The ROX3 Gene Encodes an Essential Nuclear Protein Involved in CYC7 Gene Expression in Saccharomyces cerevisiae

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The ROX3 gene was identified during a hunt for mutants with increased expression of the heme-regulated CYC7 gene, which encodes the minor species of cytochrome c in the yeast Saccharomyces cerevisiae. The rox3 mutants caused a 10-fold increase in CYC7 expression both in the presence and absence of heme, had slightly increased anaerobic expression of the heme-activated CYCI gene, and caused decreases in the anaerobic expression of the heme-repressed ANB1 gene and the aerobic expression of its heme-induced homolog. The wild-type ROX3 gene was cloned, and the sequence indicated that it encodes a 220-amino-acid protein. This protein is essential; deletion of the coding sequence was lethal. The coding sequence for β-galactosidase was fused to the 3' end of the ROX3 coding sequence, and the fusion product was found to be localized in the nucleus, strongly suggesting that the wild-type protein carries out a nuclear function. Mutations in the rox3 gene showed an interesting pattern of intragenic complementation. A deletion of the 3' coding region complemented a nonsense mutation at codon 128 but could not prevent the lethality of the null mutation. These results suggest that the amino-terminal domain is required for an essential function, while the carboxy-terminal domain can be supplied in trans to achieve the wild-type expression of CYC7. Finally, RNA blots demonstrated that the ROX3 mRNA was expressed at higher levels anaerobically but was not subject to heme repression. The nuclear localization and the lack of viability of null mutants suggest that the ROX3 protein is a general regulatory factor.

The expression of many of the nuclear genes encoding respiratory functions in the yeast Saccharomyces cerevisiae is regulated in response to both the availability of oxygen and the presence of fermentable sugars. Positive oxygen regulation serves to minimize the expression of these genes, whose products play no role during anaerobic growth. Transcription of these genes is activated through the function of at least two different transcriptional activators, HAP1 and the HAP2/3/4 complex, both of which respond to heme (5, 7, 8, 25–27, 46). The biosynthesis of which is dependent on molecular oxygen as a reactant (20). Interestingly, there are oppositely regulated isoforms for some of the respiratory genes; one of each pair is heme activated, and the other is heme repressed. It is likely that these repressed isoforms represent hpyoxic forms, proteins that function more efficiently in limiting oxygen. Such isoforms have been found for subunit V of cytochrome oxidase (10, 42), cytochrome c (7, 13, 16, 39, 53), and for reasons that are not yet clear, the translation factor eIF-5A (15, 16, 22, 35). The HEM13 gene, which encodes an oxidase during heme biosynthesis, is also subject to heme repression, ensuring that enzyme levels rise as the levels of its substrate, molecular oxygen, fall (50, 51). Similarly, the ERG11 gene, encoding cytochrome P-450, is heme repressed (44). Heme repression in all these cases is mediated by the ROX1 repressor, whose transcription is heme activated (10, 17, 18). Thus, in aerobic cells, heme accumulates and activates the expression of respiratory functions as well as the repressor of hpyoxic functions. In cells grown in limiting oxygen, heme levels fall, causing decreased transcription of the heme-induced genes, including the repressor; as a consequence, the hpyoxic genes are transcribed.

Catabolite repression is superimposed on heme regulation for many genes, serving to limit the expression of respiratory proteins. Thus, even at high oxygen tensions, most of the carbon flows through fermentation when cells are provided with glucose, resulting in the accumulation of ethanol, which may serve to toxify the environment for other microorganisms. The mechanisms of catabolite repression are not well characterized, although some of the components have been identified. The expression of both cytochrome c protein genes and the COX6 gene, encoding a subunit of cytochrome oxidase, is affected by mutations in the SNF1 (COX6 and CYC7) (48), SSN6/CYC8 (CYC1, CYC7, and COX6) (32, 48), and TUP1/CYC9 (CYC1 and CYC7) (32, 52) genes, which also regulate the expression of the catabolite-repressed invertase and the galactose catabolic enzymes (36, 43, 47), suggesting a common link between catabolite repression among these systems. However, these regulatory genes seem to play a much more general role in regulating cellular functions. Mutations in SSN6 and TUP1 render MATa cells sterile (14, 23, 34), and mutations in TUP1 cause derepression of heme-repressed and heme-activated genes (52). Thus, these genes may be involved either in circuitry which processes the input from multiple signals and transmits them to the appropriate DNA-binding proteins or in mediating interactions between specific repressors and the general transcriptional machinery.

The CYC7 gene provides one vehicle for identifying genes involved in heme regulation, since there is a strong positive
Thus, mutations in the minor species of cytochrome c, iso-2 (4), and is trascripted at low levels both aerobically and anaerobically because it contains only a weak heme activation site for HAP1 and a heme repression site for ROX1 (18, 26, 53). Thus, mutations in the HAP1 gene that generate a protein with altered specificity and null mutations in ROX1 were, in hindsight predictably, isolated by causing CYC7 overexpression (18, 45). In addition, mutations in two of the above-mentioned genes, SSN6 and TUP1, were identified during hunts for mutants with increased expression of the CYC7 gene (3, 32, 52). In the case of tap1 mutations, overexpression appears to be in part due to the lack of activity of the ROX1 repressor (52). We have continued the search for genes involved in CYC7 regulation and report here the identification of a gene encoding an essential nuclear protein. The lethality of null mutations suggests that it also plays a wider regulatory role than a simple involvement in heme regulation or catabolite repression.

**MATERIALS AND METHODS**

**Strains, growth conditions, and transformations.** The rox mutants were isolated in aGH1 (MATa trp1-289 leu2-3,112 galIΔ152) and aLR1 (MATa trp1-289 his3-11,15, galIΔ152). aGH1 is congenic with aGH1. It was derived by generating a diploid from aGH1 by transformation with a plasmid carrying the HO gene, isolating segregants that lost the plasmid, and then sporulating the diploid cells. lys2 derivatives of aGH1 and aGH1 were derived by the method of Chattoo et al. (2).

TP3-17Z (MATa ura3-52 his4-519 cyc1::CYC7/lacZ) contained the CYC7/lacZ fusion from YCP7Z integrated into the CYCI locus, disrupting the CYCI gene. i182-248 (MATa trp1-289 ura3-52 his4-519 cyc1::CYC7/lacZ), i182-363 (MATa trp1-289 ura3-52 his4-519 cyc1::CYC7/lacZ rox3-182) and i182-504 (MATa trp1-289 ura3-52 leu2-3,112 cyc1::CYC7/lacZ rox3-182) were derived from the diploid generated from a cross of TP3-17Z by aGH1-182 (aGH1 containing the rox3-182 mutation). The diploid DRZ68 was derived from a mating of RZ53-6 (MATa trp1-289 leu2-3,112 ura3-112 ade1-100) and RZ49-4 (MATa trp1 leu2-3,112 ade1-100 galIΔ152). RZ53-6hem1 was derived from RZ53-6 by one-step gene replacement using a HindIII fragment containing the HEM1 gene with an insertion of the PST1 LEU2 fragment from YEp13 into the PST1 site within the coding domain.

Genetic manipulation of yeast cells (38) and transformations (11) were carried out as described previously. S. cerevisiae was grown in YPD (2% glucose) or YPR (2% raffinose) for the preparation of RNA and β-galactosidase assays as described previously (53). The general manipulations of Escherichia coli cells used for the recombinant DNA procedures have been previously described by Maniatis et al. (19).

**Plasmids.** The vectors YCP50 (30); YEp13 (1); YEpalac12, YEplac195, YCplac33, and YCplac22 (6); pBSM13+ (Stratagene) and YIp5 (41); and the lacZ fusion vector pMC1871 (37) have been described previously. The CYC7/lacZ fusion, YCP72, has been described previously (53). The HO-containing plasmid YEPHO was obtained from R. Jensen and I. Herskind. pACT1 contained the yeast actin gene (24).

The original ROX3 clone was obtained from a yeast genomic library in YCP50 (30) by complementation and was designated YCPROX3. A map of this clone is presented in Fig. 2; in restriction sites referred to below, numbers in parentheses are the positions of the sites (in kilobases) measured from the left border of the insert. The deletion derivatives shown in Fig. 2, ΔB, ΔC, and ΔP, were obtained by cleavage with BamHI, CiaI, and PvuII, respectively, and ligation at low dilution. The BamHI (5.2)-SpH1 (5.9) fragment was inserted into the homologous sites of YCP50 to generate YCpROX3′BSp. YEpROX3′H contained the 2.7-kb HindIII (3.6 to 6.3) fragment inserted into the HindIII site of YEpplac112. YEpROX3Sp contained the 2.2-kb SpH1 (3.7 to 5.9) fragment inserted into the SpH1 site of YEplac195. YCPROX3Pv contained the PvuII (3.9 to 4.4) fragment inserted into the SmoI site of YCplac33. YlpROX3 contained the BamHI (5.2)-SalI (vector site) fragment inserted into the homologous sites of a YIp5 derivative.

The following fragments from YCpROX3 were inserted into compatible sites in the polynucleotid of pBSM13+ and resulted in the designated plasmids: HindIII (3.6 to 6.3), pBSROX3H; BamHI (5.2)-DraI (6.0), pBSROX3BD; BamHI (5.2)-EcoRI (9.6), pBSROX3BR; and BamHI (5.2)-HindIII (3.6), pBSROX3BH.

The ROX3/lacZ fusion was constructed in pBSROX3H by introducing an XhoI site into the ROX3 gene 1 bp after the last codon by site-directed mutagenesis (12) using the oligonucleotide 5'-GGAGGCTGGAGTCTCGAGCTTTACGCCA and then inserting the lacZ-containing SalI fragment of pMC1871 into the new XhoI site. The in-frame fusion junction was confirmed by sequence analysis. The HindIII fragment containing the fusion was subcloned into the HindIII site of the yeast vectors YEp13 and YCplac22, generating the plasmids YEpR3Z and YCPR3Z, respectively.

The null allele of ROX3 was constructed by replacing the pBSROX3H BglII (5.1)-SpH1 (5.9) fragment, which contains the ROX3 coding sequence (see Fig. 4), with the 2.2-kb XhoI-SalI LEU2 fragment from YEp13. The null construct was excised with HindIII, generating a fragment with ends homologous to the ROX3 locus, and transferred into the yeast genome by one-step gene replacement (31).

**Cloning the ROX3 mutant alleles.** Recovery of the mutant alleles from the yeast genome was achieved by gap repair, using the plasmid YEpROX3H-X. This plasmid contained the ROX3 HindIII fragment in which the XhoI site had been placed at the last codon subcloned into YEplac112. Digestion with XhoI and BglII excised the ROX3 coding sequence, and this gapped plasmid was transformed into yeast cells, selecting for the TRP1 marker on the plasmid. Genomic DNA was prepared from transformants and used to transform E. coli cells to ampicillin resistance (41), thereby recovering those plasmids in which the gap had been repaired by gene conversion from the mutant ROX3 locus.

**Nucleic acid blots and enzyme assays.** RNA blots using total cellular yeast RNA were carried out as described previously (53). The amount of hybridization was quantitated with a Betascan to determine the amount of radioactivity in each band normalized to an actin control. For Southern blots, genomic yeast DNA was prepared (41) and blots were carried out (40) as described previously. RNA probes were made by using the Stratagene kit and the recommended conditions.

**β-Galactosidase assays.** The levels of 3-β-galactosidase were measured as described previously (29). All data presented represent the average activities for extracts prepared from several independent transformants.

**DNA sequence analysis and primer extension.** The sequences of both strands of the ROX3 gene were determined by the dyeoxy-chain termination method of Sanger et al. 

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RESULTS

Isolation of CYC7 overexpressing mutants. The CYC7 gene encodes the iso-2-cytochrome c protein, the minor cytochrome c species in aerobically growing yeast cells (4). This gene is expressed at low levels both aerobically and anaerobically because it contains regulatory elements for both heme activation via the HAP1 transcriptional activator and heme repression through the ROX1 repressor (18, 26, 46, 53). Mutations in either of these regulatory proteins or the factors that modulate their activities may cause increased CYC7 expression, and selections for CYC7 overexpressing mutants have been carried out previously (3, 32). However, with the exception of semidominant hap1 mutants (3, 45), rox1 mutants (18), and the ccy8/ssn6 (32, 36, 43) and ccc9/tup1 (32, 52) mutants, the genes defined in these studies have not been characterized. For our studies of the heme activation/repression regulatory circuitry, we repeated this selection with a modification. To avoid excluding respiratory deficient mutants that might result from mutations in these pathways, we used a CYC7/galK fusion vector (49) in a gal1 deletion strain and selected for growth on galactose rather than selecting directly for increased CYC7 expression. Seventy-seven mutants in two strains of opposite mating type were isolated, and genetic analysis revealed five major, unlinked groups. Two of these groups identified new alleles of the ROX1 (17) and TUP1 (52) genes; the others were designated ROX3, 5, and 6. Forty-five rox1 mutants were isolated, making this the most abundant group, while the 15 rox3 mutants represented the second most abundant group. For this reason, rox3 mutants were chosen for further study.

The effects of a typical rox3 mutation, rox3-182, on the accumulation of CYC7 mRNA is shown in Fig. 1. Increased RNA levels in preparations from cells grown on glucose (catabolite repressed) both in the presence (aerobically) and, even more dramatically, the absence (anaerobically) of heme were observed. Under catabolite-repressing conditions, the effect of the rox3 mutation was less severe. To determine whether this mutation affected the expression of the heme-activated CYC1 gene and the heme-repressed ANB1 gene, the same RNA blot was probed for these transcripts. As seen in Fig. 1, the rox3 mutation had little visible effect on the expression of the CYC1 gene but caused a threefold decrease in the anaerobic expression of ANB1. The mRNA for the heme-activated ANB1 homolog, transcript tr1, is also visible on this blot because of cross hybridization with the ANB1 probe, and it showed a twofold decrease of nec expression in the rox3 mutant. Thus, the rox3 mutation caused increased expression of CYC7 under all growth conditions

but caused a decrease in the activated expression of both ANB1 and tr1.

Isolation of the ROX3 gene. The wild-type allele of the ROX3 gene was isolated by complementation of the rox3-182 mutation. For this purpose, a strain was constructed containing the rox3-182 allele and the CYC7/ lacZ fusion integrated at the CYC1 locus. This strain gave dark blue colonies on 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal) plates, reflecting the increased CYC7 expression resulting from the rox3 mutation. Cells were transformed with a yeast library constructed in the URA3-containing centromeric vector YCP50, and the resulting Ura+ transformants were replated on X-gal plates. Pale blue colonies were picked, and four contained identical plasmids capable of transforming rox3-182 cells to the wild-type CYC7 phenotype.

A restriction map of the yeast insert in the complementing clone is presented in Fig. 2. Deletions within the insert and subcloning localized the complementing region to an 800-bp region within the BamHI and SphI sites (Fig. 2). To confirm that the complementing DNA contained the ROX3 gene, the URA3 gene was integrated into the genomic locus represented by the putative ROX3 clone, and this marker was then mapped with respect to the original rox3 mutation. For this approach, the plasmid YlpROX3, which contained the URA3 gene and a 5.2-kb BamHI-Sall fragment containing the ROX3 complementing region, was constructed. The plasmid was cleaved at the unique CiaI site within the cloned sequences which directed integration to the cloned locus in i182-248, a ROX3 wild-type strain containing a ura3 mutation and the CYC7/lacZ fusion integrated at the CYC1 locus. The proper integration event was confirmed by Southern analysis (data not shown). This strain was mated with i182-504, which contained the rox3-182 allele, a ura3 mutation, and the CYC7/lacZ fusion integrated at the CYC1 locus. Fifteen tetrads were dissected and tested for the segregation of the ROX3 phenotypes by using the colony color assay on X-gal plates. The validity of the color
assay was confirmed by carrying out β-galactosidase assays on all the haploid segregants from five tetrads (data not shown). All the tetrads showed the 2:2 segregation of rox3 ura3:ROX3 URA3 expected if the cloned sequences were allelic with ROX3. Thus, the findings that the cloned DNA on a low-copy-number plasmid complemented the rox3 mutation and that the clone was derived from a locus tightly linked to the ROX3 gene strongly indicate that the clone contains the wild-type ROX3 allele.

**ROX3 Transcript.** The complementation analysis indicated that the ROX3 gene was contained within the BamHI-SphI fragment (Fig. 2). A single transcript of 900 nucleotides hybridized to probes from this region (data not shown). This RNA was transcribed from left to right on the map in Fig. 2 as determined by hybridizing single-stranded RNA probes to yeast RNA; only the probe synthesized from the DraI (800 bp rightward from the BamHI site) to the BamHI site hybridized, as seen in Fig. 3.

**Sequence of the ROX3 gene.** The 1.4-kb sequence of DNA spanning the complementing region was determined and is presented in Fig. 4. An open reading frame beginning with a methionine codon and extending 220 codons in the correct orientation to be contained within the transcript visualized from this region was found, but the 5' end extended beyond the BamHI site (at bp 52), which comprised one border of the complementing region. If the first methionine codon 3' to this BamHI site were the true initiator, then a protein of only 121 residues would be synthesized. The presence of the open reading frame and the length of the transcript suggested that a 220-amino-acid protein was encoded by this region, while the complementation data using several different constructs containing the fragments extending from the BamHI site (Fig. 2) indicated that the ROX3 protein might be smaller. A number of experiments presented below were carried out to resolve this question. First, a null mutation was constructed to determine whether a complete deletion of the entire open reading frame would give the same complementation pattern as the uncharacterized rox3-182 mutation. Second, the 5' end of the transcript was mapped to determine whether it could encode the larger protein. The results of these experiments and further analyses of other rox3 mutants are presented below and clearly implicate the larger open reading frame as encoding the ROX3 protein.

This putative protein is highly charged and quite basic in the carboxyl-terminal region. It contains no significant homology with any protein or open reading frame in the GenBank library, as determined by scanning with the tfasta program in the Wisconsin package.

**ROX3 encodes an essential product.** To determine the phenotype of a cell completely lacking the ROX3 gene product, we attempted to construct a null allele by a one-step gene replacement (31). A plasmid was constructed in which the region from the BglII site at −58 to the SphI site at 839 (176 bp 3' to the end of the coding sequence) was replaced with the LEU2 gene. Several attempts to displace the genomic wild-type ROX3 gene with this construct in haploid cells failed, suggesting that the gene might be essential. To test this hypothesis, the null allele was transformed into a diploid cell homozygous for the leu2 mutation and for the wild-type ROX3 gene, and a ROX3::rox3::LEU2 heterozygote was isolated. Southern analysis confirmed the correct construct (data not shown). This diploid was sporulated, and the resulting tetrads were analyzed with respect to the segregation of the Leu phenotype. From a total of 29 tetrads, 21 contained only two viable spores, 7 contained one viable spore, and 1 contained three viable spores. In every case, the colonies derived from the viable spores were Leu+; no viable spores containing the rox3::LEU2 allele were observed (Table 1). The original diploid was also heterozygous for the URA3 and GAL1 markers, and these segregated as
Since the original rox3 mutations affected the expression of heme-regulated genes, we investigated the possibility that the failure of rox3 null alleles to germinate was due to a defect in heme biosynthesis, which would result in a requirement for sterol. We attempted to germinate spores on medium supplemented with Tween 80 and ergosterol. Each of the nine tetrads analyzed in this manner gave only two viable spores, both of which were Leu- . Thus, the inability to germinate spores with the null allele was not due to sterol auxotrophy.

To determine whether the rox3 null allele would prevent germination or would not support vegetative growth, the heterozygote containing the null allele was transformed with the plasmid YEpROX3H (containing the TRP1 selectable marker); spores containing this plasmid and the rox3::LEU2 allele germinated. By using a haploid strain derived in this manner, the stability of the plasmid was tested. As shown in Table 1, after a long period of growth, Trp− auxotrophs were isolated from the rox3::LEU2 wild-type transformant, but no such auxotrophs could be isolated from the rox3::LEU2 mutant, indicating that ROX3 is essential for vegetative growth on rich medium. This same system was used to test the ability of the incomplete ROX3 gene contained within the BamHI subclones described above to complement the null allele. Cells containing the rox3::LEU2 null allele and the YEpROX3H plasmid were transformed with a second URA3-CEN4 plasmid containing either the original ROX3 clone or the BamHI-SphI fragment capable of complementing the rox3::LEU2 allele (Fig. 2). Several independent transformants carrying both the URA3 and TRP1 plasmids were grown and plated on nonselective medium, and individual colonies were then tested for the presence of the plasmids. As seen in Table 2, segregants lacking the TRP1 plasmid (Trp−) were obtained.

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**TABLE 1. Plasmid loss in a rox3 null mutant**

<table>
<thead>
<tr>
<th>No. of colonies</th>
<th>Trp-</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>rox3::LEU2</strong></td>
<td></td>
</tr>
<tr>
<td>2b</td>
<td>0</td>
</tr>
<tr>
<td>2c</td>
<td>0</td>
</tr>
<tr>
<td>7c</td>
<td>0</td>
</tr>
<tr>
<td>9a</td>
<td>0</td>
</tr>
<tr>
<td><strong>ROX3</strong></td>
<td></td>
</tr>
<tr>
<td>5b</td>
<td>29</td>
</tr>
<tr>
<td>9b</td>
<td>41</td>
</tr>
</tbody>
</table>

* Plasmid loss was determined by growing lightly inoculated 25-ml overnight cultures on nonselective medium (YPD), plating the cells on YPD plates, and then testing individual colonies for the Trp phenotype.

**TABLE 2. Plasmid complementation of the rox3 null allele**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>No. of colonies</th>
<th>Trp-</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TRP1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>URA3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YEpROX3H</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>YEpROX3H</td>
<td>65</td>
<td>25</td>
</tr>
</tbody>
</table>

* Complementation was measured in the rox3::LEU2 mutant 9a (see Table 1). Cells carrying two plasmids were grown under nonselective conditions and then tested for the frequency with which cells lacking either or both plasmids segregated out, as described in footnote a of Table 1.

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FIG. 4. Sequence of the ROX3 locus. The sequence of the coding strand of the ROX3 locus is presented. The bases are numbered with the A in the translational initiation codon of the long open reading frame as 1; bases toward the 3′ end are designated as positive integers, and bases toward the 5′ end are designated as negative integers. The amino acid residues are numbered to the right of each line. The Bsp11 and Spal sites deleted in the null mutant are indicated, as is the BamHI site. The sites of the point mutations in the rox3-182 and 202 alleles are each indicated by an asterisk below the mutated base; in both cases, the C was changed to a T, generating a nonsense codon.

expected; approximately equal numbers of Ura+ and Ura− and Gal+ and Gal− spores were obtained. The original diploid from which the ROX3 heterozygote was obtained was sporulated, and six tetrads were analyzed; five of them gave four viable spores, and the sixth gave three viable spores.
at a high frequency in cells containing the YCpROX3 plasmid. Clones lacking YCpROX3 (Ura-) appeared less frequently, presumably because of the presence of CEN4. As expected, no clones lacking both plasmids were obtained. On the other hand, no Trp' clones were obtained from cells carrying YCpROX3BSp, indicating that this plasmid could not complement the inviability of the rox3 null allele. These results demonstrated that while the BamHI-SphI fragment was capable of complementing the rox3-182 allele, it could not complement the null allele and therefore must not contain the intact gene.

The 5' end of the ROX3 transcript. The 5' end of the ROX3 transcript was mapped by primer extension. To obtain sufficient material, poly(A) RNA was prepared from cells transformed with YEpROX3Sp, a ROX3-containing multi-copy plasmid. The transcript synthesized from this plasmid is identical in size to that in untransformed cells (data not shown). A primer complementary to a region 3' to the methionine codon downstream from the BamHI site was used to ensure that if this AUG codon were the initiator, the 5' end could be mapped. The result of this experiment is shown in Fig. 5 and clearly indicates that the ROX3 transcript began approximately 60 nucleotides upstream from the first AUG of the large open reading frame. (Because the purpose of this experiment was to determine whether the 5' end of the mRNA was upstream from the first AUG 3' to the BamHI site or 5' from it, the primer was made complementary to a region well within the coding sequence, and the resulting cDNA was too long to map to a single nucleotide.) This result, combined with the requirement for a fragment containing the entire open reading frame for complementation of the null allele, strongly suggest that this 220-codon open reading frame encodes the ROX3 gene.

Nature of the rox3 mutations. Because the ROX3 gene product was essential and the rox3-182 mutation was complemented by a fragment of the coding sequence, we cloned and sequenced two of the original rox3 mutations, hoping to obtain some insight into the functional domains of the gene. The rox3-182 allele contained a CG-to-TA transition at bp 385 which changed codon 129 from a glutamine codon to a nonsense codon (Fig. 4). A second allele, rox3-202, was found to have a CG-to-TA transition at bp 355 which also generated a nonsense codon; this allele encoded a protein containing only the first 117 amino acids of the putative ROX3 protein. Cells carrying these mutant alleles are clearly viable; therefore, the shortened forms of these proteins are probably synthesized and are at least partially functional. The possibility that the original strains harbored a nonsense suppressor is unlikely, since these two mutant alleles were isolated in different strains. Nonetheless, to formally rule this out, the rox3-202 mutant was mated with a strain carrying a number of amber suppressible markers, and no suppression was observed in the diploid. Finally, to rule out the possibility that a low level of translational readthrough of the nonsense mutations was responsible for the viability of these mutants, a plasmid, YCpROX3Spv, was constructed. This plasmid contains the ROX3 sequences from 432 to 357; thus, it lacks the coding sequence beyond codon 119. When transformed into the strain carrying the chromosomal null allele and a plasmid carrying the wild-type gene, isolates lacking the wild-type gene could be obtained, demonstrating that the amino-terminal half of the coding sequence is all that is required to support growth.

It should be noted that the rox3-182 mutation was complemented by the BamHI-SphI fragment containing only part of the ROX3 gene. Given that this mutant probably synthesized only the amino half of the protein and that the BamHI-SphI fragment could not support vegetative growth and was missing part of the amino terminus, these results indicate that intragenic complementation occurred.

ROX3 encodes a nuclear protein. To verify that the open reading frame encodes an authentic yeast protein and to determine the cellular localization of that protein, the lacZ coding sequence was fused in frame to the 3' end of the ROX3 coding sequence. β-Galactosidase activity in cell extracts was detected (Table 3), indicating that the open reading frame could be translated in yeast cells and, combined with the sequence data and the transcript mapping data, strongly implicating the 220-amino-acid protein as the product of the ROX3 gene.

Antibody against β-galactosidase was used for in situ immunofluorescence to determine the location of the fusion protein. 4',6-Diamidino-2-phenylindole (DAPI) counterstaining was used to visualize the nuclei, and as can be seen in Fig. 6, the immunofluorescence was localized to the same region as the DAPI stain for cells carrying the ROX3/lacZ fusion plasmid (Fig. 6a and c), while in cells carrying the vector alone, no specific immunofluorescence was observed.
The ROX3 region was cloned into the vector pBC-SK+ to generate the pBC-ROX3 plasmid. To test whether heme played a role in the oxygen repression of ROX3 expression, β-galactosidase assays were carried out with extracts of heml-deficient cells carrying the ROX3/lacZ fusion plasmid contained on a centromeric plasmid. The results presented in Table 3 clearly indicate that the low aerobic expression of the ROX3 gene is not a result of heme repression. These results have been confirmed by RNA blots (data not shown).

It is not clear what role this regulation plays in ROX3 function. Since a multicopy plasmid carrying the ROX3 gene was placed in wild-type cells, the RNA accumulated at high levels under both aerobic and anerobic conditions but there was no effect on the accumulation of the CYC7, ANB1, or trl transcripts (data not shown). Thus, if the ROX3 protein also accumulated at higher levels in the aerobically grown transformants, it did not alter aerobic expression of the ROX3 target genes.

**DISCUSSION**

We describe here the initial characterization of the ROX3 gene. Mutations in this gene were identified as causing increased expression of the CYC7 gene, and the wild-type allele was cloned by complementation. The protein-coding region was identified by a combination of complementation and transcript mapping, and we demonstrated that the open reading frame in this region was translated in yeast cells by the use of a lacZ fusion. This fusion protein was found to be localized in the nucleus, strongly implying that the native ROX3 protein functions there. The protein has no significant homology to any other protein in the GenBank data base.

While the results presented here do not precisely define the function of the ROX3 protein, some inferences can be drawn. ROX3 is localized in the nucleus, and viable mutations affect the expression of some genes. These findings suggest that the ROX3 protein is a transcription factor. Viable rox3 mutations affect the expression of different genes in opposite ways; CYC7 expression is increased, while that of the oppositely regulated eIF5A homologs are decreased. These results imply that ROX3 does not function in a simple way in heme regulation, nor does it function solely as a repressor or activator of transcription. This last conclusion is also suggested by an inspection of the ROX3 protein sequence, which contains none of the motifs associated with DNA-binding proteins or transcriptional activators. In addition, unpublished gel retardation studies using wild-type and rox3 mutant extracts revealed no size differences in the complexes formed on the CYC7 ROX1 or HAP1 binding sites, further suggesting that ROX3 is not a sequence-specific DNA-binding protein. These conclusions point to a role for ROX3 as a general transcription factor, perhaps involved in mediating responses of the general transcription machinery to a family of regulatory proteins. Experiments are in progress to explore this possibility.

One of the more intriguing results obtained in this study is...
the complementation data. Although the ROX3 gene product is essential, the two point mutations sequenced contained nonsense codons halfway through the coding sequence, indicating that a protein containing only the first 118 (rox3-202) or 128 (rox3-182) amino acids is capable of carrying out the essential function of ROX3. Also, a deletion construct lacking the coding sequences 3′ from codon 119 supported growth of a null mutant, thus eliminating the possibility that very low levels of translational readthrough were responsible for the viability of the nonsense mutations. At the same time, a variety of BamHI subclones which do not contain the 5′ end of the ROX3 coding sequence (Fig. 4) complemented the rox3-182 mutant phenotype but did not alter the inviability of a rox3 deletion. The nature of the protein synthesized from this fragment is not known; the first in-frame methionine codon 3′ to the BamHI site is at codon 100, but we cannot rule out the possibility that vector initiation sites were used or that a low level of initiation at a non-AUG codon occurred. Nonetheless, these results clearly indicate that intragenic complementation occurred and that either the ROX3 protein has distinct domains that can function independently or a functional multimeric unit can be assembled from the truncated peptides. These possibilities are under investigation.

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