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ROX1 Encodes a Heme-Induced Repression Factor Regulating *ANB1* and *CYC7* of *Saccharomyces cerevisiae*

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The *ROX1* gene encodes a product implicated in the regulation of heme-repressed and heme-induced genes in *Saccharomyces cerevisiae*. The gene has been cloned and shown to code for a 1.4-kilobase transcript. The cloned gene was used to construct a null mutant to determine the role of *ROX1* in regulating the expression of several heme-regulated genes. Constitutive expression of *ANB1* (a heme-repressed gene) was observed in the null strain, indicating that *ROX1* codes for a repressor or a facilitator of repression. Enhancement of expression of *CYC7* in the null strain indicated that the *ROX1* factor is required for repression of *CYC7* to its normal low level of expression, consistent with evidence that *CYC7* has a hybrid heme-induced, heme-repressed regulatory mechanism. The null mutation had only a slight negative effect on expression of the heme-induced genes *CYC1* and *tr-1* (a heme-induced homolog of *ANB1*), suggesting that the *ROX1* factor is not directly involved in their regulation despite the existence of an unusual *rox1* mutation (*rox1-a1*) causing constitutive expression of this group. The respiratory competence of the null mutant indicates that *ROX1* is not a respiratory factor. *ROX1* expression was found to be induced by heme, indicating that the heme repression of *ANB1* and its family is the result of a cascade in which heme induces a repression factor which keeps the family of heme-repressed genes inactive during aerobic growth. The *rox1-a1* allele had earlier been shown to cause constitutive expression of the family of heme-induced respiratory genes. This allele was found to cause constitutive expression of the *ROX1* transcript itself, indicating that *ROX1* is in the major heme-induced regulon.

The transcription of a number of gene families in the yeast *Saccharomyces cerevisiae* is regulated in response to physiological conditions. One important response of this type is the facultative adaptation to variation in oxygen supply, during which the expression of a large family of nuclear genes encoding mitochondrial functions is coordinately induced by oxygen. In those cases studied, transcription of these genes is actually induced by heme (9, 24, 26, 28), the biosynthesis of which requires molecular oxygen as a substrate (19). Interestingly, there is also a class of genes which is repressed in the presence of oxygen. Studies over the past several years have revealed that a subset of these anaerobic genes is negatively regulated by heme and that this coordinate repression is mediated by the product of the *ROX1* gene (15, 17, 34; M. Cumsky, personal communication).

We assume that the products of the heme-repressed genes serve some purpose related to respiration under low oxygen tension or to the transition from the respiring to the nonrespiring state, since three of the four heme-repressed genes so far identified encode anaerobically expressed counterparts of heme-induced proteins. *COX5b* is a homolog of *COX5a*, which encodes subunit V of cytochrome oxidase (Cumsky, personal communication); the *ANB1* gene encodes a product of unknown function and has a heme-induced homolog (defined by the *tr-1* transcript [16]); and *CYC7* encodes the minor species of cytochrome *c*, homologous to the major species, the product of *CYC1*. The *CYC7* gene, unlike *ANB1* and *COX5b*, is expressed at low levels in the presence of heme, apparently because of a weak binding of the *CYP1* (*HAP1*) transcriptional activation protein to an upstream activation sequence (UAS_0) (2, 30). However, *CYC7* also contains a heme repression site (37; G. Hastings and R. S. Zitomer, manuscript in preparation) and appears to be a hybrid of the heme-repressed and heme-induced families. The heme activation and repression sites of *CYC7* act antagonistically, leading to low, near-constitutive expression (18, 31, 37). *HEM13*, encoding an oxidase involved in heme biosynthesis, is the fourth gene in this class (34), although its response to *ROX1* has not been tested. A number of other anaerobic genes which are not regulated by heme or by *ROX1* have been cloned in our laboratory (C. V. Lowry and R. S. Zitomer, manuscript in preparation), and gene products from this group may be involved in anaerobic functions unrelated to respiration.

The *ROX1* gene was identified by a selection for constitutive mutations causing aerobic expression of *ANB1* (17). However, apart from its role in regulating the heme-repressed genes, a possible role for *ROX1* in the regulation of heme-induced genes was suggested by the isolation of an unusual mutation, designated *rox1-a1*, which, in addition to causing constitutive expression of *ANB1*, also caused constitutive expression of heme-induced genes. This pleiotropic phenotype suggested a coordination between the mechanisms controlling the heme-repressed and heme-induced families. This has since been confirmed by the isolation of mutations in an additional four *ROX* genes involved in this coordination (L. Rosenblum-Vos and R. S. Zitomer, manuscript in preparation).

The *ROX1* factor is part of a complex system of adaptation to fluctuations in oxygen tension. To understand its mode of action and how it mediates heme regulation, we cloned the *ROX1* gene. We show here that its product is required for repression of the heme-repressed genes. In attempting to account for the inhibitory effect of heme and its relation to *ROX1*, we show that *ROX1* is itself induced by heme. From this we infer that the heme-repressed genes are controlled as part of a cascade: heme induces expression of the *ROX1* factor, which in turn represses the heme-repressed genes. *ROX1* appears to be expressed as part of the large regulon of

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heme-induced genes, judging from the fact that, like them, it is constitutively expressed in the *rox1-a1* mutant.

MATERIALS AND METHODS

Strains and genetic manipulations. Strains GM3C-2, GM3C-2 *rox1-b3*, and GM3C-2 *rox1-a1* have been described elsewhere (17). In some cases the mating type of GM3C-2 *rox1-b3* was switched from α to **a** by transformation with the plasmid YEpHO (12), followed by recovery of plasmid-free *MAT***a** isolates. Strain AH12, derived from GM3C-2 by integration of a *cyc7*::*LEU2* gene disruption at the *cyc7* locus, has been described previously (11). It also carries plasmid YCp*CYC1*(2.4) (16). AH12-7 was derived from strain AH12 by integration of the wild-type *CYC7* gene at the disrupted *cyc7* locus (A. Healy and R. Zitomer, manuscript in preparation). Strain Y4-121 was derived from GM3C-2 *rox1-b3* as described below. Strain LR-190 (*MAT*α *ROX1 ura3*::*ANB1-lacZ* [*URA3* disrupted with an *ANB1-lacZ* fusion insert] *leu2-3 leu2-112 trp1 gal*) and strain aLR-93 (*MAT***a** *rox1-b3 ura3*::*ANB1-lacZ leu2-3 leu2-112 trp1 gal*) were derived as haploid segregants from a cross between Y4-121 and LR-1 (Rosenblum-Vos and Zitomer, in preparation).

Tn*5* mutagenesis. Transposon mutagenesis was carried out in *Escherichia coli* HB101 Tn*5*, which was derived from a P1 phage-mediated transposition event by using RPC104 (4) (carrying Tn*5*) as the donor and HB101 as the recipient and selecting for kanamycin resistance. (This event is not a transduction, given that the host is RecA$^-$, but is presumed to result from transposition of Tn*5* into the host chromosome during an abortive transduction.) For the mutagenesis, HB101 Tn*5* was transformed with the Amp^r plasmid to be mutagenized followed by growth of a culture from a single transformant and isolation of plasmid DNA. HB101 cells were transformed with the mutagenized (frequency of ca. 10^{-4}) plasmid DNA (ca. 5 μg), and Kan^r Amp^r transformants were selected. About 150 kanamycin-resistant colonies carrying plasmids with Tn*5* inserts were pooled, and plasmid DNA was prepared and transformed into yeast cells to screen for mutant plasmids.

Plasmids and transformation. Plasmid YCpAZ6, containing an *ANB1-lacZ* fusion, was constructed by inserting the 3-kilobase (kb) *Bam*HI fragment from plasmid pMC1871 (27), which carries the major 3′ portion of the *lacZ* gene, into the coding region of the *ANB1* gene in plasmid YCp*CYC1*(2.4)B$_x$. YCp*CYC1*(2.4)B$_x$ was derived from plasmid YCp*CYC1*(2.4) by filling in the *Bam*HI site 3′ to the *ANB1* gene with Klenow fragment. The point of fusion was less than 21 codons from the translational initiation codon of *ANB1*, as indicated by the absence of the *Sal*I site near the 5′ end of *ANB1*.

Plasmid YIpAZ4 was constructed by insertion of the *Sma*I fragment of YCpAZ6 into the filled-in *Nco*I site of the *URA3* gene on plasmid YIp5 (1). The inserted fragment includes extensive 3′ (800 base pairs) and 5′ (800 base pairs) *ANB1* flanking sequences.

YCp1Z carries a fusion of the *CYC1* gene to *lacZ* (37). YCp7Z carries a fusion of the *CYC7* gene to *lacZ* (37). YCp*ROX1* was obtained by complementation of the *rox1-b3* mutation as described below. pBS-*ROX1* was constructed by inserting the 2.8-kb *Xba*I fragment containing the bulk of the *ROX1* gene into the *Xba*I site of plasmid pBSM13(+) (6). YCp*rox1*-Tn*5-6* was obtained as a mutant of plasmid YCp*ROX1* as described below.

pUC*rox1*-Tn*5-6*::*LEU2* was constructed as follows. A 13-kb fragment carrying the Tn-*5*-disrupted *ROX1* gene from YCp*rox1*-Tn*5-6* was subcloned into pUC9-Sal$_x$, a derivative of pUC9 (33) in which the *Sal*I site was destroyed. The resulting plasmid (pUC*rox1*-Tn*5-6*) was cleaved at the unique *Sal*I site in the Tn*5* segment and ligated to the 2.2-kb *Xho*I-*Sal*I fragment containing the *LEU2* gene from YEp13 (1). The resulting plasmid contained a disrupted *ROX1* gene surrounded by 3′- and 5′-flanking regions which contain *Xba*I sites, permitting excision of a fragment which could be integrated by transplacement at the *ROX1* locus.

pΔ*rox1*/*LEU2* was constructed from segments derived from plasmid pUC*rox1*-Tn*5-6*::*LEU2*. This plasmid was cleaved with *Bam*HI, and the larger of two fragments (11 kb), containing the vector portion, half of the Tn*5* element, the *LEU2* insert, and the 3′ half of the *ROX1* gene (Fig. 1), was filled in with Klenow fragment and ligated to a filled-in 2-kb *Hin*dIII fragment derived from the same plasmid containing the region starting about 0.7 kb 5′ to the *ROX1* gene (at the rightmost *Hin*dIII site in Fig. 1) and ending in the polylinker of pUC9. The resulting plasmid now carried a deletion which included approximately half of the *ROX1* gene and about 0.7 kb of 5′-flanking DNA. The region of the deletion (Fig. 1B) was flanked by an *Xba*I site (3′ to *ROX1*) and a *Bam*HI site (5′ to *ROX1*), which permitted excision of a fragment that could be transplaced at the *ROX1* locus.

Transformation of yeast cells was by the method of Klebe et al. (14). Transformation of *E. coli* was by the method of Hanahan and Meselson (10).

Integrative transformations. For integration of the *ANB1-lacZ* fusion at the *URA3* locus, YIpAZ4 was digested with *Sma*I and *Pst*I (sites within the *URA3* gene which flank the fusion) and cotransformed with plasmid YEp13 (21) into strain GM3C-2 *rox1-b3*, selecting for leucine prototrophy. Transformants were screened for uracil auxotrophy and formation of blue colonies on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) plates (indicating expression of the fusion under the rox1$^-$ phenotype). About 0.5% of the LEU$^+$ colonies had the expected cotransformed phenotype. Leu$^-$ (YEp13-free) isolates of the resulting strain, Y4-121, were used as the *ROX1* cloning strain as described above.

Strain LR-190/int-*rox1*/Tn*5-LEU2* was constructed by transformation of strain LR-190 with an *Xba*I digest of plasmid pUC*rox1*-Tn*5-6*::LEU2. Integration at the *ROX1* locus was confirmed by Southern analysis. Strain AH12-7 Δ*rox1* was obtained by transformation of AH12-7(YCpAZ6) cells with a *Bam*HI-*Xba*I digest of pΔ*ROX1*/*LEU2*. Leu$^+$ transformants were selected and screened for the formation of blue colonies on X-Gal plates. The desired transplacement was confirmed for five blue colonies by Southern analysis.

Analysis of gene expression phenotypes. Anaerobic and aerobic growth conditions for RNA analysis were as described previously (15). RNA was extracted and analyzed as described elsewhere (17). Blots were routinely reprobed with the actin gene (21) to ensure that consistent quantities of RNA were loaded on gels and to check the integrity of mRNA. Probes for Northern (RNA) blots were generated by the random-primer method (5). Single-stranded probes used to determine the orientation of transcription of the *ROX1* gene were generated by using T3 and T7 polymerases for the synthesis of labeled runoff transcripts from templates generated by digestion of pBS-*ROX1* with appropriate restriction enzymes (6). β-Galactosidase assays were performed on aerobic cultures grown in CM medium (35) or on anaerobic cultures grown overnight in CM medium supplemented with Tween 80 (0.5%) and ergosterol (10 μg/ml); 10-ml culture samples were shaken at 30°C in small flasks inside brewer jars made anaerobic with GasPaks (BBL Microbiology Sys-

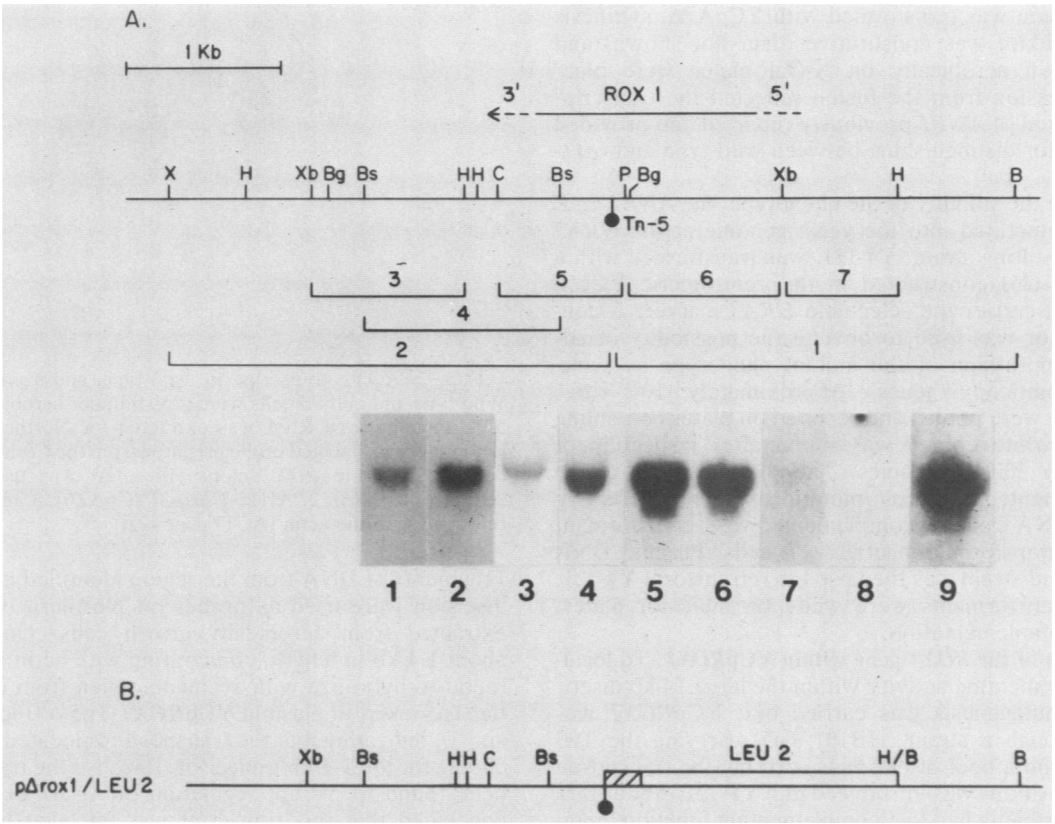

FIG. 1. (A) Map of the *ROX1* gene and transcript. The approximate location of the transcription unit was determined by probing Northern blots (lanes 1 through 7) with probes prepared by random-primer labeling from the fragments indicated by the numbered brackets below the map. The orientation of transcription was determined by probing with single-stranded RNA probes prepared by runoff transcription from plasmid pBS-*ROX1* in both orientations, sense (lane 8) and antisense (lane 9). Restriction sites are abbreviated as follows: B, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*EI; C, *Cla*I; H, *Hin*dIII; P, *Pst*I; X, *Xho*I; Xb, *Xba*I. The position of the Tn*5* element in YCp*rox1*-Tn*5-6* is also shown ( ). (B) Map of the *rox1* deletion mutation in plasmid pΔ*rox1*/*LEU2*. The 5′ end of the *ROX1* gene and approximately 1 kb of 5′-flanking DNA were deleted and replaced with a segment of DNA containing the *LEU2* gene ( ) joined to 2.5 kb of Tn*5* ( ) (not to scale). A very short segment (*Sal*I to *Bam*HI) of Tn*5* DNA is also present on the righthand side of the *LEU2* gene.

tems, Cockeysville, Md.). β-Galactosidase assays were performed by using *p*-nitrophenylgalactoside as a substrate, and data were normalized to values which would be obtained by using *o*-nitrophenylgalactoside with an empirical correction factor. X-Gal plates were prepared as described elsewhere (7). For tetrad analyses of expression phenotypes using X-Gal indicator plates, control crosses were done to be certain that mutant and wild-type phenotypes could be distinguished against the variable genetic background of the haploid segregants. In addition, β-galactosidase assays were used to check occasional ambiguous plate phenotypes. There was less variation among segregants in the liquid assay.

Materials. Restriction and DNA-modifying enzymes were obtained from Boehringer-Mannheim Biochemicals (Indianapolis, Ind.) and used according to the instructions of the manufacturer.

RESULTS

The *ROX1* gene product mediates the heme regulation of the *ANB1* gene, as indicated by the isolation of constitutive mutants insensitive to heme. In addition, certain relatively unusual *rox1* alleles also cause constitutive expression of heme-activated genes (15, 17). To investigate the nature of the *ROX1* gene product, our first step was to obtain a clone of the gene. Since there is no known phenotype associated with the expression of the *ANB1* gene, we constructed an *ANB1-lacZ* fusion in order to detect complementation of a *rox1* mutation by a cloning plasmid carrying the wild-type gene. The 5′ end of the *ANB1* gene was fused in frame to the *lacZ* gene; when this construction was introduced into a wild-type strain (GM3C-2) on a centromeric plasmid (YCpAZ6), anaerobically grown transformants were found to accumulate about 100 times as much β-galactosidase as did aerobically grown cells (Table 1), and, as expected, white colonies were formed during aerobic growth on X-Gal plates. The congenic strain (GM3C-2 *rox1-b3*) carries the *rox1-b3* allele, which causes constitutive *ANB1* expression.

TABLE 1. Expression of β-galactosidase from the *ANB1-lacZ*, *CYC1-lacZ*, and *CYC7-lacZ* fusions in the *rox1* null mutant

Strain	Fusion	β-Galactosidase (U)	
		Aerobic	Anaerobic
AN12-7(YCp1Z)	*CYC1-lacZ*	31.2	1.4
AH12-7 Δ*rox1*(YCp1Z)	*CYC1-lacZ*	23.0	0.9
AH12-7(YCp7Z)	*CYC7-lacZ*	1.8	0.3
AH12-7 Δ*rox1*(YCp7Z)	*CYC7-lacZ*	3.6	0.3
AH12-7(YCpAZ6)	*ANB1-lacZ*	0.4	35.6
AH12-7 Δ*rox1*(YCpAZ6)	*ANB1-lacZ*	30.2	39.4

When this strain was transformed with YCpAZ6, synthesis of β-galactosidase was constitutive (data not shown) and colonies grown aerobically on X-Gal plates were blue. Hence, expression from the fusion reflected the transcriptional regulation of *ANB1* previously reported and provided a phenotype for distinguishing between wild-type and *rox1-b3* cells.

To enhance the stability of the phenotype, the *ANB1-lacZ* fusion was integrated into the yeast genome at the *URA3* locus. The resulting strain, Y4-121, was transformed with a yeast library (25) constructed in the centromeric YCp50 vector, which carries the selectable *URA3* marker. A centromeric vector was used to obviate the possibility of adventitious suppression of the mutant phenotype by overexpressed multicopy genes. Approximately 10^5 Ura^+ transformants were pooled and replated on plates containing X-Gal. One white colony was found after inspection of approximately 30,000 colonies. The plasmid (YCp*ROX1*) that complemented the *rox1* mutation was recovered by isolation of DNA from the complemented yeast transformant followed by transformation of *E. coli* cells. Plasmid DNA from the *E. coli* strain was then used to retransform Y4-121. All of the transformants were white on indicator plates, confirming complementation.

Localization of the *ROX1* gene within YCp*ROX1*. To localize the complementing activity within the large 14-kb insert, transposon mutagenesis was carried out. YCp*ROX1* was passaged through a strain (HB101 Tn*5*) carrying the Tn*5* transposon, and a pool of plasmids carrying the transposon at different positions was introduced into Y4-121. About 15% of the transformants had lost complementing function (compared with none for the nonmutagenized plasmid), indicating Tn*5* insertion within the putative *ROX1* gene. One such plasmid (p*ROX*-Tn*5-6*) was recovered in bacterial cells, and the position of the transposon (13) (and hence that of the complementing gene) was determined by restriction analysis (Fig. 1).

Genetic confirmation of complementation. To confirm genetically that the clone obtained in our search contained the *ROX1* gene, the cloned segment was used to direct a disrupting selectable marker into the putative *ROX1* chromosomal locus. This construct would then permit a demonstration of linkage between the locus of the cloned segment and that of the *rox1-b3* mutation. For this purpose, the *LEU2* gene was inserted into the Tn*5* element contained in plasmid YCp*ROX1*-Tn*5-6*, in which the *rox1*-complementing gene had been found to be functionally disrupted (Fig. 1). Integration of this construction at the locus of the clone was then selected for by transformation of the *leu2 ROX1*$^+$ strain LR-190 to LEU^+, followed by Southern blot analysis to confirm replacement of the wild-type segment by the disrupted one (data not shown). The phenotype of transformants carrying the disrupted segment was found to be $rox1^-$, i.e., constitutive for *ANB1-lacZ* expression as determined by enzyme assay and plate phenotype; this permitted us to follow the disrupted allele through segregation analysis. The disrupted strain (LR-190/int-*rox1*/Tn*5-LEU2*) was mated with Y4-121, and the diploid was subjected to tetrad analysis, with each haploid segregant assayed for *ANB1-lacZ* expression. All of 10 tetrads analyzed in this way segregated 4:0 for the $rox1^-$ phenotype, indicating linkage between the locus of the disruption and the *rox1-b3* mutation carried in Y4-121.

Location and orientation of the *ROX1* transcript. More precise localization of the *ROX1* gene was accomplished by mapping its transcript within the cloned segment (Fig. 1).

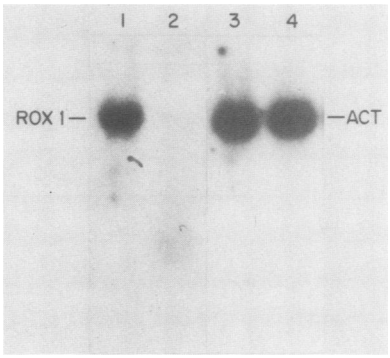

FIG. 2. *ROX1* transcript in wild-type and *rox1* null strains. Wild-type and mutant cells were grown under aerobic conditions in YPD medium, and RNA was extracted for Northern analysis. A random-primer-labeled probe prepared from the *Cla*I-*Bgl*II fragment derived from the *ROX1* clone (Fig. 1A) was used. Lanes: 1, AH12-7(YCpAZ6); 2, AH12-7 Δ*rox1*(YCpAZ6); 3 and 4, same blot reprobed with the actin (ACT) gene (21).

Fragments of DNA from the region identified by transposon insertion were used as probes on Northern blots of RNA extracted from aerobically grown cells. One transcript, about 1.4 kb in length (comigrating with actin mRNA), was found to hybridize with segments taken from either side of the Tn*5* insert in plasmid YCp*ROX1*-Tn*5-6* (Fig. 1A, lanes 1 and 2), indicating that the transposon is located in the region coding for the 1.4-kb transcript. Because the transposon had been found to disrupt regulation of *ANB1* expression, we concluded that this transcript was encoded by the *ROX1* gene. Weak or negligible hybridization by fragments located near the 5′ or 3′ end, compared with strong hybridization by fragments within the transcribed region, roughly defined the boundaries of the transcript (Fig. 1A, lanes 3 through 7). The orientation of transcription was determined by use of single-stranded RNA probes (Fig. 1A, lanes 8 and 9).

***ROX1* protein required for repression of *ANB1*.** The observed *ANB1*-constitutive phenotype of the Tn*5-LEU2* disruption of *ROX1* indicated that the *ROX1* gene product is either a repressor of *ANB1* or required for its repression. However, it was conceivable that the *ROX1* factor was an activator and that the constitutive phenotype arose from the production of a protein truncated at the carboxy-terminal end (as presumably occurs in the Tn*5* disruption). To rule this out, it was desirable to determine the phenotype of a null allele created by deletion of the amino-terminal portion coded on the 5′ side of the Tn*5* insert. The deletion was constructed with the *LEU2* gene inserted to permit selection of transformants carrying the disrupted segment at the *ROX1* locus (Fig. 1B). The gene transplacement was carried out in strain AH12-7, generating strain AH12-7 Δ*rox1*. Evidence that this strain carried a null allele was obtained by Northern blot analysis (Fig. 2). The 1.4-kb *ROX1* transcript was missing in the null mutant and replaced by a very faint diffuse smear of about 0.7 kb, presumably corresponding to low-level transcription of the 3′ segment initiated within the adjacent Tn*5* element (Fig. 2, lane 2). Since it is unlikely that this species would be translated efficiently and since the 5′ end is eliminated, we presume that the *ROX1* protein is absent in this strain. Further support for this assumption came from the observation that a deletion of the region bounded by the *Xba*I sites, which includes all but at most a small portion of the 5′ end, conferred the same phenotype (data not shown) with respect to *ANB1* expression as did the Tn*5* insertion and the deletion shown in Fig. 1B.

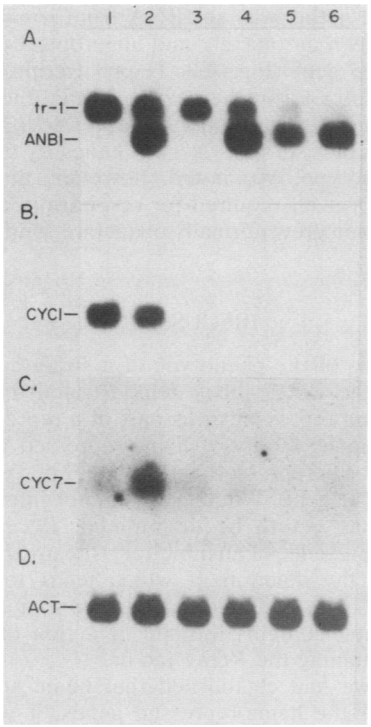

FIG. 3. Expression of the *ANB1*, *CYC1*, *tr-1*, and *CYC7* genes in wild-type and *rox1* null strains. Cells were grown aerobically to a density of 10^7/ml. Portions were then shifted to anaerobic growth conditions for 30 or 90 min or continued in aerobic growth for 30 min before being harvested for RNA extraction and Northern analysis. Lanes: 1, 3, and 5, strain AH12-7(YCpAZ6); 2, 4, and 6, strain AH12-7 Δ*rox1*(YCpAZ6). Lanes 1 and 2, Aerobic growth; lanes 3 and 4, 30 min of anaerobic growth; lanes 5 and 6, 90 min of anaerobic growth. (A) Probe for *ANB1* and *tr-1*: random-primer-labeled *Xho*I-*Bam*HI fragment carrying the *ANB1* gene derived from YCp*CYC1*(2.4). (B) Probe for *CYC1*: random-primer-labeled *Eco*RI-*Hin*dIII fragment carrying part of the *CYC1* gene derived from YCp*CYC1*(2.4). (C) Probe for *CYC7*: *Kpn*I-*Xho*I fragment carrying a portion of the *CYC7* gene derived from YCp*CYC7* (31). (D) Blot shown in panel C, reprobed with the actin (ACT) gene.

Expression of *ANB1* was tested in the various *rox1* null strains and was found to be constitutive: the *ANB1* transcript, which was missing in aerobic wild-type cells (Fig. 3, lane 1), was present in the null mutant (lane 2) at levels even slightly greater than in anaerobic wild-type cells (lane 5). Possibly there was a low level of *ROX1* protein present in the wild-type cells after the 90-min period of anaerobic growth which caused a slight repression compared with the null mutant. Assay of β-galactosidase confirmed the constitutive expression of *ANB1* in the null mutants (Table 1). The fact that elimination of the *ROX1* gene product prevented the repression of *ANB1* normally observed during aerobic growth indicates that the *ROX1* factor plays an obligatory role in repression.

Role of *ROX1* in the expression of heme-induced genes. Most of the nucleus-coded mitochondrial genes studied so far show induction strongly dependent on the presence of heme. We refer to these as heme-induced genes. Induction of these genes is mediated by the heme-responsive *CYP1* (*HAP1*) protein (3, 30). The *ROX1* gene was earlier tentatively implicated in their regulation by observations of the phenotype of the *rox1-a1* allele, which causes semiconstitutive expression under anaerobic conditions. To determine if the *ROX1* gene product plays a direct role in the regulation of the heme-induced genes, we examined expression of *CYC1* and *tr-1*. Analysis of RNA extracted from mutant cells grown in the presence or absence of oxygen revealed that expression of *CYC1* was almost normal; *CYC1* mRNA was almost undetectable in both the wild-type and *rox1* null strains after 30 min of anaerobic growth (Fig. 3B, lanes 3 and 4) and was present at nearly equal levels during aerobic growth, with a slight decrease in the null strain (lanes 1 and 2). Assays of β-galactosidase levels in cells carrying a *CYC1-lacZ* fusion plasmid affirmed the RNA blot data (Table 1). A similar slight effect was seen in the expression of the *tr-1* gene (Fig. 3A, lanes 1 and 2). (Higher levels of *tr-1* mRNA under anaerobic conditions suggest that it is more stable than *CYC1* mRNA, which turns over rapidly [36].) We have difficulty in interpreting the minimal effect of the *rox1* null mutation on expression of these two heme-induced genes. Clearly the *ROX1* gene product is not required for their regulation, despite the semiconstitutive phenotypes caused by the *rox1-a1* mutation (17) and by other *rox1* alleles (Rosenblum-Vos and Zitomer, in preparation). It is paradoxical that the *ROX1* factor is susceptible to recessive mutations which disturb the regulation of the heme-induced genes while actual elimination of the factor produces a much less noticeable effect (see Discussion).

Regulation of *CYC7* by *ROX1*. We had earlier observed effects of *rox1* mutations on expression of *CYC7*, both in enhanced mRNA levels (C. V. Lowry, L. Rosenblum-Vos, and R. S. Zitomer, unpublished observations) and in an altered distribution of transcriptional start sites (Healy, Lowry, and Zitomer, manuscript in preparation). Hence, it was of interest to determine the effect of the absence of the *ROX1* factor on expression of this gene. The null mutation substantially enhanced expression; higher levels of *CYC7* mRNA were observed in the null strain during aerobic growth (Fig. 3C, lanes 1 and 2) than in the wild type. The mutation had no apparent effect on the low levels observed in anaerobic cells. β-Galactosidase assays of cells carrying a *CYC7-lacZ* fusion indicated the same pattern, although the enhancement was less pronounced (Table 1). The effect of the null mutation implies that the *ROX1* gene product is required for repression of *CYC7* expression to its normal low level, which earlier work had shown was the net result of repression and activation mechanisms operating at different sites in the upstream region (31, 37). One of these sites has been identified as a heme repression site (Hastings and Zitomer, in preparation), and our present finding suggests that the *ROX1* factor is involved in the heme repression mechanism, presumably acting in the same fashion as it does in the regulation of *ANB1* and *COX5b*.

***ROX1* regulated by oxygen via heme.** In earlier work we had found that aerobic inhibition of *ANB1* expression is due to negative regulation by heme (15), which is synthesized only in the presence of oxygen (19). To account for the effect of heme, there were two alternative explanations: either heme interacts as a negative coeffector with the *ROX1* factor to inhibit expression, or heme acts indirectly, stimulating the production of a repressor. In the latter case, *ROX1* transcription might be expected to be induced during aerobic growth. The *ROX1* transcript was present in aerobic cells (Fig. 4A, lane 1) but was almost completely eliminated after 90 min of anaerobic growth (lane 3), showing that *ROX1* was induced in the presence of oxygen. To demonstrate that heme is the signal for the aerobic induction of *ROX1*, we showed that the loss of *ROX1* expression in anaerobic cells could be reversed by the addition of heme (Fig. 4B, lanes 1 and 2). These

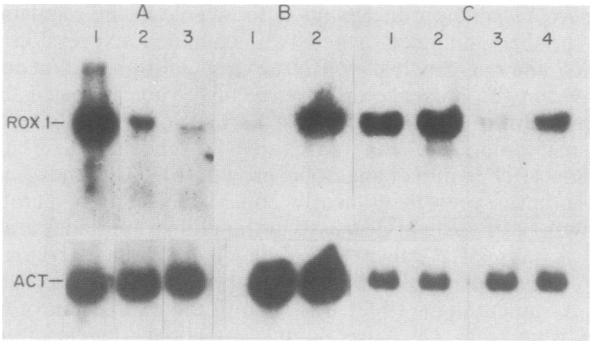

FIG. 4. Expression of the *ROX1* gene. Various strains derived by integrative or plasmid transformation from strain GM3C-2 were grown under various conditions for RNA extraction and Northern analysis. The *ROX1* probe was prepared as in Fig. 2. (A) Strain AH12-7(YCpAZ6) grown as in Fig. 3. Lanes: 1, aerobic growth; 2, 30 min of anaerobic growth; 3, 90 min of anaerobic growth. (B) Strain GM3C-2(YCp*CYC1*(2.4) grown for 90 min under anaerobic conditions without (lane 1) or with (lane 2) addition of heme (50 μg/ml). (C) Strain GM3C-2 and the congenic *rox1-a1* mutant GM3C-2 *rox1-a1* grown aerobically and anaerobically. Lanes: 1, GM3C-2, aerobic; 2, GM3C-2 *rox1-a1*, aerobic; 3, GM3C-2, anaerobic; 4, GM3C-2 *rox1-a1*, anaerobic. Lower panels, Same blots, reprobed with the actin (ACT) gene.

observations indicate that expression of *ANB1* is inhibited in aerobic cells as part of a regulatory cascade initiated by heme: heme signals the expression of a number of respiratory genes and simultaneously induces the *ROX1* factor, which functions to repress *ANB1*.

In the simplest form of this model, the *ROX1* factor acts alone (i.e., without a coeffector) to repress *ANB1*, which is induced under anaerobic conditions only after the factor has been degraded. For *ANB1* induction to occur within 1 h of a shift to anaerobic growth (15), the turnover of the *ROX1* protein would have to be much more rapid than that of most yeast proteins, which were found in one study to have half-lives of about 5 h (29). Although the induction of *ROX1* by heme would appear sufficient to explain the inhibitory effect of heme on *ANB1* expression, it is still possible that heme also functions as a negative coeffector. However, recent evidence indicates that heme is not required for repression: a low constitutive level of *ROX1* expression was observed to occur in a *rox4* mutant grown under anaerobic conditions (where heme would be absent), and *ANB1* expression was simultaneously inhibited (Rosenblum-Vos, Lowry, and Zitomer, unpublished observations).

***ROX1* coregulated with other heme-induced genes.** The fact that *ROX1* expression is heme induced suggested that this gene is in the same regulon as are the heme-induced respiratory genes (that is, that common *trans*-acting factors mediate its induction). This arrangement would permit parsimonious coordination of expression of the heme-induced and heme-repressed gene families, since *ROX1* induction would not require a separate regulatory mechanism. One defining characteristic of the heme-induced respiratory regulon, aside from heme induction, is constitutive expression caused by the pleiotropic *rox1-a1* mutation. Cells carrying this allele have been found to be constitutive for several nucleus-encoded mitochondrial genes (all of six tested so far [17; C. V. Lowry, R. S. Zitomer, and L. Grivell, unpublished observations]). Hence, constitutive regulation of the *ROX1* gene itself in a *rox1-a1* background would imply that the gene is coregulated with the family of respiratory genes. To determine if this was so, RNA from *rox1-a1* and wild-type cells grown aerobically and anaerobically was probed with the *ROX1* gene (Fig. 4C). The transcript was semiconstitutively expressed in the *rox1-a1* strain under anaerobic conditions (lanes 2 and 4), implying that *ROX1* is coregulated with the class of respiratory genes defined by the pleiotropic *rox1-a1* phenotype. We noted, however, that the *ROX1* factor was not itself required for respiration: cells carrying the *rox1* deletion grew normally on lactate, a nonfermentable energy source.

DISCUSSION

Our analysis of the phenotype of a strain carrying a null mutation at the *ROX1* locus demonstrated that the *ROX1* factor acts as a repressor or as part of a repression mechanism affecting the family of heme-repressed genes; *ANB1* expression was derepressed, independent of oxygen, in cells lacking a *ROX1* gene. Whether this factor interacts directly with DNA has yet to be determined. Recent work has revealed the presence of an inhibitory site upstream from the *ANB1* gene, the deletion of which leads to constitutive expression (Lowry and Zitomer, in preparation). It may be that this region contains the site of action of a repressor complex containing the *ROX1* factor.

Although we had established that heme acts to inhibit expression of the heme-repressed genes, it was unknown how the inhibition was mediated. Since we found that heme induced the *ROX1* repression factor, it is reasonable to conclude that the negative effect of heme is simply due to that induction; i.e., the presence or absence of the *ROX1* repressor (a function of heme induction or protein degradation, respectively) may be sufficient to achieve regulation. Whether heme plays an additional role as a coeffector with *ROX1* remains to be determined. Another example of a gene family which is regulated by a repressor which is itself regulated was provided in studies on the *RME1* factor, which represses sporulation (*SPO*) genes and which is itself repressed by the concerted action of **a**1 and α2 factors (20). Interestingly, just as *ROX1* appears to be a member of the family of heme-induced genes, *RME1* is a member of the family of haploid-specific genes (*HSG*); thus, in each case, mutually exclusive regulons (heme repressed and heme induced, *HSG* and *SPO*) are coordinately expressed by inclusion of a repressor of one set in the other set.

Because one unusual *rox1* allele, *rox1-a1*, caused constitutive expression of the heme-induced genes (17), we earlier hypothesized that the *ROX1* factor played a direct role in regulating that family. Hence, it was surprising that elimination of the *ROX1* factor in a null mutant caused only a modest effect on the expression of *CYC1* and *tr-1*. This indicates that *ROX1* has no obligatory role in regulation of the respiratory genes despite the observed effect of the *rox1-a1* mutation. Several explanations of the apparent paradox are possible. One which we entertain as a working hypothesis supposes that the *ROX1* factor is part of a multifactor complex. This complex would bind heme and in this state have two separate functions. The first function, not requiring *ROX1*, would be to activate factors (e.g., *HAP1*) which induce the respiratory genes. The second function of the complex, requiring *ROX1*, would be to repress the heme-repressed genes or to activate their repressor. In this scheme, the mutant product of the *rox1-a1* allele would distort the complex, preventing repression of heme-repressed genes and also causing aberrant induction of heme-induced genes under anaerobic conditions. The recessive-

ness of the phenotype (Lowry and Zitomer, unpublished observations) would be explained by a higher affinity of the wild-type protein for the complex. The *rox1* null mutation would not affect expression of the heme-induced genes because the *ROX1* factor is not involved in the heme induction function of the complex; i.e., the absence of the protein would not affect heme induction, but the presence of a mutant protein could still alter the function of the complex in that respect.

To explain the enhancing effect of the *rox1* null mutation on *CYC7* expression, we tentatively conclude that *ROX1* participates in repression of *CYC7* through the same mechanism that represses the anaerobic genes *ANB1* and *COX5b* and possibly *HEM13*. The *CYC7* gene is normally expressed at a low level in the presence of oxygen (as signaled by heme) and at a reduced level in the absence of oxygen. Aerobic expression of *CYC7* is dependent on *CYP1* (*HAP1*) (2, 3, 23, 30, 37) and on an upstream activation sequence (UAS_0) (2, 23, 37), as is the expression of the heme-induced genes (3, 8), but other *cis*- and *trans*-acting elements are involved in a complex mechanism, which appears to be a hybrid of heme induction, heme repression, and nonspecific activation and repression elements. The products of the *ROX3*, *ROX4*, *ROX5*, and *ROX6* genes have been implicated in regulation of *CYC7* (Rosenblum-Vos and Zitomer, in preparation), as have factors encoded by the set of *CYC7* regulatory genes identified by Matner and Sherman (18). Our conclusion that the *ROX1* factor is involved in repressing *CYC7* by a mechanism common to the heme-repressed genes is based on the data presented here and on the identification of a heme repression element in one segment of the *CYC7* regulatory region; this segment caused anaerobic induction when fused to the *GAL1* gene or when placed upstream to a modified form of the *CYC7* gene in which the heme induction element (UAS_0) was eliminated (37; Hastings and Zitomer, in preparation).

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