acids is strongly regulated at the level of transcription by nutrient fatty acids. This appears to be a major component of the previously observed elevation of OLE1 enzyme activity by saturated fatty acids and its repression in response to unsaturated acids (1). Essential transcription activation and unsaturated FAR elements appear to be located in short 111-bp region located approximately 500 bp upstream of the OLE1 transcribed region. That fragment is sufficient to activate and confer unsaturated fatty acid repression on an unrelated gene that contains only the basal promoter elements and no upstream transcription activating sequences. Saturated fatty acids, however, do not activate vectors containing only the FAR region elements, suggesting that sequences that respond to those stimuli lie in another region of the promoter. Taken together these observations suggest that there are at least two independent systems that regulate OLE1 transcription in response to fatty acids.

The identification of transcription factors that act through the FAR region is critical to our understanding of the mechanisms of fatty acid-mediated repression. The data presented here indicate that a specific 9-bp sequence at the distal end of the 100-bp FAR fragment is essential for fatty acid-mediated repression and plays some role in activation (Figs. 3 and 4). A region at the opposite end of that fragment is essential for activation. If the latter sequences are targets for transcription-activating DNA binding proteins, then they must also play a role in FAR region-mediated unsaturated fatty acid repression.

The data presented here are consistent with either of two models for control of OLE1 transcription (21). One is that repression is mediated by an unsaturated fatty acid-responsive DNA binding protein. When activated, it competes with transcription factors for binding to FAR element sequences. A second model is that repression is triggered by an unsaturated fatty acid-activated auxiliary transcription factor that interacts with DNA binding proteins occupying the FAR region.

Associated with the essential regulatory sequence at the 5' boundary of the FAR region is a series of symmetric GC-rich elements. These include a pair of CCCGGG sequences followed by an inverse GGGGCC sequence within a 30-bp region. A homologous pair of GC-rich sequences was found in the transcription-activating region of the promoter of the sterol biosynthetic gene ERG11 (22). That gene encodes a microsomal cytochrome P-450 enzyme responsible for the demethylation of lanosterol. The significance of the GC-rich sequences is further reinforced by the occurrence of homologous paired sequences in the upstream noncoding region of the ERG3 gene, which encodes the sterol C-5 desaturase (23, 24). All three genes encode intrinsic enzymes that act at the endoplasmic reticulum surface. The existence of homologues to the OLE1 sequences in another lipid biosynthetic pathway suggests they may be involved with a type of transcriptional control for lipidogenesis that has yet to be identified.

The effect of the combined faa1, faa4 disruption in blocking repression of OLE1 transcription is intriguing and suggests that repression is related to the availability of acyl-CoA species formed from the exogenous fatty acids. The two genes that affect the repression of OLE1, FAA1 and FAA4, account for approximately 99% of the cellular 14:0 CoA and 16:0 CoA synthetase activities in wild-type cells grown in glucose medium (29). Furthermore, both genes are responsible for almost all of the activation of imported 14:0 and 16:0. It is not clear, however, whether they are also the primary activators for unsaturated species such as 16:1, 18:1, or 18:2. Disruption of FAA1 and FAA4 results in a striking reduction in the incorporation of these fatty acids into glycerolipids and a sharp increase in cellular fatty acids (27). One possibility is that the OLE1 regulatory circuit responds to the size or the composition of the intracellular acyl-CoA pool generated by the two synthetases. Alternatively, Faa1p and Faa4p may be involved in the intracellular transport of exogenous fatty acids or in their partitioning to cellular locations that are accessible to the regulatory sensor.

The acyl-CoA binding protein appears to play a significant role in OLE1 expression. Disruption of this abundant and highly specific binding protein increases OLE1 transcription greater than 5-fold, which accurately correlates with a 5-fold increase in OLE1 mRNA levels. Although disruption of the ACBP gene does not produce a significant phenotype with respect to growth, it appears to cause an increase in the levels of unsaturated fatty acids relative to saturated species in total cellular lipids. One interpretation of this response might be that the regulatory sensor that detects available substrate for desaturase responds to saturated ACBP-bound acyl-CoAs. The absence of ACBP-bound substrate may elicit a cellular response ordinarily used to monitor levels of available saturated substrates for the enzyme. In the absence of this signal, OLE1 expression is increased to compensate for the perceived reduction in substrate. If the actual acyl-CoA levels are not rate-limiting, this increase in OLE1 expression could have the concomitant result of increasing levels of cellular unsaturated fatty acids.

Hap1p (28) appears to be one of several transcription activators that recognize FAR region elements. Analysis of reporter gene activity in Hap1p-deficient cells indicates that it is responsible for more than half of the OLE1 transcription activity in wild-type cells grown under fermentative conditions. Disrup-
tion of HAP1, however, does not appear to affect either the balance of cellular saturated and unsaturated species or the relative levels of fatty acids found in those cells. This indicates that, in the absence of HAP1p, other transcription factors activate the OLE1 FAR element to produce sufficient mRNA to maintain normal membrane fatty acyl lipid composition. We are currently attempting to identify these unknown activation and repressor elements by the isolation of regulation defective mutants.

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REFERENCES

1. Bossie, M. A., and Martin, C. E. (1989) J. Bacteriol. 171, 6409–6413
2. Kamiryo, T., Parthasarathy, S., and Numa, S. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 386–390
3. Numa, S., and Tanabe, T. (1984) in Fatty Acid Metabolism and Its Regulation (Numa, S., ed) pp. 1–27, Elsevier, Amsterdam
4. Hablacher, M., Ivessa, A. S., Paltauf, F., and Kohlwein, S. D. (1993) J. Biol. Chem. 268, 1096–1098
5. Struhl, K. (1998) Bio/Technology 9, 35–78
6. Turi, T. G., and Loper, J. C. (1992) J. Biol. Chem. 267, 2046–2056
7. Smith, S. J., and Parks, L. (1993) Yeast 9, 1177–1187
8. Arkington, B. A., Bennett, L. G., Skaftrud, P. L., Guynn, C. J., Barbuch, R. J., Ulbright, C. E., and Bard, M. (1991) Gene (Amst.) 102, 39–44
9. Schneider, J. C., and Guarente, L. (1991) Mol. Cell. Biol. 11, 4934–4942
10. Rosenthal, J., Ertbjerg, P., and Knudsen, J. (1993) Biochem. J. 290, 321–326
11. Johnson, D. R., Knoll, L. J., Levin, D. E., and Gordon, J. I. (1994) J. Cell Biol. 127, 751–762
12. Guarente, L. (1992) in The Molecular and Cellular Biology of the Yeast, Saccharomyces, Vol. 2, Gene Expression (Jones, E. W., Pringle, J. R., and Broach, J. R., eds) pp. 49–98, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
13. Miller, J. H. (1972) Experiments in Molecular Genetics, pp. 352–355, Cold Spring Harbor, Cold Spring Harbor, NY
