

Mga2p Processing by Hypoxia and Unsaturated Fatty Acids in *Saccharomyces cerevisiae*: Impact on LORE-Dependent Gene Expression

Yide Jiang,¹ Michael J. Vasconcelles,¹ Sharon Wretzel,¹ Anne Light,¹ Laura Gilooly,¹ Kevin McDaid,¹ Chan-Seok Oh,² Charles E. Martin,² and Mark A. Goldberg^{1*}

Hematology Division, Department of Medicine, Brigham & Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115,¹ and The Nelson Biological Laboratory, Bureau of Biological Research, Department of Cell Biology and Neuroscience, Busch Campus, Rutgers University, Piscataway, New Jersey 08854²

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In *Saccharomyces cerevisiae*, *OLE1* encodes a $\Delta 9$ fatty acid desaturase, an enzyme that plays a critical role in maintaining the correct ratio of saturated to monounsaturated fatty acids in the cell membrane. Previous studies have demonstrated that (i) *OLE1* expression is repressed by unsaturated fatty acids (UFAs) and induced by low oxygen tension, (ii) a component of this regulation is mediated through the same low oxygen response element (LORE) in the *OLE1* promoter, and (iii) Mga2p is involved in LORE-dependent hypoxic induction of *OLE1*. We now report that LORE-*CYC1* basal promoter-*lacZ* fusion reporter assays demonstrate that UFAs repress the reporter expression under hypoxic conditions in a dose-dependent manner via LORE. Electrophoretic mobility shift assays show that UFAs repress the hypoxia-induced complex formation with LORE. Studies with a construct encoding a truncated form of Mga2p support the hypothesis that both hypoxia and UFA signals affect the processing of Mga2p and the UFA repression of *OLE1* hypoxic induction is mediated through Mga2p. Data from Western blot assays provide evidence that under normoxic conditions, Mga2p processing produces approximately equimolar levels of the membrane-bound and processed forms and is unaffected by UFAs. Hypoxic induction of *OLE1*, however, is associated with increased processing of the protein, resulting in an approximately fivefold increase in the soluble active form that is counteracted by exposure of the cells to unsaturated fatty acids. Data from this study suggest that the Mga2p-LORE interaction plays an important role in *OLE1* expression under both normoxic and hypoxic conditions.

Saccharomyces cerevisiae is a facultative aerobe that can utilize oxygen for energy production and therefore has developed a number of mechanisms by which to sense and respond to changes in oxygen availability. The regulatory pathways that are involved with the response to hypoxia (low oxygen) have been studied for decades, and there has been significant progress in this field (3, 23, 43).

In mammalian cells, the best-studied hypoxia signal transduction pathway is mediated by hypoxia-inducible factor 1 (HIF-1) (3, 32). HIF-1 is a heterodimer comprised of HIF-1 α and ARNT, two basic helix-loop-helix proteins in the PAS family (39). ARNT mRNA and protein levels are not significantly affected by ambient oxygen tension. HIF-1 α mRNA levels are also not appreciably affected by oxygen tension, but HIF-1 α protein is only minimally present in normoxia and is rapidly degraded by the ubiquitin-proteasome pathway under this condition (15, 16, 21, 30). This process is dependent on the von Hippel-Lindau tumor suppressor protein (pVHL) (24), which serves as the recognition component of a ubiquitin ligase that promotes ubiquitin-dependent proteolysis of HIF-1 α and directly interacts with a highly conserved region within the HIF-1 α oxygen-dependent degradation domain (7, 22, 28, 35).

Recent studies have indicated that a proline hydroxylation within that conserved region is responsible for targeting HIF-1 α for pVHL-mediated destruction (17, 18). In hypoxic cells, HIF-1 α degradation is suppressed, leading to heterodimer formation with ARNT. It can then interact with other DNA-binding proteins, such as HNF4 and p300, which provide tissue and developmental specificity and enable enhanced transcription of target genes in response to hypoxia (3, 10).

The *S. cerevisiae* *OLE1* gene encodes the $\Delta 9$ fatty acid desaturase, an enzyme that forms monounsaturated palmitoleic (16:1) and oleic (18:1) fatty acids from palmitoyl (16:0) or stearoyl (18:0) coenzyme A. Unsaturated fatty acids (UFAs) are essential for maintaining optimal cell membrane fluidity and expansion in growing cells and serve as major components of storage lipids in stationary-phase cells. *OLE1* is highly regulated in response to various physiologically important signals. It has been shown that *OLE1* transcription is regulated by fatty acid pools. Its transcription is only weakly induced (1.6-fold) by exogenous saturated fatty acids, but UFAs strongly repress *OLE1* transcription, by as much as 60-fold (6, 25). Systematic *OLE1* promoter deletion-*lacZ* fusion analysis indicates that fatty acid response (FAR) elements located 466 to 576 nucleotides upstream from the translation start ATG within the *OLE1* promoter contribute to *OLE1* repression by UFAs (6).

Studies also indicate that *OLE1* expression can be induced under hypoxic conditions, by cobalt and iron chelation, similar to several mammalian hypoxia-inducible genes (23, 38). We

* Corresponding author. Mailing address: Hematology Division, Department of Medicine, Brigham & Women's Hospital, Harvard Medical School, 221 Longwood Ave., Boston, MA 02115. Phone: (617) 732-5841. Fax: (617) 739-0748. E-mail: Mark.Goldberg@genzyme.com.

TABLE 1. *S. cerevisiae* strains and plasmids used in this study

Strain or plasmid	Genotype or plasmid description	Source or reference
RZ53-6	<i>MATa trp1-289 leu2-3, 112 ura3-52 ade1-100</i>	R. Zitomer
BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	RG ^a
BY4741Δ <i>mga2</i>	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ΔMGA2</i>	RG
p62::934	<i>OLE1</i> promoter- <i>lacZ</i> fusion	5
p62::396	<i>OLE1</i> promoter- <i>lacZ</i> fusion	5
pAM4	LORE in <i>OLE1</i> promoter contains mutations in p62::934	38
pTBA30	Basal <i>CYC1</i> promoter- <i>lacZ</i> fusion	A. K. Vershon
pAM6	Tandem repeat LORE-basal <i>CYC1</i> promoter- <i>lacZ</i> fusion	38
pAM23	Centromeric plasmid; contains 5.1-kb fragment that contains <i>MGA2</i>	19
pAM30	Derivative of pAM23 with deletion of 244 aa ^b in <i>MGA2</i> C terminus	This study
pAM31	Derivative of pAM23 with deletion of 172 aa in <i>MGA2</i> C terminus	This study
pRS315	Parental centromeric plasmid of pAM23	ATCC ^c
YEplac181- <i>myc</i> MGA2	2μm plasmid; contains triple-myc-tagged <i>MGA2</i>	14

^a RG, Research Genetics, Inc. (40).

^b aa, amino acids.

^c ATCC, American Type Culture Collection.

recently identified and characterized a *cis* transactivation element, LORE (low oxygen response element), in the promoter region of *OLE1* (38). A similar, but longer element has also been described by Nakagawa et al. (26). In addition, data from both our laboratory and Nakagawa et al. suggest that the same LORE is involved in the UFA repression of *OLE1*. Genetic analyses have demonstrated that *MGA2* or *SPT23* is required for *OLE1* transcription (42).

Hoppe et al. (14) have identified a novel processing pathway in *S. cerevisiae* termed regulated ubiquitin-proteasome-dependent processing. The crucial factors in this pathway are Spt23p and Mga2p, which are initially made as dormant precursors (p120) that are firmly anchored in the endoplasmic reticulum-nuclear envelope membranes by their C-terminal tails. It was demonstrated that this pathway controls the level of UFAs in *S. cerevisiae* by regulating *OLE1* expression (14). A shortage of UFAs leads to the Rsp5p-mediated ubiquitination of Spt23p and Mga2p, the release of their N-terminal transcription factor domains (processed forms, p90) into the cytosol, and enhanced expression of the Ole1p, Δ9 fatty acid desaturase. Addition of UFAs that contained more than one double bond almost completely blocked Spt23p precursor processing.

Recently, we have demonstrated that *MGA2* is involved in LORE-dependent hypoxic induction of genes in *S. cerevisiae* (19). We hypothesized that UFAs also repress LORE-dependent *OLE1* hypoxic induction through Mga2p. We further hypothesized that Mga2p processing is regulated by both hypoxia and UFA signals but that the two signals work in opposing manners.

Here we provide both direct and indirect evidence that strongly supports the hypothesis that Mga2p processing is regulated by both hypoxia and UFAs and demonstrate that LORE is involved in the UFA repression of *OLE1* expression through *MGA2* in *S. cerevisiae*. We demonstrate that the Mga2p-LORE interaction plays an important role in not only the hypoxic induction but also the UFA repression of *OLE1* expression.

MATERIALS AND METHODS

Media, chemicals, and enzymes. Yeast strains were grown in YPD medium (Bio 101, Inc., Carlsbad, Calif.) or SC dropout medium, depending on the plasmid-selectable markers. Luria-Bertani medium was used to grow bacteria.

Ampicillin (U.S. Biochemical Corp., Cleveland, Ohio) was used as necessary at 50 μg/ml unless indicated otherwise. Radiolabeled compounds were purchased from Perkin-Elmer (Boston, Mass.). ULTRAhyb was bought from Ambion (Austin, Tex.). Salmon sperm DNA was from GIBCO BRL (Rockville, Md.). CoCl₂, NiCl₂, 1,10-phenanthroline, Tween 80 (oleic acid, approximately 70%; balance primarily linoleic, palmitic, and stearic acids), linoleic acid (L.A.), arachidonic acid, oleic acid, γ-linolenic acid (γL.A.), stearic acid, tergitol, ergosterol, and NP-40 were obtained from Sigma Chemical Co. (St. Louis, Mo.). T4 polynucleotide kinase and deoxynucleoside triphosphates were purchased from Promega Corporation (Madison, Wis.). Shrimp alkaline phosphatase and *Taq* polymerase were purchased from Roche Molecular Biochemicals (Indianapolis, Ind.); other restriction enzymes were from New England BioLabs (Beverly, Mass.). All enzymes were used in accordance with the manufacturers' instructions.

Plasmid and plasmid constructions. The plasmids used in this study are listed in Table 1. The construction of several of the *OLE1* promoter-*lacZ* fusion deletion series was described previously (6, 38). Plasmid p62::934 is the reporter with the *OLE1* promoter sequence (from -934 relative to the ATG translational start codon with the A of the codon designated +1)-*lacZ* fusion. Plasmid p62::396 is the reporter with the *lacZ* fusion with the *OLE1* promoter sequence from -396. pAM4 is p62::934 with mutations in the LORE of the *OLE1* promoter (38). Reporter plasmid pAM6 contains a tandem repeat of *OLE1* LORE sequences (-347 to -328 relative to the ATG translational start codon with the A of the codon designated +1) in front of *CYC1* basal promoter-*lacZ* fusion vector pTBA30. A centromeric plasmid, pAM23, containing *MGA2* was constructed previously (19). Two truncated forms of Mga2p were constructed by introducing a stop codon into pAM23 at the positions encoding amino acids 941 and 869, respectively, of Mga2p, thereby generating proteins Mga2p-Δ172 (pAM31) and Mga2p-Δ244 (pAM30), which had deletions 172 and 244 amino acids, respectively, from the C terminus of Mga2p. A 2μm plasmid with a *LEU2* selection marker, YEplac181-*myc*MGA2, contains a triple repeat of a myc epitope in front of *MGA2*. This was a gift from S. Jentsch, Department of Molecular Cell Biology, Max Planck Institute for Biochemistry, Martinsried, Germany (14).

Strains and growth conditions. The strains used in this study are listed in Table 1. Yeast cells containing *lacZ* fusion plasmids were grown at 30°C on uracil dropout medium containing dextrose (37). For UFA repression analysis, yeast cells were grown in medium supplemented with 1% tergitol as described previously (6). Cells were grown in the presence of UFAs for 6 h prior to the β-galactosidase assays. Plasmid amplifications and bacterial transformations were performed with *Escherichia coli* strain DH5α (Invitrogen Corp., Carlsbad, Calif.). Yeast transformations were performed by the method of Elble (11). Preparative cultures were grown aerobically in a shaker (Innova 4000 incubator shaker; New Brunswick Scientific, Edison, N.J.) at 200 rpm and 30°C to mid-logarithmic phase. For experiments assessing yeast under hypoxic conditions, the procedure was as described previously (38). Growth was monitored by measuring the yeast optical density at 600 nm (OD₆₀₀) at the completion of each experiment.

β -Galactosidase assays. Assays of cells containing plasmids derived from the *OLE1* promoter-*lacZ* fusion p62 constructs were performed as described previously (31). Cell densities for these assays were determined by measurement of OD₆₀₀. The β -galactosidase activities reported here are the results of at least two independent experiments. Each experimental assay was performed in quadruplicate. When appropriate, data from replicate experiments are presented as the mean \pm the standard error.

Yeast extract preparation. Haploid yeast cells were cultured in 1-liter flasks containing 200 ml of YPD (1% yeast extract, 2% peptone, 2% dextrose) under either normoxic or hypoxic conditions, harvested at mid-log phase (OD₆₀₀ of 0.8), and lysed by vortexing with glass beads as previously described (29). Following addition of ammonium sulfate to 40% and incubation on a rocker table at 4°C for 30 min, the precipitate was collected by centrifugation at 12,000 \times g in a microcentrifuge at 4°C for 10 min. The pellet was resuspended in storage buffer (20 mM HEPES [pH 8.0], 5 mM EDTA; 20% [vol/vol] glycerol, 1 mM phenylmethylsulfonyl fluoride, 7 mM β -mercaptoethanol) and stored frozen at -80°C. The soluble protein concentration was determined with a Bradford dye-binding assay (Bio-Rad).

EMSAs. Electrophoretic mobility shift assays (EMSAs) were performed essentially as described previously (38), with paired synthetic oligonucleotides as a LORE probe (-347 to -328 relative to the ATG translational start codon with the A of the codon designated +1). Synthetic paired oligonucleotides were end labeled with polynucleotide kinase and purified with a Sephadex G-25 spin column (Roche Molecular Biochemicals) to remove unincorporated nucleotide. Probes made by PCR were purified away from labeled primers [γ -³²P]ATP and *Taq* polymerase with a QIAquick spin PCR purification kit (Qiagen, Santa Clarita, Calif.). In each reaction mixture, 10 to 20 ng of probe was used. Binding reaction mixtures were in 40 μ l of buffer H [25 mM HEPES (pH 7.5) at room temperature, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM MgCl₂, 1 mM CaCl₂, 50 mM NaCl, 7% glycerol, 1% NP-40, 15 ng of poly(dA-dT) per μ l] for 20 min at room temperature. Proteins were diluted in binding buffer on ice immediately before use. Reaction mixtures were loaded onto 5% acrylamide gels (acrylamide/bisacrylamide ratio, 29:1), electrophoresed in 0.5 \times Tris-borate-EDTA, and run for 3 h at 4°C and 15 V/cm. Gels were dried and exposed to X-OMAT AR film (Kodak) to visualize the shifted bands.

RNA isolation and Northern blot analysis. Total yeast RNA was isolated as described previously (8). Equal amounts (15 μ g) of total RNA were analyzed by Northern blots in accordance with standard procedures for separation of RNA with 1% formaldehyde gels. RNA from the gels was transferred to Nytran Plus membranes (Schleicher & Schuell Inc., Keene, N.H.) in 10 \times SSC (1 \times SSC is 0.15 M sodium citrate plus 0.015 M NaCl) overnight. Prehybridization, hybridization, and washing of membranes were performed as previously described (2). Northern blots were quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.), and autoradiographs were also prepared on X-OMAT AR film (Kodak).

To make radiolabeled cDNA probes for *ACT1* and *ATF1*, yeast genomic DNA prepared by the rapid isolation of yeast chromosomal DNA protocol (13) was subjected to PCR with appropriate pairs of primers for the particular genes of interest. The PCR products were first purified with a QIAquick spin PCR purification kit (Qiagen), separated by agarose gel electrophoresis in 1 \times Tris-acetate-EDTA (TAE), and then purified with a Qiagen gel extraction kit (Qiagen) in accordance with the manufacturer's recommendations.

To detect *OLE1* mRNA, a radiolabeled DNA probe was made with a 0.5-kb *EcoRI* fragment from the *OLE1* protein coding sequence. DNA fragments were separated by agarose gel electrophoresis in 1 \times TAE and purified with a Qiagen gel extraction kit (Qiagen) in accordance with the manufacturer's recommendations. The purified DNA fragments were labeled to high specific activity with [³²P]dCTP (Perkin-Elmer) by the random primer extension method with Ready to Go DNA labeling beads (Amersham Pharmaceutical Biotech, Piscataway, N.J.) reaction kit. Unincorporated nucleotides were removed from the sample with a Sephadex G-50 spin column (Roche Molecular Biochemicals). The specific activity of labeled probes was determined by liquid scintillation counting.

Biotinylated probe pull-down and Western blot assays. Both oligonucleotides containing the LORE sequence (5'-CGGACGTTGAACACTCAACAACCTTATCTAG-3') and one containing a mutated LORE sequence (5'-CGGACGTTGAACAtaCgACAACCTTATCTAG-3'; the lowercase letters signify mutations), biotinylated at the 5' end, were synthesized and purified by Integrated DNA Technologies, Inc., Coralville, Iowa, and equimolar amounts of complementary strands were annealed, respectively. Dynabeads M-280 Streptavidin (DynaL, Inc., Lake Success, N.Y.) were prepared in accordance with the manufacturer's instruction. Probe immobilization was performed by mixing 0.5 ml of Dynabeads M-280 Streptavidin with 1 nmol of the double-stranded oligonucleotides containing LORE or the one containing mutated LORE in accordance

with the manufacturer's instructions. Finally, the beads were resuspended in 0.5 ml of binding buffer (see EMSA). For the pull-down experiments, 2 mg of whole-yeast crude extract (CE) from strain BY4741 Δ *mga2* transformed with YEplac181-*mycMGA2* in 0.5 ml of binding buffer was incubated with 0.5 ml of beads at room temperature for 30 min with regular mixing to keep the beads in suspension. The beads were then concentrated with a magnetic rack and washed three times with 0.5 ml of binding buffer. The second wash used the binding buffer containing 5 μ g of poly(dA-dT). Finally, the beads were eluted with 100 μ l of binding buffer containing 1 M NaCl. A 10- μ l volume of these final eluates was diluted with 30 μ l of binding buffer without NaCl and subjected to EMSA and Western blot analyses.

For Western blot analyses, a total of 30 μ g of CE in a 20- μ l volume of 1 \times sodium dodecyl sulfate loading buffer was boiled for 5 min and resolved by sodium dodecyl sulfate-7.5% polyacrylamide gel electrophoresis. Ready-Gels (Bio-Rad, Hercules, Calif.) were transblotted overnight to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech) with a Mini Trans-Blot electrophoresis transfer cell (Bio-Rad). A Western blotting kit (SuperSignal West Dura; Pierce, Rockford, Ill.) was used. The membrane was probed with a 1:1,000 monoclonal anti-c-myc antibody (clone 9E10; Sigma). Antigen-antibody complexes were visualized by enhanced chemiluminescence (SuperSignal; Pierce) in accordance with the manufacturer's instructions and detected by Hyperfilm ECL (Amersham Pharmacia Biotech). For quantitation of gels, films were scanned with a Personal Densitometer (Molecular Dynamics) and analyzed with ImageQuant software (Molecular Dynamics).

RESULTS

Tween 80 represses hypoxia-induced *OLE1* expression. UFA (Tween 80) and ergosterol are required exogenous substrates in yeast cultured under anaerobic conditions (9). Previous studies of the induction of *OLE1* expression under low oxygen tension (i.e., hypoxic but not anaerobic conditions) were done with yeast strains grown in medium supplemented with Tween 80 and ergosterol (23). However, when we tested hypoxia-induced reporter expression with an *OLE1* promoter-*lacZ* fusion in yeast in medium containing Tween 80, we found that the robust hypoxic induction of the reporter gene was dramatically repressed (Fig. 1A) whereas the basal reporter expression was not affected. Similar experiments with medium supplemented only with ergosterol showed no effect on *OLE1* expression under normoxic and hypoxic conditions (data not shown). This initial observation led us to pursue the identification of the *cis* elements that may be responsible for the repression. Previous experiments demonstrated FAR elements (-466 to -576) within the *OLE1* promoter that contributed to *OLE1* repression by UFA (6). To test whether these FAR elements are involved in Tween 80 repression, we performed an *OLE1* promoter-*lacZ* reporter assay with the *OLE1* promoter in which the FAR elements were deleted (p62::396). Surprisingly, the hypoxic induction of this reporter construct was still strongly repressed by Tween 80, as shown in Fig. 1A. Because the major components of Tween 80 are various UFAs and it was previously recognized that UFAs exert substantial repression of *OLE1* expression, we postulated that the repressive effects of Tween 80 might be mediated by UFAs specifically. As shown in Fig. 1B, when the UFA L.A. was added to the medium, it strongly repressed the expression of the p62::396 reporter as well. These results suggested that there might be another *cis* element 3' to -396 of the *OLE1* promoter that is critical for *OLE1* UFA regulation under hypoxic conditions.

Dose-dependent repression of *OLE1* induction under hypoxic conditions by UFAs. Northern blot analyses were performed to examine the effects of different concentrations of

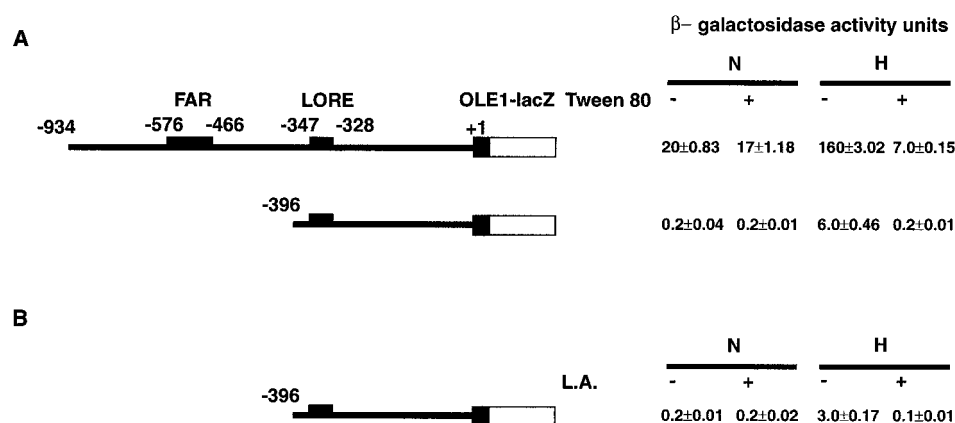


FIG. 1. Repression effects of UFAs on the β -galactosidase activity of the *OLE1* promoter-*lacZ* fusion. Constructs p62::934 and p62::396 are shown on the left. The narrow line represents the *OLE1* promoter sequence. The number above each narrow line indicates the position of the deletion end point with respect to the ATG start codon of the wild-type base sequence with the A of the codon designated +1. The solid black bar represents the amino-terminal 27 amino acids of the *OLE1* coding sequence fused to *E. coli lacZ* (blank bar). The small solid bar above the promoter indicates the FAR and LORE elements, which are also labeled. The values to the right are β -galactosidase activities under normoxic (N) and hypoxic (H) conditions, which were determined as described in Materials and Methods. The values (units of activity) shown are averages of at least three independent experiments that were performed in quadruplicate. (A) Tween 80 repression of β -galactosidase activity of reporters p62::934 and p62::396. (B) L.A. (100 μ M) repression of β -galactosidase activity of reporter p62::394.

UFAs on *OLE1* expression at the mRNA level. Figure 2 shows that the induction of *OLE1* expression under hypoxic conditions was repressed by L.A. in a dose-dependent manner. The hypoxia-induced *OLE1* mRNA levels were increasingly repressed by the addition of increasing amounts of L.A. to the medium. At 25 μ M L.A., the *OLE1* mRNA level under hypoxic conditions was about half of that in the control in which no L.A. was added to the medium. At 100 μ M L.A., the basal *OLE1* mRNA expression under normoxic conditions was repressed to a similar degree as under hypoxic conditions (data not shown). At 1 mM L.A., *OLE1* mRNA expression was not detectable by Northern blot analyses, consistent with our previous observation (38). Another UFA, γ L.A., was also tested and produced similar results (data not shown).

Dose-dependent repression of *OLE1* hypoxic induction by UFAs via LORE. LORE is a previously identified *cis* transactivation element that plays a critical role in hypoxic induction of *OLE1* (38). It is located between -347 and -328 in the *OLE1* promoter region. The LORE-*CYC1* promoter-*lacZ* fusion reporter pAM6 was utilized to demonstrate that LORE is involved in L.A. repression in our previous studies (38). Here, we further demonstrate the dose-dependent repression of LORE-mediated hypoxia-induced reporter expression (pAM6) by various UFAs (Fig. 3). Yeast cells were grown under hypoxic conditions in medium supplemented with various concentrations of four different UFAs. All of the UFAs examined demonstrated a clear dose-dependent repression of hypoxia-induced reporter gene expression. However, arachidonic acid and γ L.A., which possess four *cis* double bonds (C-5, C-8, C-11, and C-14) and three *cis* double bonds (C-6, C-9, and C-12), respectively, exert greater repression of reporter expression (as indicated by the lower β -galactosidase activities), with a 50% inhibitory concentration of \sim 5 μ M, compared to a 50% inhibitory concentration of \sim 20 μ M for oleic acid and L.A., which contain one (C-9) and two (C-9 and C-12) double bonds in *cis*, respectively. Notably, there was almost complete repression of reporter expression with all four UFAs tested at 100

μ M. However, stearic acid, a saturated fatty acid, did not have significant effects at this concentration. These results suggest that UFAs specifically repress *OLE1* hypoxic induction via LORE and that, at low concentrations, the number of double bonds in the UFAs affects the intensity of UFA repression, regardless of their position and stereochemistry.

The effects of UFAs on hypoxia-induced complex formation involving LORE were investigated by EMSA. CEs from yeast grown under normoxic and hypoxic conditions with or without increasing amounts of L.A. were utilized for EMSAs with the end-labeled double-stranded LORE oligonucleotides as a probe. As shown in Fig. 4, specific hypoxia-induced complex B2 was increasingly repressed by CEs from yeast cells grown in medium supplemented with increasing amounts of L.A. It appears that complex B2 from normoxic CEs was also repressed. Another UFA, γ L.A. (100 μ M), was also tested, and it produced similar results (data not shown).

EMSA were also performed with hypoxic CEs and addition of increasing amounts of L.A. to the binding reaction mixture *in vitro*. At increasing concentrations of up to 1 mM, L.A. did not significantly affect the formation of the specific hypoxia-induced complex (B2) (data not shown).

Other *cis* elements besides LORE are involved in *OLE1* expression under normoxic conditions. *OLE1* promoter-*lacZ* reporter assays were performed to test whether other *cis* elements besides LORE are involved in *OLE1* expression under normoxic conditions. As shown in Fig. 5, γ L.A. exhibited repressive effects on reporter gene expression (p62::934) under normoxic conditions. When the reporter pAM4 was used, in which mutations were introduced into the LORE region of the full-length (>900-bp) *OLE1* promoter, decreased basal reporter gene expression was observed, consistent with our previous report (38) suggesting that LORE contributes to *OLE1* expression under normoxic conditions. However, when γ L.A. was added, further repression of reporter gene expression was observed, implying that there are additional *cis* elements in the

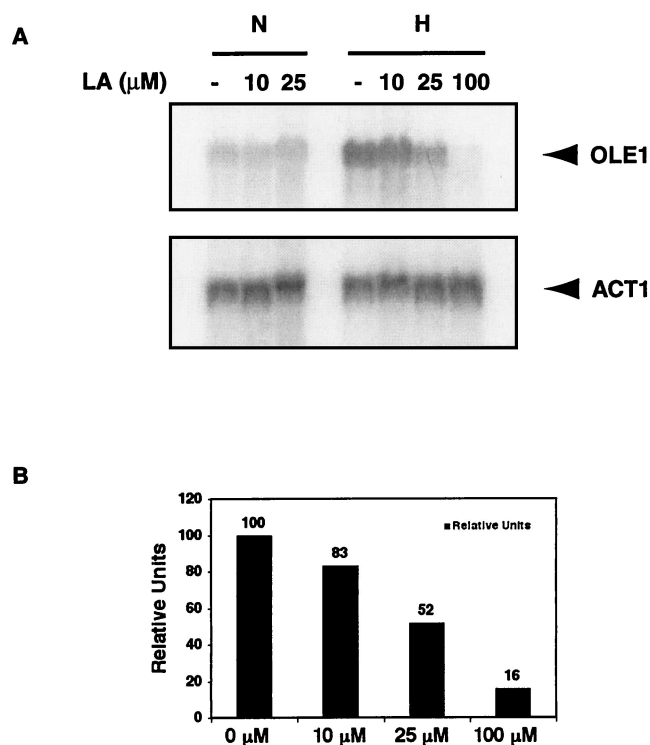


FIG. 2. (A) Northern blot analysis under L.A.-treated conditions. Total RNA was extracted from yeast cells grown in increasing concentrations (10, 25, and 100 μM) of L.A. for 6 h. The RNA blot was first hybridized with a specific *OLE1* probe, stripped, and rehybridized with a control *ACT1* probe. The experiment was done under normoxic (N) and hypoxic (H) conditions. The positions of the *OLE1* and *ACT1* mRNAs are shown. (B) Quantitation of *OLE1* mRNA in the Northern blot under hypoxic conditions in panel A. Signal intensity was quantitated with a Molecular Dynamics PhosphorImager. Transcript levels were normalized to the level of *ACT1* mRNA and are presented as 100 relative units for *OLE1* mRNA extracted from yeast cells grown without L.A.

OLE1 promoter that play a role in UFA repression. An obvious candidate element is the previously described FAR element (6). These results are consistent with our previous conclusion that UFA repression of *OLE1* transcription depends on the FAR element under aerobic conditions (6).

Mga2p, a factor required in LORE-dependent hypoxic induction, is also involved in UFA repression. Previously, we have shown that *MGA2* is involved in the LORE-dependent hypoxic induction of genes in *S. cerevisiae* (19). *CEN*-based plasmid pAM23, containing the wild-type *MGA2* gene, was transformed into the BY4741 Δ *mga2* strain and was able to fully restore the hypoxic induction of *OLE1* in a reporter assay, as well as in Northern and EMSA analyses (19). Dose-dependent repression of *OLE1* hypoxic induction by UFAs was also tested in strain BY4741 Δ *mga2* and the parental strain. Medium containing 100 μM L.A. almost completely repressed *OLE1* expression under normoxic and hypoxic conditions in reporter and Northern assays with both strains BY4741 and BY4741 Δ *mga2* transformed with pAM23 (data not shown).

We have previously shown that a soluble, truncated form of Mga2p is regulated by UFA under normoxic conditions when it is independently expressed in an *spt23* Δ *mga2* Δ strain (5). To

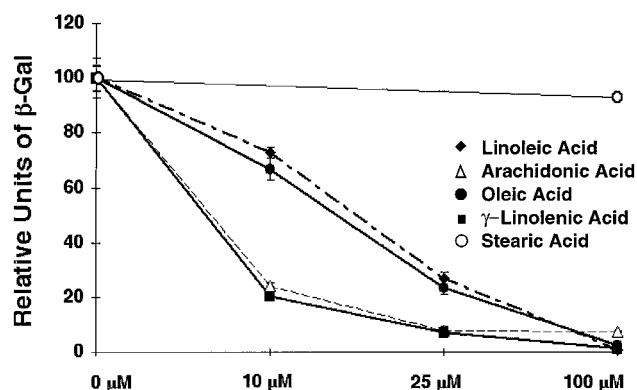


FIG. 3. Dose-dependent repression by UFAs of hypoxia-induced reporter gene expression via LORE. β -Galactosidase assays were performed as described in Materials and Methods. Yeast strains carrying reporter pAM6 were grown in increasing concentrations of fatty acids as indicated in the diagram (0, 10, 25, and 100 μM) for 6 h under hypoxic conditions. The β -galactosidase activity of each strain grown without UFAs under hypoxia was designated 100 U. The values shown are averages of two independent experiments that were performed in quadruplicate.

test the hypothesis that Mga2p processing is regulated by hypoxia and UFAs, we constructed two truncated forms of Mga2p by introducing a stop codon at amino acid positions 941 and 869 of *MGA2* in pAM23 to generate Mga2- Δ 172 (pAM31) and Mga2- Δ 244 (pAM30), respectively. The resultant translated Mga2p proteins had deletions of 172 and 244 amino acids, respectively, from the C terminus of wild-type Mga2p. It was hypothesized that these truncated forms of Mga2p might function like endogenously processed Mga2p (p90). If UFA-mediated proteolytic processing of Mga2p is the cause of LORE-dependent UFA regulation, then hypoxia or UFAs should exert little or no additional effect on *OLE1* expression in yeast when either truncated form of Mga2p is expressed in BY4741 Δ *mga2*. Thus, *CEN*-based plasmids pAM23, pAM30, and pAM31 were transformed into the BY4741 Δ *mga2* strain carrying the tandem repeat LORE-*CYC1* promoter-*lacZ* fusion, pAM6, and in vivo reporter analyses were performed. As shown in Fig. 6, when the *MGA2*-containing plasmid (pAM23) was transformed into BY4741 Δ *mga2*, the hypoxic induction of β -galactosidase activity was dramatically repressed by the addition of either 100 μM or 1 mM L.A. (data not shown) to the medium. Consistent with previous observations, the basal reporter activity under normoxic conditions was also reduced. However, the reporter activities in the BY4741 Δ *mga2* strain containing either truncated form of Mga2p, Mga2- Δ 172 (pAM31) or Mga2- Δ 244 (pAM30), increased significantly under normoxic conditions and were similar to the levels found under hypoxic conditions. Additionally, the β -galactosidase activities under both conditions were slightly reduced by addition of 100 μM L.A. to the medium. However, dramatic (>70%) repression of reporter gene expression was observed in the two strains carrying the truncated Mga2p plasmids when 1 mM L.A. was used (data not shown). These data suggest that mechanisms other than Mga2p proteolytic processing are also involved in UFA-mediated *OLE1* regulation under normoxic and hypoxic conditions. Additionally, the data suggest that the

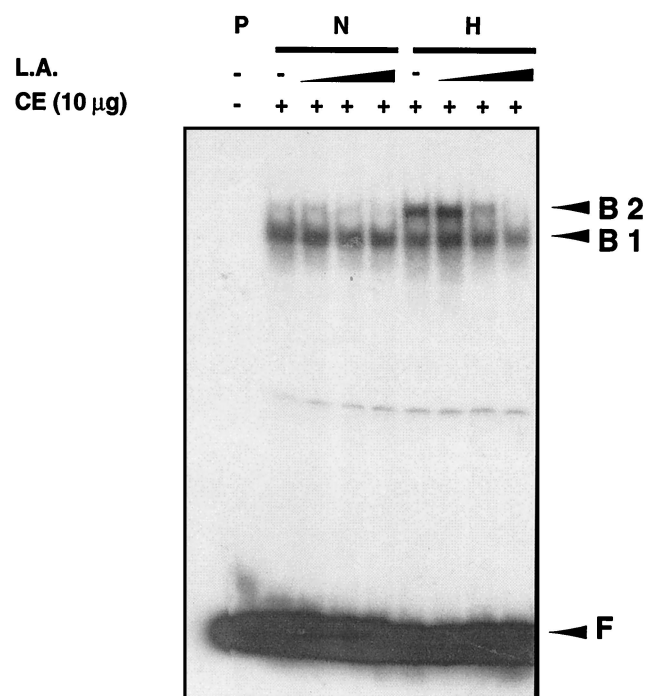


FIG. 4. L.A. repression of hypoxia-induced complex formation in LORE. EMSAs were performed as described in Materials and Methods. A 10- μ g sample of CE from *S. cerevisiae* grown under normoxic (N) or hypoxic (H) conditions was incubated for 20 min at 25°C with a 32 P-labeled LORE and then subjected to electrophoresis at 4°C for ~3 h. Free probe (F) and bound complexes (B1 and B2) were detected by autoradiography. A minus sign indicates that no CE was added or that the CE was from cells grown without L.A. A plus sign indicates that CE was added. The letter P represents the DNA probe. CEs from yeast cells grown in increasing concentrations of L.A. (10, 25, and 100 μ M) were used as indicated.

C-terminally truncated forms of Mga2p, Mga2p- Δ 172 and Mga2p- Δ 244, do not function as robustly under hypoxic conditions as naturally processed Mga2p.

Northern blot analysis was performed as shown in Fig. 7. The *OLE1* mRNA levels under normoxia were increased in BY4741 Δ mga2 transformed with pAM30 compared with those in the wild type, as expected on the basis of the reporter assays. Hypoxia did not further induce *OLE1* mRNA levels in BY4741 Δ mga2 transformed with pAM30. Addition of L.A. had no dramatic effects on the mRNA levels under either normoxic or hypoxic conditions. Experiments with BY4741 Δ mga2 transformed with pAM31 did not show significantly increased levels of *OLE1* mRNA under normoxic conditions. Further, no hypoxic induction or L.A. repression of *OLE1* mRNA levels were observed. This is consistent with data from the reporter assays (Fig. 6), in which lower overall reporter activity was observed.

Another LORE-dependent hypoxic gene, *ATF1*, was also studied. Previously, we demonstrated that *ATF1*, encoding an alcohol acetyltransferase, requires *MGA2* for hypoxic induction (19, 38). Furthermore, *ATF1* expression is repressed by UFAs (12). As shown in Fig. 7, similar to *OLE1* expression, the *ATF1* mRNA levels of BY4741 Δ mga2 transformed with either truncated Mga2p plasmid pAM30 or pAM31 were significantly increased under normoxic conditions. Hypoxia had no signifi-

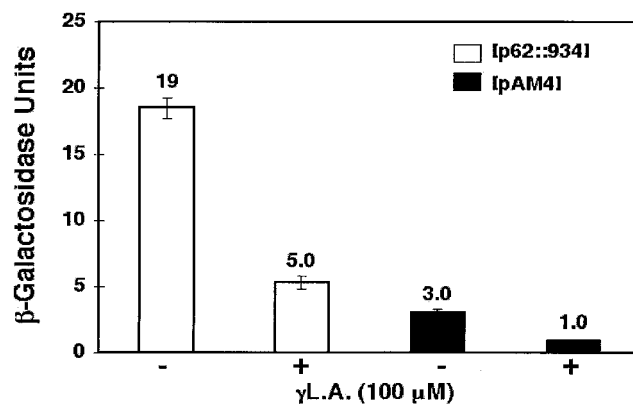


FIG. 5. Effect of γ L.A. on expression of reporter activity under normoxic conditions. β -Galactosidase assays were performed as described in Materials and Methods. Yeast strains carrying reporter p62::934 or pAM4, which possesses mutations in LORE, were grown in medium with or without 100 μ M γ L.A. for 6 h under normoxic conditions. The values shown are absolute units of β -galactosidase activity, which are averages of two independent experiments that were performed in quadruplicate.

cant effect compared with normoxia, and addition of 100 μ M L. A. had no significant effect under either condition.

In vitro EMSAs were also performed with CEs from BY4741 Δ mga2 transformed with plasmids containing wild-type *MGA2* or truncated Mga2p and grown under various conditions. As shown in Fig. 8, slower-migrating complex B3 from the CE containing either truncated Mga2p protein was virtually the same under normoxic and hypoxic conditions with or without 100 μ M L.A., consistent with the results from the reporter assay and Northern blot analyses. A slightly different migration pattern was observed with CEs containing the two truncated Mga2p proteins. In CE containing Mga2p- Δ 244, B3 migrated slightly faster than in CE containing Mga2p- Δ 172. This may reflect the 72-amino-acid difference between these two truncated proteins. Additionally, the complexes from CEs with either protein migrated slower than B2, which represents wild-type Mga2p. The migration difference between complexes B2 and B3 may result from the amino acid differences among Mga2p90, Mga2p- Δ 244, and Mga2p- Δ 172. When a mutated LORE sequence (mLORE) was used as a probe, the complexes from the CEs containing either wild-type or truncated Mga2p disappeared although the nonspecific B1 complex remained intact (data not shown). This suggests that B2 and B3 are functionally equivalent, specific LORE-binding complexes associated with the various Mga2p proteins studied.

Mga2p processing is regulated by hypoxia and UFA. To directly test the hypothesis that Mga2p processing is regulated by hypoxia and UFA, we transformed 2 μ m-based plasmid YEplac181-^{myc}MGA2 into BY4741 Δ mga2. CEs and RNA for Western and Northern blot analyses, respectively, were prepared. As shown in Fig. 9A, a Western blot with the anti-myc antibody demonstrated that the Mga2p120/Mga2p90 ratio varies depending upon whether the cells are grown under normoxic or hypoxic conditions. In normoxic cells, levels of membrane-bound Mga2p were about 80% of those of the proteolytically processed p90 form. This was increased to about 90% of the processed form in cells that were exposed to

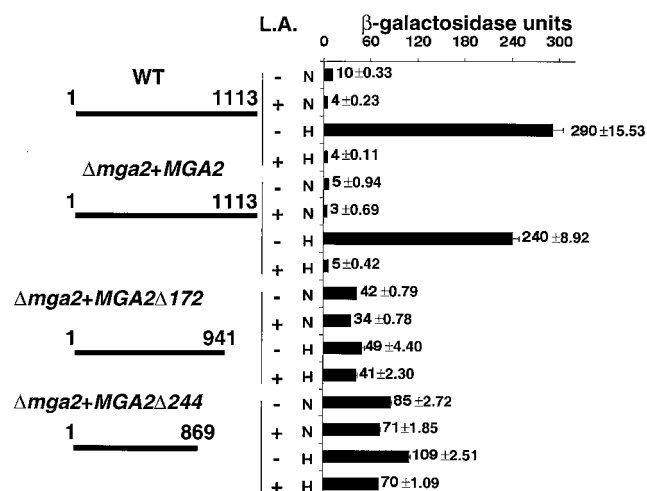


FIG. 6. Activities of truncated Mga2p constructs under different conditions. The truncated constructs are shown on the left. The narrow line represents the Mga2p amino acid sequence. The numbers above each narrow line indicate positions in the amino acid sequence with respect to the N terminus on the left and the C terminus on the right. WT indicates that reporter (pAM6) analyses were performed with wild-type strain BY4741. $\Delta mga2+MGA2$ indicates that reporter (pAM6) analyses were performed with the BY4741 $\Delta mga2$ strain transformed with pAM23 containing full-length MGA2. $\Delta mga2+MGA2\Delta 172$ indicates that strain BY4741 $\Delta mga2$ transformed with pAM31 was used. $\Delta mga2+MGA2\Delta 244$ indicates that strain BY4741 $\Delta mga2$ transformed with pAM30 was used. L.A. indicates growth medium containing 100 μ M L.A. dissolved in 1% ethanol. A plus sign indicates that L.A. was added; a minus sign indicates that no L.A. was added. The diagram on the right shows β -galactosidase activities under normoxic (N) and hypoxic (H) conditions. The absolute units of activity indicated are averages of at least three independent experiments that were performed in quadruplicate.

100 μ M L.A. This slight effect of UFAs on the p120/p90 ratio under normoxia is consistent with the previous report of Hoppe et al. (14), who observed that Mga2p processing is only moderately influenced by UFAs.

Hypoxia significantly increased the relative proportion of p90 (p120/p90 ratio, 0.27 under hypoxia versus 0.84 under normoxia). When UFA was added under hypoxia, a restoration of the p120/p90 ratio toward that observed in normoxia was seen (from 0.27 to 0.70). Northern blot assays were also performed to assess *OLE1* mRNA expression. As shown in Fig. 9B, similar hypoxic induction and UFA repression patterns were observed under the same conditions. Again, the UFA repression of mRNA levels under normoxic conditions was not as dramatic.

p90 Mga2p forms a complex with LORE. To directly test the hypothesis that the p90 form of Mga2p complexes with LORE, we performed biotinylated probe pull-down assays with probes containing either wild-type LORE or mutated LORE. As shown in Fig. 10, the eluates from beads with LORE primarily contained the p90 form of Mga2p when previously incubated with either hypoxic or normoxic CEs. The lower intensity of the p90 band in normoxic CEs is consistent with the lower levels of processed p90 under normoxic conditions. Importantly, no bands were detected from the eluates obtained from beads with mutated LORE that had been incubated with either hypoxic or normoxic CEs. Similar shifted bands were observed

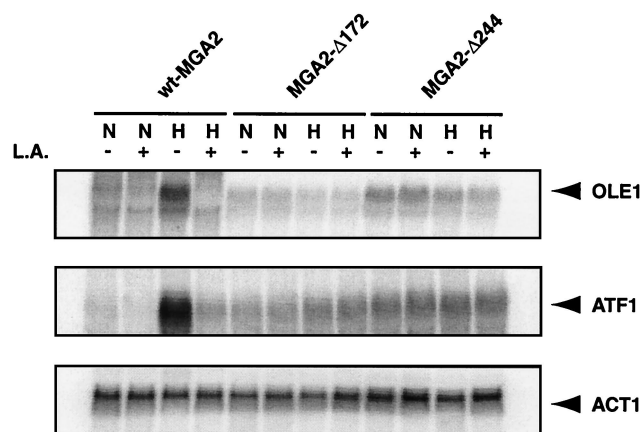


FIG. 7. Effect of truncated Mga2p on *OLE1* gene expression as assessed by Northern blot analysis under various conditions. L.A. indicates growth medium containing 100 μ M L.A. dissolved in 1% ethanol. A plus sign indicates that L.A. was added; a minus sign indicates that no L.A. was added. The letters N and H indicate that yeast cells were grown under normoxic and hypoxic conditions, respectively, as described in Materials and Methods. The positions of the *OLE1*, *ATF1*, and *ACT1* mRNAs are shown. wt, wild type.

when the same LORE-bead elutes from both hypoxic and normoxic CEs were used in EMSAs (data not shown).

DISCUSSION

The degree of fatty acid unsaturation in cell membrane lipids plays a major role in determining membrane fluidity. Alterations of the ratio of saturated fatty acids to mono-UFAs have been implicated in a variety of diseases, including diabetes, obesity, hypertension, cancer, and neurologic, vascular, and heart diseases, in mammals (27, 33, 36). In yeast, it has been suggested that this ratio is related to the heat shock response, ethanol tolerance, and mitochondrial movement and inheritance (1, 4, 33, 34).

OLE1, which encodes a $\Delta 9$ fatty acid desaturase in *S. cerevisiae*, is an enzyme that plays a critical role in maintaining the correct ratio of saturated fatty acids to mono-UFAs in the cell membrane. Two well-studied signals known to significantly affect *OLE1* expression are UFAs and hypoxia, which, respectively, repress and activate *OLE1* expression at the transcriptional level (23, 25, 26, 38). We hypothesized that there may be communication between these different signal pathways. Here we provide evidence that the *cis* element LORE and the *trans* factor Mga2p are involved in both hypoxic induction and UFA repression of *OLE1*.

LORE-*CYC1* basal promoter-*lacZ* fusion reporter (pAM6) assays and Northern blot analyses clearly demonstrated that UFAs repress hypoxia-induced expression in a dose-dependent manner (Fig. 2 and 3). The data suggest that, at low concentrations of fatty acids, the number of *cis* double bonds in a UFA is a major factor in determining the potency of UFA repression. Results of EMSAs further confirmed that UFAs repress LORE-dependent gene expression, as indicated by inhibition of hypoxia-induced complex formation with LORE (Fig. 4). This differential repression might be caused by changes induced in membrane fluidity by the more highly un-

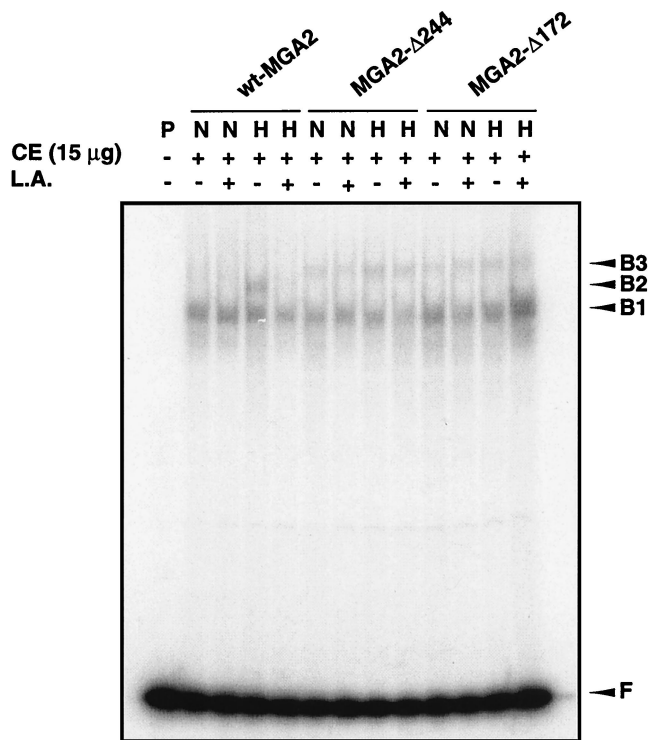


FIG. 8. EMSA analyses of truncated Mga2p CE. EMSAs were performed as described in Materials and Methods. A 15- μ g sample of CE from yeast cells grown under normoxic (N) or hypoxic (H) conditions with (+) or without (-) 100 μ M L.A. was incubated for 20 min at 25°C with 32 P-labeled LORE and then subjected to electrophoresis at 4°C for ~3 h. Free probe (F) and bound complexes (B1, B2, and B3) were detected by autoradiography. The letter P represents the DNA probe. wt, wild type.

saturated species or by differences in the rates of fatty acid import or intracellular movements that produce different concentrations of the various species in the vicinity of the sensors that trigger those regulatory changes. Given the suggestion here that fatty acids affect *OLE1* expression by more than one pathway, it is conceivable that Mga2p-mediated expression might respond to changes in membrane lipid composition, as well as internal free fatty acid or fatty acyl coenzyme A pools.

Northern analyses, LORE-dependent reporter assays, and EMSAs with two truncated forms of Mga2p mimicked the processed p90 form of Mga2p and support the hypothesis that both hypoxia and UFA signals affect the processing of Mga2p and that both UFA repression and hypoxic induction of *OLE1* are mediated through the same factor, Mga2p, that acts via LORE. One of the truncated Mga2p forms used in this study, Mga2- Δ 172, is similar to one studied before (41). Zhang et al. demonstrated that if the C-terminal 173 codons were deleted from *MGA2*, in either *CEN*- or 2 μ m-based plasmids, the truncated gene was able to suppress the Ty-induced mutation *lys2-61*. Another C-terminally truncated Mga2p protein from a 2 μ m-based plasmid could suppress *ufd1-2* (a temperature-sensitive mutant form of *ufd1*, an essential gene of unknown function that was identified in a screen for yeast mutants that stabilize an artificial ubiquitin fusion protein [20]), similar to Spt23p and its C-terminally truncated forms (14). It is note-

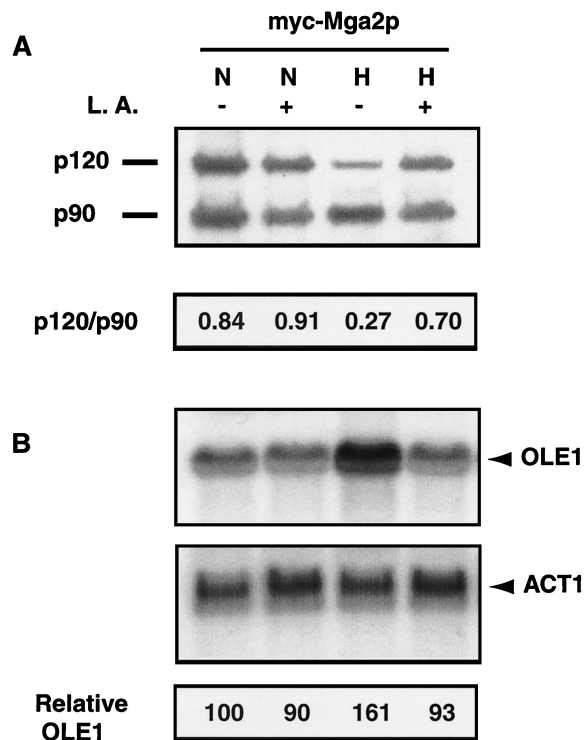


FIG. 9. Hypoxia and UFA affect Mga2p processing and *OLE1* expression. (A) Western blot assay. BY4741 Δ *mga2* cells transformed with YEplac181-*mycMGA2* containing a *myc-MGA2* fragment (myc-Mga2p) were grown under different conditions. CE were prepared as described in Materials and Methods after incubation of the yeast cells under normoxic (N) or hypoxic (H) conditions with (+) or without (-) 100 μ M UFA (L.A.). At the bottom are p120/p90 ratios determined by densitometric measurement of p120 and p90 band densities. A Western blot assay was performed as described in Materials and Methods and probed with a monoclonal antibody to c-myc. (B) Northern blot assay. The positions of the *OLE1* and *ACT1* mRNAs are shown. At the bottom is a quantitative analysis of *OLE1* mRNA expression in the Northern blot above. Signal intensity was quantitated with a Molecular Dynamics PhosphorImager. Transcript levels were normalized to the level of *ACT1* mRNA and are presented as relative units with respect to the *OLE1* mRNA extracted from strain BY4741 Δ *mga2* transformed with YEplac181-*mycMGA2* containing the *myc-MGA2* fragment (Δ *mga2*+*MGA2*) and grown without L.A. under normoxic conditions.

worthy that previous studies demonstrated that Spt23p processing can be strongly regulated by UFAs, which repress *OLE1* expression (14). *SPT23* could be crucial in general UFA repression under normoxic conditions via a LORE-independent element, for example, the FAR element, which plays an important role in *OLE1* normoxic expression.

Finally, Western blot analysis (as shown in Fig. 9) directly demonstrated that both UFAs and hypoxia affect the processing of Mga2p and that the effects of these two signals can counteract one another. These observations regarding Mga2p processing are consistent with the data from Northern blot analyses. Interestingly, results from previous studies have suggested that UFAs have only a modest influence on Mga2p processing (14), and these results are consistent with our findings. We believe that at least part of the reason for this is the much higher levels of expression in the 2 μ m-based myc-MGA2 plasmid. The results of Northern blot assays in which

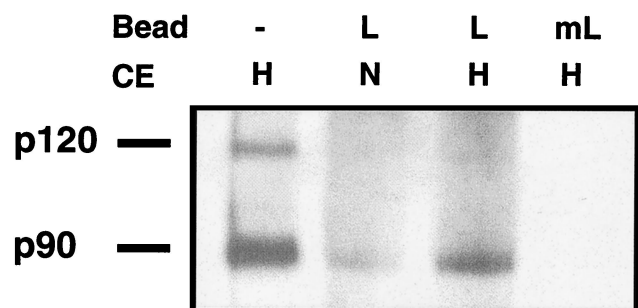


FIG. 10. p90 Mga2p forms a complex with LORE. A biotinylated probe pull-down assay was done as described in Materials and Methods. Strain BY4741 Δ *mga2* transformed with YEplac181-*mycMGA2* containing a *myc-MGA2* fragment (*myc-Mga2p*) was grown under different conditions. CEs were prepared as described in Materials and Methods after incubation of yeast cells under normoxic (N) or hypoxic (H) conditions and incubated with double-stranded, biotinylated LORE (L) or mutated LORE (mL) bound to streptavidin-coated magnetic beads. Protein complexes were purified with a magnet, washed, eluted, and analyzed by Western blotting as described in Materials and Methods, except in lane 1 (no beads), for which the CE from cells grown under hypoxic conditions was used directly in the Western blot. The blot was probed with a monoclonal antibody to c-*myc*. The positions of the p120 and p90 forms of Mga2p are indicated.

BY4741 Δ *mga2* was transformed with a *CEN*-based *MGA2* plasmid (Fig. 7), compared to a 2 μ m-based *myc-MGA2* plasmid (Fig. 9B), indicate that the UFA repression of the *myc-MGA2* plasmid is less dramatic than that of *CEN*-based *MGA2* plasmid pAM23. The moderate effects of UFA on blocking of Mga2p processing, as assessed by Western analysis, especially under normoxic conditions, support this hypothesis. It is also possible, although unlikely, that the *myc* tag contributes to these effects. In summary, both direct and indirect evidence from this study strongly supports the hypothesis that both UFA and hypoxia signals affect the processing of Mga2p. The interesting question of exactly how these signals affect the processing of Mga2p remains unsolved.

The results of biotinylated-probe pull-down assays (Fig. 10) provide direct evidence that the processed p90 form of Mga2p, not the precursor p120, is a component of the LORE complex. This could explain the different migration of the two truncated Mga2p forms compared with that of wild-type Mga2p in Fig. 8. The slower migration of the shifted bands from truncated Mga2p may result from the difference in the number of amino acids between the truncated and wild-type forms of Mga2p. Truncated Mga2p may still be bigger than p90.

It is worth noting that the effect of the ubiquitination-proteasomal degradation pathway on a crucial transcription factor of a hypoxia signal transduction pathway provides a potential common theme for hypoxic regulation of gene expression between yeast and mammalian cells. In yeast, the ubiquitin-proteasome-dependent pathway is involved in the processing of Spt23p and Mga2p (14). We have shown that *MGA2* is an important transcription factor involved in the LORE-dependent hypoxic induction of genes in *S. cerevisiae* (19). Although we did not directly address in this study the question of whether the processing of Mga2p is facilitated by hypoxia in a ubiquitin-proteasome-dependent manner, it is quite possible that the mechanism of Mga2p processing under hypoxia is

similar to that of UFA repression, in which the processing of Mga2p depends on the ubiquitin-proteasome-dependent pathway. In the case of the mammalian hypoxic response, HIF-1 α is usually immediately destroyed by the ubiquitin-proteasome pathway under normoxic conditions and is stabilized by hypoxia (3, 32). Significantly, the proteasomal degradation of Mga2p is only partial and probably occurs under hypoxic conditions to produce higher levels of the active protein form, whereas for HIF-1 α , protein degradation is complete under normoxic conditions.

The present study provides another example of how cells may translate different signals to cellular responses, perhaps via the same pathway. The details of the mechanism by which *OLE1* expression is regulated by two signals, UFAs and oxygen, are under active investigation.

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Y.J. and M.J.V. contributed equally to this work.

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