Identification and Characterization of a Low Oxygen Response Element Involved in the Hypoxic Induction of a Family of *Saccharomyces cerevisiae* Genes

IMPLICATIONS FOR THE CONSERVATION OF OXYGEN SENSING IN EUKARYOTES*

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An organism’s ability to respond to changes in oxygen tension depends in large part on alterations in gene expression. The oxygen sensing and signaling mechanisms in eukaryotic cells are not fully understood. To further define these processes, we have studied the ΔΦ fatty acid desaturase gene *OLE1* in *Saccharomyces cerevisiae*. We have confirmed previous data showing that the expression of *OLE1* mRNA is increased in hypoxia and in the presence of certain transition metals. *OLE1* expression was also increased in the presence of the iron chelator 1,10-phenanthroline. A 142-base-pair (bp) region 3’ to the previously identified fatty acid response element was identified as critical for the induction of *OLE1* in response to these stimuli using *OLE1* promoter-*lacZ* reporter constructs. Electromobility shift assays confirmed the presence of an inducible band shift in response to hypoxia and cobalt. Mutational analysis defined the nonamer sequence ACTCAACAA as necessary for transactivation. A 20-base pair oligonucleotide containing this nonamer conferred up-regulation by hypoxia and inhibition by unsaturated fatty acids when placed upstream of a heterologous promoter in a *lacZ* reporter construct. Additional yeast genes were identified which respond to hypoxia and cobalt in a manner similar to *OLE1*. A number of mammalian genes are also up-regulated by hypoxia, cobalt, nickel, and iron chelators. Hence, the identification of a family of yeast genes regulated in a similar manner has implications for understanding oxygen sensing and signaling in eukaryotes.

Regardless of an organism’s complexity, its ability to adapt to changes in environmental oxygen tension is critical to its survival. In many circumstances, the adaptive response to low oxygen tension requires alterations in the expression of specific genes. In mammals, genes encoding proteins such as erythropoietin and vascular endothelial growth factor, critical molecules in system-wide functions such as erythropoiesis and blood vessel formation, respectively, were among the first shown to be regulated by hypoxia (reviewed in Refs. 1 and 2). The regulation of these genes by hypoxia has served as a paradigm for the subsequent identification of hypoxia-regulated genes involved in basic intracellular metabolic and biochemical pathways. For example, the hypoxia responsiveness of genes involved in sugar transport (e.g. GLUT1 and GLUT3) and energy production (e.g. genes encoding certain glycolytic enzymes) is mediated by hypoxia inducible factor-1 (HIF-1) (reviewed in Ref. 8), the same transcription factor that plays a critical role in erythropoietin and vascular endothelial growth factor activation in response to low oxygen tension (3, 4). HIF-1 is a heterodimeric protein comprised of α and β subunits, both of which are basic helix-loop-helix proteins in the PAS family of transcription factors. The previously identified β subunit is ARNT, the aryl hydrocarbon receptor nuclear translocator. ARNT mRNA and protein levels are not significantly affected by ambient oxygen tension. In contrast, although HIF-1α mRNA levels are not appreciably affected by oxygen tension, HIF-1α protein is only minimally present in normoxia, being rapidly degraded by the ubiquitin-proteasome pathway in this condition. However, HIF-1α accumulates in response to hypoxia, certain transition metals (e.g. cobalt and nickel), and iron chelators such as desferrioxamine. Once the heterodimer is formed under these conditions, it can interact with other DNA-binding proteins (such as HNF4) which function in part to provide tissue and developmental specificity (reviewed in Refs. 1 and 2). HIF-1 has also been shown to associate with scaffold proteins such as p300/CBP (5), which have been shown to interact with the basal transcription machinery (6, 7). The interactions between the proteins in this complex have been the focus of several investigators, and have been reviewed previously (reviewed in Ref. 2).

Although a substantial understanding of the structure and function of HIF-1α exists, the mechanism by which mammalian cells sense hypoxia remains poorly defined. In certain prokaryotes, a heme protein has been shown to serve as an oxygen sensor (reviewed in Refs. 1 and 8). Evidence supports

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1 The abbreviations used are: HIF-1, hypoxia inducible factor-1; LORE, low oxygen response element; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay; FAR element, fatty acid-regulated element; UFA, unsaturated fatty acid; LBP, low oxygen response element-binding protein; bp, base pair(s); TEMED, N,N,N,N'-tetramethylethylenediamine.
the role for a heme protein in mammalian oxygen sensing as well (9). Certain transition metals such as cobalt and nickel appear to mimic hypoxia and induce several hypoxia-regulated genes. It has been proposed that these metals may replace iron in the heme moiety of such a protein, thereby altering its conformation to a “deoxy” state (9). Alternatively, these transition metals may disrupt the production of reactive oxygen species which may serve as important signaling molecules in the oxygen response pathway (reviewed in Refs. 1 and 10). A recent study provided evidence against metal substitution in heme (11).

Iron chelation also mimics hypoxia. Just as certain metals may interfere with intracellular Fenton chemistry in some manner, decreased intracellular iron may also decrease reactive oxygen species within the cell, leading to altered gene expression (reviewed in Ref. 2). HIF-1α has been shown to be sensitive to these perturbations in intracellular oxygen-free radicals (12). Finally, carbon monoxide inhibits the expression of hypoxia-induced genes and HIF-1α activation. Carbon monoxide can bind reversibly to heme proteins, and may lead to a change in conformation to the “oxy” state (12). A novel human cytosolic flavohemoprotein with cytochrome b₅ and b₅ reductase domains and functional NAD(P)H oxidoreductase activity has recently been cloned (13). This protein fulfills all of the criteria for an oxygen sensor, although definitive data in this regard remains lacking.

The yeast, Saccharomyces cerevisiae, a single-celled euarkyote, is a facultative anaerobe which respires in the presence of oxygen but ferments under anaerobic conditions. Therefore, it may interfere with intracellular Fenton chemistry in some manner, decreased intracellular iron may also decrease reactive oxygen species within the cell, leading to altered gene expression (reviewed in Ref. 2). HIF-1α has been shown to be sensitive to these perturbations in intracellular oxygen-free radicals (12). Finally, carbon monoxide inhibits the expression of hypoxia-induced genes and HIF-1α activation. Carbon monoxide can bind reversibly to heme proteins, and may lead to a change in conformation to the “oxy” state (12). A novel human cytosolic flavohemoprotein with cytochrome b₅ and b₅ reductase domains and functional NAD(P)H oxidoreductase activity has recently been cloned (13). This protein fulfills all of the criteria for an oxygen sensor, although definitive data in this regard remains lacking.

The yeast, Saccharomyces cerevisiae, a single-celled euarkyote, is a facultative anaerobe which respires in the presence of oxygen but ferments under anaerobic conditions. Therefore, it is not surprising that yeast have evolved sophisticated molecular mechanisms involving oxygen-dependent gene regulation. Several yeast genes, exemplified by ANB1, have been shown to be up-regulated by complete anaerobiosis, mediated in large part through the ROX1p protein, a DNA-binding protein which functions as a repressor (reviewed in Ref. 14). There is no evidence that any factor that mimics hypoxia in mammalian cell lines induces ANB1 expression in yeast. However, recently it has been shown that other genes in S. cerevisiae exhibit increased expression above baseline at low oxygen tensions, before complete anaerobic conditions are reached (15). One gene in particular, OLE1, encodes the Δ9 fatty acid desaturase and is critical for unsaturated fatty acid biosynthesis. Incubation in the presence of cobalt or nickel leads to increased expression of OLE1, mimicking mammalian hypoxia-regulated genes (15). In this study, we have further defined the regulation of OLE1 by hypoxia, certain transition metals, and iron chelation. The cis element responsible for hypoxia induction of OLE1 expression was identified and characterized using reporter gene studies and electromobility shift assays. This low oxygen response element, or LORE, was used to search the S. cerevisiae genome. This search led to the identification of a family of yeast genes regulated in a similar manner. Taken together, the evidence presented supports the hypothesis that critical aspects of the oxygen sensing mechanism are highly conserved among all eukaryotes.

MATERIALS AND METHODS

Media, Chemicals, and Enzymes—Yeast strains were grown in YPD medium (Bio 101, Inc., Carlsbad, CA) or SC dropout medium, depending on the plasmid selectable markers. LB was used for bacteria growth purposes. Ampicillin (U.S. Biochemical Corp.) was used as necessary at 50 μg/ml unless indicated otherwise. o-Nitrophenyl-β-galactopyranoside was obtained from ICN Biochemicals Inc. or Sigma. Radiolabeled compounds were purchased from PerkinElmer Life Sciences. Formamid, dextran sulfate, and Denhardt's solution were bought from American Bioanalytical. Acrylamide, bisacrylamide, TEMED, and protein molecular mass markers were from Bio-Rad. Ammonium sulfate, phenylmethylsulfonlfluoride, CoCl₂·6H₂O, 1,10-phenanthroline, and Nonidet P-40 were obtained from Sigma. SeaKem ME-agarose was from FMC Bioproducts. T4 polynucleotide kinase and dNTPs were purchased from Promega Corp. Shrimp alkaline phosphatase and T4 polynucleotide kinase and dNTPs were purchased from Boehringer Mannheim. Restriction enzymes were from New England BioLabs. All enzymes were used according to the manufacturer's instructions.

Oligonucleotide Synthesis—Oligonucleotides were synthesized by Integrated DNA Inc. When necessary restriction sites for cloning were added at the 5' ends of primers and were preceded by 3 to 6 nucleotides for efficient digestion. Paired oligonucleotides were used for direct cloning purposes. Paired oligonucleotides used for direct cloning purposes contained a phosphate group at the 5' end. Table I shows the nucleotide sequences used for polymerase chain reaction (PCR), Northern blot assay, EMSA, cloning, and site-directed mutagenesis.

Plasmid and Plasmid Construction—Plasmids used in this study are shown in Table II. The construction of several of the OLE1 promoter-lacZ fusion deletion series was described previously (16). Construction of pAM6, pAM7, pAM10, and pAM16 vectors containing the OLE1 promoter-lacZ fusions (~347 to ~328) to the ATG translational start codon with the A of the codon designated as +1) was performed by inserting the synthetic paired oligonucleotides (10'-5' and 10'-3') into the Xhol restriction site of pTBA30. The CYC1 bas promoter-lacZ fusion vector obtained from Dr. A. Vershon. pAM16 contains one LORE copy in the ~347 to ~328 forward or (+) orientation 5' to the basal CYC1 promoter-lacZ fusion. pAM7 has one LORE copy in the ~328 to ~347 reverse or (−) orientation. pAM6 contains a tandem repeat of the LORE in the (+) orientation. pAM10 was generated by inserting the synthetic paired oligonucleotides (yd-19 and yd-20) into the XhoI restriction site of pTBA30. The LORE in this plasmid has three mutations. Plasmid pAM4 is the p62:934 derivative with three nucleotide substitutions in the LORE region (~C342T, ~T341A, and ~A339G) prepared utilizing three-step PCR with oligonucleotides containing site-directed muta-

| Name          | Sequencea |
|---------------|-----------|
| Oligonucleotides for EMSA (LORE identification) |
| 1'–3'         | `AAATTTCTCTCCATGTTGGTTTA` |
| 2'–5'         | `AAATTTCTCTCCATGTTGGTTTA` |
| 3'–5'         | `AAATTTCTCTCCATGTTGGTTTA` |
| 4'–5'         | `AAATTTCTCTCCATGTTGGTTTA` |
| 5'–3'         | `AAATTTCTCTCCATGTTGGTTTA` |
| 6'–5'         | `AAATTTCTCTCCATGTTGGTTTA` |
| 7'–5'         | `AAATTTCTCTCCATGTTGGTTTA` |
| 8'–5'         | `AAATTTCTCTCCATGTTGGTTTA` |
| 9'–5'         | `AAATTTCTCTCCATGTTGGTTTA` |
| 10'–5'        | `AAATTTCTCTCCATGTTGGTTTA` |
| 11'–5         | `AAATTTCTCTCCATGTTGGTTTA` |
| 12'–5         | `AAATTTCTCTCCATGTTGGTTTA` |
| 13'–5         | `AAATTTCTCTCCATGTTGGTTTA` |
| 14'–5         | `AAATTTCTCTCCATGTTGGTTTA` |
| 15'–5         | `AAATTTCTCTCCATGTTGGTTTA` |

a Numbers at 5' and 3' ends of oligonucleotides indicate the position of the nucleotide of OLE1 promoter with respect to the start codon (A of ATG is +1).

b Underlined indicates the restriction site.

c Lower case letter indicates mutation in sequence.

2 M. Vasconcelles, unpublished data.

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Table II

Yeast (S. cerevisiae) strains and plasmids

| Strains and plasmids | Genotype | Plasmid description | Source |
|----------------------|----------|---------------------|--------|
| RZ53–6               | a trp1–289, leu2–3, 112, ura3–52, ade1–100 | Numbers following :: indicate the position of the nucleotide with respect to the start codon (A of ATG is +1) in lacZ fusion constructs (Choi et al. (14).) | This study |
| [p62–934]            |          | The OLE1 promoter contains -C342T, -T341A, and -A339G mutations in p62–934 | This study |
| [pTBA30]             |          | Basal CYC1 promoter-lacZ fusion | This study |
| [pAM6]               |          | A tandem (+) repeat LORE-basal CYC1 promoter-lacZ fusion | This study |
| [pAM7]               |          | (-) LORE-basal CYC1 promoter-lacZ fusion | This study |
| [pAM10]              |          | (+) mutant LORE-basal CYC1 promoter-lacZ fusion | This study |
| [pAM16]              |          | (+) LORE-basal CYC1 promoter-lacZ fusion | This study |
| RZ53–6Δrox1          | a trp1–289, leu2–3, 112, ura3–52, ade1–100, rox1:::leu2 |          | R. Zitomer |
| [p62–934]            |          |          | This study |

*Numbers indicate the position of the nucleotide of OLE1 promoter with respect to the start codon (A of ATG is +1); the letter in front of the number represents the wild type nucleotide and the letter after the number is the nucleotide substituted.

Strains and Growth Conditions—Table II contains the yeast strains used in these studies. Yeast cells containing lacZ fusion plasmids were grown at 30 °C on uracil dropout medium containing dextrose (17). For unsaturated fatty acid repression analysis, yeast was grown in medium supplemented with 1% Ergot as described previously (16). Cells were grown in the presence of UFAs for 6 h prior to the β-galactosidase assays. Plasmid amplifications and bacterial transformations were performed using Escherichia coli strain DH5 (Invitrogen Corp.). Yeast transformations were performed by the method of Elble (18). Preparative cultures were grown aerobically in a shaker at 200 rpm (Innova 4000 incubator shaker, New Brunswick Scientific) at 30 °C to mid-logarithmic phase. For experiments assessing yeast under hypoxic conditions, mid-logarithmic phase preparative cultures were used to inoculate special air-tight flasks with inlet and outlet ports to allow for equilibration with the appropriate gas mixtures. Cultures were exposed to a continuous flow of hydrated medical grade nitrogen (BOC Gases, Murray Hill, NJ), unless otherwise specified, for 6 h after inoculating the medium via the inlet port. Of note, medical grade nitrogen is contaminated with trace amounts of O2 (less than 1%). The percentage of saturated O2 in each flask was confirmed by using an oxygen monitor (G. C. Industries, Inc.) attached to the outlet port of each culture. For experiments assessing yeast exposed to cobalt, cobalt chloride was resuspended in storage buffer (20 mM HEPES, pH 8.0, 5 mM EDTA, 20% (v/v) glycerol, 7 mM phenylmethylsulfonyl fluoride, 7 mM β-mercaptoethanol) and stored frozen at −80 °C. The soluble protein concentration was determined using a Bradford dye binding assay (Bio-Rad).

EMSA—EMSA were performed essentially as described by Carey (21) utilizing synthetic paired oligonucleotides (e.g. 10′-5′ and 10′-3′) as a probe or a probe containing the LORE sequence made by PCR using p62-934 as the template with 32P-labeled oligonucleotides 1-5′ and yr-10-397 to 234′ as the primer. Synthetic paired oligonucleotides were end labeled using polynucleotide kinase and purified using a Sephadex G-25 spin column (Roche Molecular Biochemicals) to remove unincorporated nucleotides. Probes made by PCR were purified away from labeled primers, [γ-32P]ATP and Taq polymerase using a QiAquick spin PCR purification kit (Qiagen). The fmol DNA sequencing system (Promega Corp.) was used for sequencing according to its technical manual. Reactions were run on 6% sequencing gels, which were dried and exposed to X-Omat AR film (Kodak) to visualize the sequence.

Yeast Extract Preparation—Haploid yeast (S. cerevisiae, strain RZ53–6) were cultured in 1-liter flasks containing 200 ml of YPD (1% yeast extract, 2% peptone, 2% dextrose) either under normoxic or hypoxic conditions, harvested at midlog phase (A600 = 0.8), and lysed by vortexing with glass beads according to published protocols (20). 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 14,000 rpm 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% (v/v) glycerol, 7 mM phenylmethylsulfonyl fluoride, 7 mM β-mercaptoethanol) and stored frozen at −80 °C. The soluble protein concentration was determined using a Bradford dye binding assay (Bio-Rad).

β-Galactosidase Assays—Assays of cells containing plasmids derived from the OLE1 promoter-lacZ fusion p62 constructs were performed as described previously (19). Cell densities for these assays were determined by measurement at A600. Transformants were assayed for each of the plasmid constructs listed in Table II. β-Galactosidase activities reported here are the results of at least two independent experiments. Each experimental assay was performed in quadruplicate.

DNA Sequencing—Plasmid templates for sequencing were isolated using a QiAprep spin purification kit (Qiagen). The fmol DNA sequencing system (Promega Corp.) was used for sequencing according to its technical manual.

E. coli preparation and sequencing: E. coli was grown to mid-log phase in LB (1% peptone, 0.5% yeast extract, 0.5% NaCl) at 30 °C, then transferred into M9 minimal media (10 mM Na2HPO4, 5 mM KH2PO4, 8 mM NH4Cl, 50 μM MgCl2) at 1000 A600. Transformants were assayed for each of the plasmid constructs listed in Table II. β-Galactosidase activities reported here are the results of at least two independent experiments. Each experimental assay was performed in quadruplicate.

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RESULTS

Hypoxia, the Transition Metals Cobalt and Nickel, and the Iron Chelator 1,10-phenanthroline Increase OLE1 Expression by Northern Analysis and β-Galactosidase Reporter Assay—Previous studies have shown that OLE1 mRNA transcript levels are increased at low oxygen (O₂) tensions, below 0.5 μmol.

Maximal expression, approaching a 4-fold increase over baseline normoxia, was observed following 8–10 h of anoxia (15, 26). First, we confirmed that OLE1 mRNA is maximally expressed in the presence of trace O₂ (<1%) concentrations (data not shown). Subsequently, we extended this finding utilizing a plasmid (p62::934) in which 934 bp of the OLE1 promoter is fused in-frame with the lacZ gene. Less than a 2-fold induction over baseline levels in normoxia was observed in 1% O₂ although a 6-fold induction occurred at an extremely low O₂ tension (data not shown). In a similar fashion, we have verified previous studies demonstrating increased levels of OLE1 mRNA following incubation of S. cerevisiae with increasing concentrations of cobalt chloride (CoCl₂) and nickel chloride (NiCl₂) in normoxia (15) (data not shown). The concentrations were similar to those used in the study of hypoxia-regulated mammalian genes such as erythropoietin (9). These data were confirmed with experiments utilizing the OLE1 promoter-lacZ reporter assay. The degree of induction approached that which has been previously reported (15), even though the metal concentrations were almost 10-fold lower. At 800 μM CoCl₂ and 450 μM NiCl₂, significant differences in the growth rate of the yeast compared with control cultures were observed in our experiments, presumably due to direct toxicity of the culture to the metal on S. cerevisiae. It is not immediately apparent why the significantly lower metal concentrations used in the present studies appear to exert the same effect on OLE1 expression as the much higher concentrations employed in previous reports (15); differences in exposure time may be relevant.

Several mammalian hypoxia and transition metal-inducible genes are also up-regulated by the iron chelator, desferrioxam-
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OLE1 Promoter Deletion –lacZ Constructs Define a 142-bp Region (−255 to −396 Relative to the Transcription Start Site) Critical for Induction by Hypoxia and CoCl₂—A series of OLE1 promoter–lacZ fusion reporter constructs were transformed into the RZ53-6 strain and incubated in hypoxia, CoCl₂, or 1,10-phenanthroline. The β-galactosidase activities of these reporter constructs are shown in Fig. 2. The removal of bases −567 through −488 resulted in an 80-fold drop in enzyme activity under normoxia, suggesting the presence of an activating sequence in this region. Previous work has identified the fatty acid-regulated element in this region. Previous work has identified the fatty acid-regulated element in this region. In contrast, removal of bases −396 to −234 produced a small reduction in reporter gene expression under normoxia, hypoxia, and cobalt-treated conditions (within 2.7-fold). Deletions 3’ to base −488 resulted in low basal reporter gene activities under normoxic conditions. However, removal of bases −488 through −396 did not dramatically affect the hypoxia- and cobalt-induced reporter gene expression. In contrast, the 142-bp region between −396 and −255 proved to be critical. Its removal essentially abolished the hypoxia induction and caused a significant reduction of CoCl₂-induced reporter gene expression. The observation that the deletion of sequence between −567 and −488 also resulted in about a 2-fold reduction of CoCl₂-induced reporter gene expression implies that additional regulatory elements necessary for complete CoCl₂-induced OLE1 gene expression may reside in this region.

Hypoxia-Induced Activation Complex Formation with Lore—The analysis of the OLE1 promoter–lacZ fusion deletion series strongly suggests that the −255 to −396 region of the OLE1 promoter contains a cis element responsible for hypoxic induction. The possibility of an activation complex formed in hypoxic conditions was tested in EMSA using crude cell extracts from normoxia, hypoxia-, and cobalt-treated yeast. A wild type OLE1 promoter DNA fragment containing base pairs −234 to −396 was generated using PCR as a probe for the assay. Fig. 3 shows that two shifted bands (B1 and B3) were present in hypoxia and cobalt, although the CoCl₂-treated extracts were of somewhat lower intensity. Band B2 was constitutive and could be displaced by nonspecific DNA (data not shown). To further define the DNA region responsible for the hypoxia-inducible complex formation, a series of double stranded nucleotides 20 base pairs in length covering the entire −255 to −396 region of OLE1 DNA were synthesized and used as cold competitors for the hypoxia-inducible shifted bands (B1 and B3) in EMSA. A double-stranded oligonucleotide 10/3′ (Table I, number 10) could effectively compete out the shifted bands as shown in Fig. 3, whereas the remainder of the double-stranded oligonucleotides from 1-5/1-3′ (number 1) to 14-5/14-3′ (number 14) could not. Fig. 3 is a representative gel containing paired oligonucleotides numbers 8, 9, 10, and 11. Therefore, oligonucleotide 10 appears to contain a site(s) for hypoxia- and CoCl₂-induced protein(s) binding and was designated as the Lore. To further investigate the role of oligonucleotide number 10 in hypoxia- and CoCl₂-induced complex formation, EMSAs were performed using end-labeled oligonucleotide number 10 as a probe. Fig. 4 demonstrates that crude extracts from both hypoxia- and cobalt-treated yeast form a specific complex with oligonucleotide number 10 in vitro. Of note, when this shorter radiolabeled probe is used, band B2 is prominent whereas B3 is no longer present. This is consistent with the hypothesis that B3 complex formation requires additional element(s) outside of oligonucleotide 10. Furthermore, when mutations were introduced in oligonucleotide 10 (m#10), the intensity of the nonspecific B2 was further enhanced. Shortened exposure time,
however, confirmed that the specific hypoxia-induced B1 complex formation was not observed. No specific hypoxia- or CoCl₂-induced complex formation was observed using probe number 8, confirming the previous EMSA competition assay.

**LORE Is Required for Hypoxia-induced OLE1 Expression in Vivo—**A lacZ fusion reporter pAM4 was constructed such that the full-length OLE1 promoter possessed three mutations in the LORE region (−328 to −347). The requirement of an intact LORE for hypoxia-induced OLE1 expression was tested using the β-galactosidase assay in yeast containing the pAM4 reporter. In this reporter assay, an 8-fold decrease in the basal level of expression in normoxia compared with the nonmutated LORE reporter was observed. The mutated LORE sequence eliminated the 6-fold hypoxic induction seen with the wild type reporter. This result is consistent with EMSA data, which reveal no hypoxia-induced band shift when the same mutated fragment was utilized as a probe (data not shown). However, the CoCl₂-dependent induction by reporter assay was not affected (data not shown).

**LORE Is Sufficient for Hypoxia-induced Gene Expression under the Control of a Heterologous Promoter—**The LORE was fused to the basal CYC1 promoter-lacZ fusion plasmid pTBA30. As shown in Fig. 5, pAM6, carrying two copies of the LORE in tandem, possesses robust transcriptional activation under both hypoxic and cobalt-treated conditions with about 44- and 10-fold increases, respectively. The plasmid carrying one copy of the LORE in both orientations also substantially stimulated the reporter gene expression under both hypoxic and cobalt-treated conditions. In contrast, the plasmid pAM10 containing the CYC1 heterologous promoter with three mutations in LORE did not show induction by either hypoxia or cobalt. Considerable variability in the basal expression of these constructs may be due to differences in sequences related to orientation and copy number of insert.

**LORE Is Involved in OLE1 Repression under Hypoxic Conditions by Unsaturated Fatty Acids (UFA)—**Previous experiments demonstrated FAR elements (−466 to −576) within the OLE1 promoter contribute to OLE1 repression by UFA in normoxic conditions (16). However, when an OLE1-lacZ fusion containing a deleted FAR element was tested under hypoxia, the transactivation repression by UFA was still observed (data not shown). That observation led us to examine whether the LORE plays a role in UFA induced OLE1 gene repression. Fig. 6A shows that the UFA linoleic acid can strongly repress normoxia-, hypoxia-, and cobalt-induced expression of the lacZ reporter plasmid pAM6 which contains two copies of the LORE in tandem. Inhibition showed a dose-response with an IC₅₀ ~ 20 µM. Similar dose-response inhibition was obtained using the unsaturated fatty acids γ-linolenic acid, oleic acid, and arachidonic acid but not with the saturated fatty acid steric acid (data not shown). Consistent with the β-galactosidase assay, the OLE1 mRNA level was also dramatically repressed by linoleic acid as shown in Fig. 6B by Northern blot analysis. This re-

**Fig. 5.** Expression of reporter activity from the heterologous pTBA vector containing the LORE. The diagram on the left indicates the LORE (vertical solid bar) inserted into the heterologous constructions employing the yeast CYC1::lacZ constructions. The small arrow in the vertical bar indicates the orientation of the inserted LORE. The horizontal blank and solid bars represent the CYC1::lacZ gene fusion. The diagram on the right indicates the normalized β-galactosidase activity under normoxic (N), hypoxic (H), and cobalt (C)-treated conditions. The number in parentheses indicates the fold of induction relative to N. The actual β-galactosidase units for normoxia samples are: pTBA30, 0.028; pAM6, 1.06; pAM10, 0.045; pAM16, 0.041; pAM7, 0.813, which are the average of two independent assays performed in quadruplicate.

**Fig. 6.** The effects of unsaturated fatty acid on OLE1 gene expression under hypoxic conditions. In all three panels, N, H, and C indicate yeast grown under normoxic-, hypoxic-, and cobalt-treated conditions as described under “Materials and Methods.” E and EtOH represent the growth medium containing 1% ethanol, L.A. indicates the growth medium containing 1 mM linoleic acid dissolved in 1% ethanol. Panel A, histogram of β-galactosidase activity of reporter pAM6 under varying conditions. The units of activity are the average of two independent assays. Panel B, Northern blot assay of OLE1 gene expression under varying conditions. ACT1 cDNA probe was prepared using PCR (see "Materials and Methods"). In the diagram OLE1 and ACT1 indicate OLE1 and ACT1 mRNAs, respectively. Panel C, EMSA for the crude extracts from yeast grown under different conditions. + indicates CE added; − indicates no CE or EtOH or linoleic acid added. P represents DNA probe. On the right side of this panel, B1 and B2 indicate the specific and nonspecific shifted bands, respectively; F represents the free probe.
expression in normoxia is consistent with previous studies (16). Again, UFA repression could not be overcome by incubation in hypoxic or cobalt-containing conditions. Crude extracts from linoleic acid-treated yeast were utilized for EMSA. As shown in Fig. 6C, the hypoxia-induced LORE complex formation was significantly suppressed with disappearance of binding complex B1. The intensity of the basal expression of the B1 complex under normoxic condition was also repressed, implying that LORE may be involved in the basal expression of OLE1 as well. The nonspecific band B2 was not affected.

Role of ROX1 in OLE1 Expression under Hypoxic Conditions—ROX1 plays a significant role in the regulation of many anoxia-inducible yeast genes (reviewed in Refs. 1 and 14). Several studies have provided evidence that ROX1p functions as a repressor of anoxia-inducible gene expression under normoxic conditions (Ref. 28, reviewed in Refs. 29 and 30). Previous studies (28, 30, 31) had postulated that ROX1 may contribute to OLE1 induction under anoxic conditions based on predicted potential ROX1p-binding sites in the OLE1 promoter region. A ROX1 deletion mutant strain of RZ53-6 (RZ53-6Δrox1) was, therefore, utilized to investigate the ROX1 effects on OLE1 expression under hypoxic conditions. The results of in vivo β-galactosidase assays of reporter p62::934 in strains RZ53-6 and RZ53-6Δrox1 showed that the basal expression of the reporter gene under normoxic conditions is essentially the same in the ROX1 deletion strain as in its parental strain (data not shown). This suggests that ROX1 does not play a role as a repressor in the low basal level expression of OLE1 under normoxic conditions. Moreover, significant induction was still observed under hypoxic conditions (4.2-fold increase) and cobalt-treated conditions (5.1-fold increase) in the ROX1 deletion strain. In the Northern blot analysis the OLE1 mRNA level was induced in both strains under hypoxia- and cobalt-treated conditions. Consistent with the in vivo transactivation data, it was also shown that the relative mRNA levels of hypoxia- and cobalt-treated ROX1-deleted yeast were similar to that of wild type. The basal OLE1 mRNA expression under normoxia was similar in both strains as well (data not shown). Another hypoxic gene ATF1 (see later “Results”) showed similar results.

Sequence Specificity of the LORE Binding Activity—To further define the sequence requirements in LORE, we made a series of single base pair substitutions in the site (Fig. 7A) and assayed the effects of the mutations on DNA binding in vitro (Fig. 8, A and B). Many of the mutant LOREs altered DNA binding ability; in particular, those shown in Fig. 7B are representative of the observed range of EMSA responses. The effects on DNA binding varied from the absence of a detectable specific complex formation B1 for mutant C337A, to a reduction for mutant A346C, to about the same as the wild type LORE for mutant G347T, to an increase in DNA binding for mutant T331G. Another group of mutant LOREs (e.g. G347C and A335C) demonstrated altered complex formation with reduction of B1 but also creation of new shifted bands, suggesting that a new protein-DNA complex may have been created. The DNA binding ability of all the mutant LOREs is summarized in Fig. 8A. Substitutions that show large decreases in specific binding complex formation are concentrated in the center of the LORE. DNA binding was sensitive to single nucleotide substi-
The figure. A representative gene containing potential LOREs in their promoter region. Names of genes are listed on the left. The core sequences are shadowed in the center of the sequence. B, a representative EMSA for potential LOREs. 10 μg of crude extract (CE) from normoxic (N)- and hypoxic (H)-treated yeast were incubated for 20 min at 25 °C with 32P-labeled wild type (OLE1) and potential LOREs from promoters of the genes indicated, then subjected to electrophoresis in the cold for ~3 h. Pairs of oligonucleotides for putative LORE sequences were used as indicated in A. The lane marked — indicates no CE was added; the increased amount of CE (5, 10, and 50 μg) was shown by the triangle. Free probe (F), specific bound complex B1, and nonspecific bound complex B2 were detected by autoradiography. C, Northern blot analysis of several hypoxia- and cobalt-inducible yeast genes. Certain genes containing a putative LORE in their promoter region were tested for hypoxia- and cobalt-induced gene expression by Northern blot analysis. Cells were grown to midexponential phase and total RNA was extracted as described under “Materials and Methods.” The RNA blot was first probed with one specific probe and then after the blot was stripped and reprobed with another. The SUT1, TRX2 cDNA probes were prepared using PCR (see “Materials and Methods”). In the figure OLE1, ATF1, TRX2, SUT1, ACT1, and PGK1 indicate OLE1, ATF1, TRX2, SUT1, ACT1, and PGK1 mRNAs, respectively. PhosphorImaging of the resulting blots are shown in the figure.

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Identification of a Family of Genes under Similar LORE Control—A search of the S. cerevisiae genome for LORE core sequences present in the promoter regions of other genes was carried out using DNA Pattern (32) and PatMatch3 web-based tools. Fig. 8A illustrates alignment of putative LOREs in the promoter region of several genes. Some of the potential LOREs (from the promoter sequences of TRX2, FKH1, FTR1, RPL35A, and MET22) have exactly the same nine core nucleotides. The potential LOREs from the ATF1 and TIR1 promoter regions each have one nucleotide mismatch in the core region. Another potential LORE from SUT1 possesses two mismatches in this region. It is worth noting that the expression of ATF1, TIR1, and SUT1 is increased at low oxygen tensions (34–37). The hypoxia-induced complex formation of all potential LOREs from the listed genes was tested in vitro using EMSAs as shown in Fig. 8B. The results demonstrate that, like the wild type LORE from the OLE1 promoter, a clear hypoxia-induced complex is formed with the potential LOREs from the promoters of the genes indicated. The fact that potential LOREs from RPL35A, TRX2, and MET22 share the same core sequence but exhibit varied degrees of complex formation ability, based on the intensity of the B1 band shift in Fig. 8B, implies that the nucleotides outside the core also play an important role in DNA binding. On the other hand, the potential LOREs from ATF1, TIR1, FTR1, and SUT1 with one or two nucleotide mismatches still showed hypoxia-induced DNA binding (even increased DNA binding in the case of ATF1), suggesting that certain positions in the core sequence may be varied yet still function as a LORE in vitro.

To test the possibility that these genes may be hypoxia-inducible, Northern blot analyses of certain genes (ATF1, TRX2, SUT1, FTR1, and RPL35A) were performed as shown in Fig. 8C. Consistent with previous data (36), ATF1 expression was significantly induced under the hypoxic conditions employed. This study shows for the first time that ATF1 and TRX2 are induced by cobalt treatment as well, similar to OLE1. About a 3-fold hypoxia and 2.5-fold cobalt induction of TRX2 mRNA was observed. The hypoxia-induced SUT1 expression was confirmed; however, there was no significant induction by cobalt. FTR1 mRNA was also examined and it was confirmed that its level was decreased under hypoxic conditions, consistent with previous studies (data not shown) (38). The RPL35A mRNA level was not changed under hypoxic conditions (data not shown).

RAP1p and LORE-binding Protein(s) (LBP)—RAP1p is a yeast multifunctional protein involved in transcriptional activation/repression, and telomere function (Ref. 39, reviewed in Refs. 40 and 41). Previous studies on the regulation of the ATF1 gene identified an 18-bp element essential for transcriptional activation in vitro (36). This element also contains a putative LORE. A purified glutathione S-transferase-RAP1p fusion was utilized for in vitro EMSA using a probe from the ATF1 promoter containing the 18-bp element. The results

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showed that RAP1p could form a complex with the ATF1 promoter DNA sequence. To test the possibility of RAP1p involvement in binding to the LORE, EMSAs were performed. The results demonstrate that the putative LORE from ATF1 forms a complex (B1) with the crude extracts from hypoxia-treated yeast cells analogous to the LORE from OLE1 (data not shown). On the other hand, a constitutive, strong binding band (B3) was observed using the RAP1p binding sequence from the PGK1 promoter as a probe. Additional EMSAs were performed using a very well characterized RAP1p binding sequence and mutants from the TPI promoter as probes (42). These results confirm that the B3 complex binds RAP1p (data not shown). A series of EMSAs were done to investigate the relationship between B3 and B1 (data not shown). Unlabeled LORE from either ATF1 or OLE1 could not compete out the B3 and vice versa, suggesting that the constitutive B3 complex involved with RAP1p binding is different from the hypoxia-induced B1 complex involving the LORE from ATF1 and OLE1. Unlabeled LORE of ATF1 could effectively compete out the complex formed with radiolabeled OLE1 LORE and vice versa (data not shown), which together with the previous B3 competition EMSA results suggest that the complex formed with the OLE1 LORE and the ATF1 LORE are similar. Because there is only 1-bp difference in the core region of the LORE between OLE1 and ATF1, a mutated LORE T341C from OLE1 which corresponds to the putative ATF1 LORE was examined. This single base change caused a constitutive complex formation in crude extracts from normoxia and hypoxia. The subsequent cold probe competition EMSAs suggested that that complex was the same as the B3 complex formed by the RAP1p binding sequence from PGK1 (data not shown). The B1 complex could still be observed using hypoxic crude extract, and could be displaced by unlabeled OLE1 LORE probe. In summary, these in vitro results support the hypothesis that the putative LORE of ATF1 functions like the LORE of OLE1 in the regulation of gene expression by hypoxia. Moreover, these data do not support a role for RAP1p binding to the LORE sequence during hypoxic induction.

**DISCUSSION**

OLE1 encodes the Δ9 fatty acid desaturase, an enzyme involved in the formation of unsaturated fatty acids. This enzyme introduces a double bond between carbons 9 and 10 of substrate palmitoyl (16:0) or stearoyl (18:0)-CoA with molecular O2 serving as an electron acceptor to form palmitoleic (16:1) or oleic (18:1) acid, respectively. Previous studies have demonstrated that OLE1 is up-regulated under hypoxic conditions. Its induction under hypoxic conditions may be in response to the limitation of O2 as a substrate.

The experiments presented in this paper have confirmed previous data showing that the expression of OLE1 mRNA is increased in hypoxia and in the presence of the transition metals cobalt and nickel under aerobic conditions (15). Data presented here have shown that these stimuli induce an OLE1 promoter-lacZ reporter gene as well. Aerobic incubation with the iron chelator 1,10-phenanthroline also leads to increased OLE1 expression as evidenced by Northern blot and reporter assays. Subsequently, using reporter gene assays and EMSAs, a LORE, which functions as a transcriptional activation cis element, was identified. The LORE, about 20 bp in length, is necessary and sufficient for OLE1 hypoxia-induced gene expression and is also sufficient for hypoxia-induced gene expression when placed upstream of a heterologous promoter. Further studies demonstrated that the same LORE sequence is involved in OLE1 repression by UFA in normoxic, hypoxic, and cobalt containing conditions. A family of genes containing a similar LORE in their promoter regions was identified by searching the S. cerevisiae genome using the 9-nucleotide DNA core binding sequence (ACTCAACAA), which was determined by performing EMSAs using a series of single nucleotide substitutions in the OLE1 LORE in vitro. Among them, ATF1, TRX2, SUT1, and TIR1 may be under similar LORE control for hypoxia-inducible gene expression.

Extensive studies over the past decade have defined the transcriptional repression mechanism for the regulation of anoxia-inducible genes (reviewed in Refs. 14 and 26) in S. cerevisiae. This mechanism is illustrated by the regulation of ANB1, a prototypic anaerobic-induced yeast gene. Both genetic and biochemical evidence have demonstrated that the nongenomic repression of this gene is mediated by the ROX1p repressor through its binding to the ANB1 operator site. The full repression by ROX1p requires two general transcription mediators, SSN6p and Tup1p (43–45). In an anaerobic environment, ROX1 expression is decreased, ROX1p levels decline, and eventually the repression of anoxia-regulated genes is released. Heme and Hap1 are involved in ROX1 expression in normoxic conditions (44–47). Based on the consensus binding sequence of ROX1p, putative ROX1p binding sequences in the promoter regions of many other genes in S. cerevisiae, including OLE1, have been identified. The OLE1 promoter region contains three putative ROX1p-binding sites at −130, −260, and −272 relative to the first nucleotide of the translation start codon (28, 30, 31). Previous studies suggested that OLE1 is only slightly derepressed in a ROX1 disruptant under aerobic conditions (31, 48). The results from the current study confirm that ROX1 does not play a significant role in the basal aerobic expression of the OLE1 gene. There were no significant changes in either reporter gene or OLE1 mRNA expression in a S. cerevisiae strain in which the ROX1 gene had been disrupted. Moreover, both hypoxia- and cobalt-induced OLE1 gene expression was observed in this strain; the expression of the ROX1-mediated anaerobic yeast gene ANB1 was not up-regulated by cobalt. Of note, the hypoxic induction in the ROX1 deletion strain was not as dramatic as its parental strain (4.2-fold versus 9.1-fold increase) in the reporter assay although the cobalt induction in the same strain was similar (4.2-fold versus 5.1-fold increase). This suggests that ROX1 may be involved in the OLE1 hypoxic induction pathway in some capacity; however, our data provide no compelling evidence that ROX1p serves as a repressor of OLE1 expression in normoxia.

Multiple pathways involved in regulating hypoxic and anoxic gene expression in yeast may exist (49). Studies of several other hypoxic/anaerobic genes including SUT1 (34), GDP2 (50), PAU (51), and DNA1 (52) have demonstrated ROX1p-independent hypoxic/anaerobic induction. Another hypoxic/anaerobic gene, TIR/SPR1, has variably been reported to be ROX1p-independent and ROX1p-dependent (35, 37). The identification of a LORE in this study indicates that transcription activation is crucial to the increased expression of certain yeast genes in response to extremely low oxygen tension (versus complete anaerobiosis). The identification of the putative transcriptional activator(s), i.e. the LBP, is under active investigation.

It is also demonstrated in this study that the LORE is involved not only in the hypoxic induction of OLE1 and ATF1, but also in cobalt (Fig. 5) induction. Mutations in the LORE dramatically decrease the reporter expression in both hypoxic and cobalt-treated conditions. However, some cobalt-induced reporter expression is still observed (~4.5-fold induction), suggesting that other as yet undefined elements may be involved. This result is consistent with observations utilizing the OLE1 promoter deletion-lacZ fusion assays (Fig. 2). In the shortest construct (p62::255) there is still a 2.4-fold induction for cobalt-
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treated yeast, despite no hypoxic induction.

The S. cerevisiae genome-wide search for LOREs in putative promoter regions identified many genes which are potentially regulated by low oxygen tension. One of these genes, the anaerobically-induced gene, TIR1, encodes a stress-induced cell wall mannoprotein, and is also a member of the serinepepe family (35, 37, 53). The in vivo function of a putative LORE in its promoter region (–344 to –325) is not known. Another gene, SUT1, encodes a small molecule transporter, and is up-regulated in anaerobic conditions to facilitate the uptake of sterols when sterol biosynthesis is blocked by the absence of oxygen (34). The in vivo significance of a putative LORE found in the SUT1 promoter region (–377 to –358 relative to the translational start codon), is also unclear at this point. Yet another gene identified in the genomic search for potential LOREs in promoter regions is the TRX2 gene, which encodes a small sulfydryl-rich protein thioredoxin. About a 3-fold hypoxic and 2.5-fold coalt induction of TRX2 mRNA was observed in Northern blot analysis (Fig. 8C). Confirming these data, in vitro EMSA demonstrated that the potential LORE obtained from the TRX2 promoter sequence could indeed form a hypoxia-specific complex. Nonetheless, the role of hypoxic induction of TRX2 in vivo still requires further investigation.

The precise physiological function of thioredoxin is not clearly understood; it appears to be part of a cellular nonenzymatic defense system in response to oxidative stress (reviewed in Ref. 54). Evidence for the involvement of reactive oxygen species in mammalian oxygen sensing and signaling exists (reviewed in Refs. 1 and 2). HIF-1, the mediator of physiological and pathophysiological responses to hypoxia, is subject to complex redox control mechanisms (Refs. 12 and 55, reviewed in Ref. 56). Therefore, it would not be surprising to identify reactive oxygen species involved in oxygen sensing and signaling in yeast.

Interestingly, transcription of the FTR1 gene, which functions as an iron permease mediating high affinity iron uptake in yeast, was reported to be induced by oxygen (38). We have confirmed this result, demonstrating that the FTR1 mRNA level was decreased under hypoxic conditions (data not shown). This suggests that the potential LORE in its promoter region is either nonfunctional or serves as a repression cis element under hypoxic conditions. It is likely that additional factors such as adjacent transcription factor-binding sites, spacing of the binding site, or a lower factor binding affinity in vivo may be critical in the regulation of genes possessing LOREs in their promoter regions.

OLE1 appears to be induced maximally by hypoxia as opposed to anoxia; a genome-wide transcriptional analysis of aerobic and anaerobic chemostat cultures of S. cerevisiae demonstrated that OLE1 has only a marginal (1.3-fold) increase under stringent anaerobic conditions (57). Thus, OLE1 represents a yeast gene that is regulated by hypoxia, certain transition metals (15), and iron chelation, strikingly similar to several hypoxia-inducible mammalian genes, such as erythropoetin, vascular endothelial growth factor, the glucose transporters GLUT1 and GLUT3, and several glycolytic enzymes. Given the importance of the ability to adapt to hypoxic stress throughout evolution, it is not surprising to find yeast and mammalian genes which are similarly regulated. Studies of several hypoxia-inducible mammalian genes have led to the identification of a hypoxia responsive element (58–61) and the heterodimeric hypoxia-inducible factor HIF-1 which binds to it (62). Although functionally the LORE and hypoxia responsive element are similar, sequence analysis does not reveal any similarity. Differences with respect to CoCl2-mediated induction of LORE-containing yeast genes provide additional support to the notion that additional factors most likely exist in S.

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