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Combinatorial Repression of the Hypoxic Genes of *Saccharomyces cerevisiae* by DNA Binding Proteins Rox1 and Mot3

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The hypoxic genes of *Saccharomyces cerevisiae* are transcriptionally repressed during aerobic growth through recruitment of the Ssn6/Tup1 general repression complex by the DNA binding protein Rox1. A second DNA binding protein Mot3 enhances repression of some hypoxic genes. Previous studies characterized the role of Mot3 at the hypoxic *ANB1* gene as promoting synergy among one Mot3 site and two Rox1 sites comprising operator A of that gene. Here we studied the role of Mot3 in enhancing repression by Rox1 at another hypoxic gene, *HEM13*, which is less strongly regulated than *ANB1* and has a very different arrangement of Rox1 and Mot3 binding sites. By assessing the effects of deleting Rox1 and Mot3 sites individually and in combination, we found that the major repression of *HEM13* occurred through three Mot3 sites closely spaced with a single Rox1 site. While the Mot3 sites functioned additively, they enhanced repression by the single Rox1 site, and the presence of Rox1 enhanced the additive effects of the Mot3 sites. In addition, using a Rox1-Ssn6 fusion protein, we demonstrated that Mot3 enhances Rox1 repression through helping recruit the Ssn6/Tup1 complex. Chromatin immunoprecipitation assays indicated that Rox1 stabilized Mot3 binding to DNA. Integrating these results, we were able to devise a set of rules that govern the combinatorial interactions between Rox1 and Mot3 to achieve differential repression.

The response of the budding yeast *Saccharomyces cerevisiae* to hypoxia and anaerobiosis involves the induction of multiple regulons. One of these, characterized by a rapid induction of many oxygen utilizing functions, consists of about 70 Rox1, Ssn6/Tup1 repressed genes (21, 45, 46). Tup1 and Ssn6 form an in vivo general repression complex (33, 39, 40) that represses multiple, otherwise unrelated regulons. Repression is effected by at least two mechanisms. First, Tup1 binds to the amino-terminal tails of histone H3 and H4 and recruits the histone deacetylase Hda1, thereby phasing a nucleosome over the TATA region of a promoter and blocking TATA binding protein recruitment (4, 12, 34, 41). Second, there is a chromatin-independent mechanism that is less well characterized (14, 16, 30, 43). At many genes, these mechanisms are redundant (29, 44).

The Ssn6/Tup1 complex has no intrinsic DNA binding activity and is targeted to specific regulons through recruitment by regulon-specific DNA binding proteins (6, 17, 24, 31, 32, 36, 37). For the hypoxic genes, regulation is controlled by the DNA binding protein Rox1 and, for many but not all hypoxic genes, Mot3 (3, 20, 46). Rox1 recruits Ssn6/Tup1 by an interaction with the TPR (tetratricopeptide repeat) domain of Ssn6 (38). Repression of the hypoxic genes is directly related to the level of Rox1 protein in cells (8). The *ROX1* gene is transcriptionally induced aerobically and repressed anaerobically by the DNA binding protein Hap1 which senses oxygen levels through cellular heme levels (22). Heme biosynthesis requires molecular oxygen in two tandem steps, first as an electron acceptor for oxidative decarboxylation and then for oxidation of two methylene groups to methenyl groups. The first of these two steps is rate limiting under hypoxic conditions (42). The Rox1 protein is highly labile, rapidly disappearing from the cell when *ROX1* transcription is repressed at the onset of hypoxia. Finally, *ROX1* is also auto-regulated, which tightly controls the protein's cellular levels (8).

The role of Mot3 in hypoxic gene regulation is less clear. Mot3 affects the expression of a large, eclectic collection of genes (15, 28). It appears to play a major role in the aerobic repression of a true anaerobic set of genes (35), but its role in repression of the Rox1 hypoxic regulon is secondary for some genes, while others are not affected by Mot3 at all (20). Mot3 does not bind cooperatively with Rox1 in vitro (20), and chromatin immunoprecipitation experiments indicated that it can recruit Ssn6 in the absence of Rox1 (29). However, at *ANB1*, this Rox1-independent Ssn6 recruitment does not result in a repression-competent complex.

Our previous analysis has focused on one hypoxic gene, *ANB1* which is repressed over 200-fold. In the absence of Rox1, *ANB1* is fully derepressed, while a *mot3* deletion results in only a fourfold depression (20). The *ANB1* regulatory region contains two operators, A and B, each of which contains two Rox1 sites. Operator A also contains a single Mot3 site, and it is this operator which is responsible for the bulk of aerobic repression (8, 20). The full Rox1, Mot3, Ssn6/Tup1 complex results in the positioning of a nucleosome over the *ANB1* TATA box; while in the absence of Mot3, this positioned nucleosome is lost, although repression is maintained through a chromatin-independent mechanism (20). Not all hypoxic genes are expected to be regulated in the same manner. First, not all hypoxic genes are repressed to the same extent. *ANB1* encodes the a subunit of eukaryotic initiation factor 5 (eIF-5a), an essential translation factor, but there is an aerobic homologue, *TIF51A* (19). As a result, *ANB1* can be completely

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TABLE 1. Strains used

Strain	Genotype	Source or reference
RZ53-6	α *trp1-289 leu2-3 leu2-112 ura3-52 ade1-100*	3
RZ53-6Δ*rox1*	RZ53-6 *rox1*::*LEU2*	8
RZ53-6Δ*tup1*	RZ53-6 *tup1*::*URA3*	8
RZ53-6Δ*ssn6*	RZ53-6 *ssn6*::*LEU2*	8
RZ53-6Δ*mot3*	RZ53-6 *mot3*::*kanMX*	20
RZ53-6Δ*r*Δ*m*	RZ53-6 *rox1*::LEU2 *mot3*::*kanMX*	20
MZ22-4Δ*rox1*	**a** *trp1 his3 gal1*-Δ152 *rox1*::*LEU2 ura3*::*AZ4*	20
MZ22-4Δ*r1*Δ*m3*	MZ22-4Δ*rox1* with *mot3*::*kanMX*	20
MZ22-4PC	MZ22-4 with *rox1*::*ROX1-galK tup1*::*TRP1*	23
MZ65-88	**a** *trp1 his3 leu2 gal1-Δ152 ssn6*::*LEU2 ura3*::*AZ4 rox1*::*ROX1-galK*	This study
KZ211-21	α *trp1 leu2 ade1 ura3*::AZ4 *rox1*::*LEU2 ssn6*::*LEU2*	This study
KZ211-14	**a** *trp1 leu2 ade1 ura3*::AZ4 *rox1*::*LEU2 ssn6*::*LEU2 mot3*::*kanMX*	This study

repressed. While aerobic homologues exist for a number of hypoxic genes, others are unique. For these, some level of expression is required under aerobic conditions and repression is less severe. Second, the arrangement and numbers of Rox1 and Mot3 sites are quite different in different genes.

HEM13 is a less strongly regulated hypoxic gene. It encodes the enzyme coproporphyrinogen III oxidase, which catalyzes the rate-limiting step in heme biosynthesis (42). The hypoxic derepression of this gene allows the cell to continue heme biosynthesis under limiting oxygen. Since *HEM13* is the only gene encoding coproporphyrinogen oxidase, it cannot be as strongly repressed as the duplicated *ANB1* gene, and consequently, it is regulated over a much narrower range than *ANB1*.

HEM13 contains five putative Mot3 sites interspersed among four widely spaced Rox1 sites. A previous study involved a detailed analysis of the activation sequences of *HEM13* and repression by the three coding-sequence-proximal Rox1 sites (1). The authors concluded that the most distal one was responsible for most of the repression. Interestingly, this site is closely flanked by three of the five Mot3 sites in the regulatory region of *HEM13*. In this study, we further investigated the repression at this gene, focusing on the relationship between the Mot3 and Rox1 sites and the role of Mot3 in repression. We report here a combinatorial repression mechanism involving the cooperative recruitment of the Ssn6/Tup1 complex by Rox1 and Mot3. The combination of sites determines the strength of repression.

MATERIALS AND METHODS

Strains and growth conditions. The strains used in this study are listed in Table 1. KZ211-21 and KZ211-14 were meiotic products of the mating of RZ53-6Δ*r*Δ*m* with MZ65-88 by standard yeast genetics (18). Yeast cultures were grown at 30°C, in rich yeast extract-peptone-dextrose medium or synthetic complete medium lacking the appropriate nutrient to select for plasmid maintenance. Anaerobic growth was achieved by bubbling N_2 gas through the medium. Yeast transformations were performed by using standard techniques (18).

Plasmids. All plasmid constructions were carried out by using standard techniques as described previously (2). Genomic sequences were obtained from the *Saccharomyces* Genome Database maintained at Stanford University (http://www.yeastgenome.org). The sequences for all genes discussed are numbered with the adenine of the start codon numbered +1, bases 5′-wards numbered negatively, and bases 3′-wards numbered positively. The sequences of oligonucleotides used are available upon request.

(i) YCpAZ33. The *ANB1-lacZ* fusion plasmid YCpAZ33 has been described previously (13).

(ii) *HEM13-lacZ* fusions. The upstream region of *HEM13* from −983 to +3 was PCR amplified from genomic DNA prepared from RZ53-6 as described previously (18), generating EagI and PstI restriction sites at the 5′ and 3′ ends, respectively. This promoter region was inserted as an EagI-PstI fragment into YCp(33)*ROX1-lacZ* (8), replacing the *ROX1* promoter with that of *HEM13* to generate the *lacZ* fusion plasmid YCp(33)*HEM13Z*. Deletions of consensus Rox1 and Mot3 binding sites in this region were generated by PCR-based mutagenesis, where each binding site was replaced by a restriction site. Table 2 shows the relevant sequences before and after the site deletions. Multiple deletions were created by combining individual deletions in a stepwise manner, with the exception of the R1R2 deletion, which also deleted all the sequences between the two sites.

(iii) YCp(22)*MOT3*. YCp(22)*MOT3* was constructed by cloning the BamHI-HindIII fragment from pBS*MOT3* (20), containing the *MOT3* sequence from −775 to +1960, into YCplac22 (13).

(iv) YCp(22)pT1-M3-HA. YCp(22)pT1-M3-HA was constructed by fusing the *TUP1* promoter, −2080 to +4, from YEp(181)TUP1BBg (5) to a *MOT3*-hemagglutinin (HA) derivative (29) with a SalI site at +4 in the vector YCplac22. The resulting construct expressed the HA epitope-tagged *MOT3* constitutively from the *TUP1* promoter.

(v) p*MOT3*-HA. *MOT3*-HA was PCR amplified from YCp(23)*MOT3*-HA, which has the native *MOT3* 5′ regulatory and coding sequence joined to five copies of the HA epitope tag plus the 400 bp of *TUP1* 3′ sequences (29). The amplification introduced an EagI site at +4 and a SacI site 500 bases into the *TUP1* 3′ sequences. The product was inserted into the EagI-SacI sites of pIVEX2.4d (RTS System; Roche). This construction introduced six histidine residues and a factor Xa protease site preceding the *MOT3* sequences.

(vi) YCp(22)*R1-S6e*. The c-*myc* (9E10) epitope-tagged *SSN6* coding and 3′ sequences (+4 to +3272) were PCR amplified with the addition of an XhoI site at the 5′ end and an EcoRI site at the 3′ end. This fragment was ligated into the XhoI (immediately following codon 99) EcoRI sites of YCp(22)*ROX1*Δ*Q* (7). This construct resulted in the *ROX1* promoter and high mobility group DNA binding domain coding sequence (−1290 to +297), followed by an XhoI site fused to the *SSN6* coding sequence (+2 to +2901) plus the c-*myc* epitope tag and *SSN6* 3′ sequences (+2902 to +3272).

Protein purification. His-*MOT3*-HA was expressed in BL21 codon-plus cells (Stratagene). Cells were grown at 37°C to mid-exponential phase in terrific broth (2) supplemented with 50 μg of ampicillin/ml and 34 μg of chloramphenicol/ml. The cultures were shifted to 18°C, and protein expression was induced overnight by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to 0.2 mM. Cells were chilled, harvested by centrifugation for 5 min at 4,000 × *g*, and resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, adjusted to pH 8.0 with NaOH) containing 1 μg of pepstatin/ml, 1 μg of leupeptin/ml, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride. After freezing at −80°C and then thawing, cells were lysed with three 15-s cycles of sonication alternating with 2 min on ice. The cell lysate was clarified by centrifugation for 30 min at 12,000 × *g*. Clarified extracts were then batch bound to Ni-nitrilotriacetic acid agarose (QIAGEN) for 1 h with continuous gentle inversion at 4°C. Beads were batch washed with 20 bed volumes of lysis buffer containing 20 mM imidazole. Elutions were carried out in 250 mM imidazole in lysis buffer. The His tag was cleaved from the Mot3-HA with factor Xa (New England Biolabs) per the manufacturer's recommendations.

Maltose binding protein-Rox1 was purified as described previously (20) with

TABLE 2. Binding site deletions

Deletion type	Distance[a]	Sequence[b]	Binding site and restriction site
Rox1 consensus site deletions	−603	cactgttgggAAAAACAATACGcctaattcgt	R4
		TCG	SalI replacement
	−484	tattttaattTCAATTGTTTAGaaagtgcctt	R3 XbaI replacement
	−259	acgctccagcTTGAACAAAGCAtaagactgca	R2
		G	XhoI replacement
	−195	gcttgctttgCCCATTGTTCTCgtttcgaaag	R1
		CTCGA	XhoI replacement
Mot3 consensus site deletions	−733	cactttccagAAGGCAtagccttgcc	M5
		CTCGAG	XhoI replacement
	−503	aaagttactaTAGGCAcggtatttta	M4
		CTAG	XbaI replacement
	−468	gtttagaaagTGCCTTcacaccatta	M3
		CGAC	SalI replacement
	−433	attaccgtcaTAGGCActttctgctg	M2
		CCGGTACC	AgeI-KpnI replacement

[a] Numbers represent distance 5′ from the beginning of the coding sequence.
[b] The wild-type DNA sequence is shown with the Rox1 or Mot3 binding site in uppercase. The sequences deleted are underlined, and the sequences presented below are substituted to create the restriction site indicated. In M3 and M2, the lowercase base underlined represents a deleted base discovered after sequence analysis.

minor modifications. Expression of the fusion was induced with 1 mM IPTG for 3 h.

Electrophoretic mobility shift assays. Gel shifts assays were performed as described previously (20) with restriction fragments that separated Mot3 consensus binding sites. The regions used are indicated in Fig. 2. DNA restriction fragments were labeled by Klenow-mediated fill-in of the restriction enzyme generated single-stranded ends in the presence of [α-^{32}P]dATP. Binding was carried out in 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM $MgCl_2$, 1 μg of dI-dC/ml, and 10% glycerol. Protein was incubated with DNA for 5 min at room temperature and then loaded directly on a 6 or 8% polyacrylamide gel. Specific competition was accomplished by adding 130 ng of synthetic 55-bp DNA containing the known Mot3 binding sequence from *ANB1* (20). For nonspecific competition, 130 ng of an equal length synthetic DNA lacking Mot3 binding sequences was used.

β-Galactosidase assays. Assays were performed as described previously (18). The effects of the individual deletions were small, and to minimize experimental error, the assays were performed with pooled plates of transformants. We found that the largest contribution to error was the substantial variation from performing assays with several individual transformants, perhaps due to variations in copy number. These errors disappeared when several hundred colonies from individual transformant plates were pooled and used to start overnight cultures for a single assay. The errors presented in Tables 3, 4, and 5 show one standard deviation from the mean for assays repeated at least three times, each with a different pool of transformants.

RNA blots, immunoblots, and chromatin immunoprecipitation (ChIP) analyses. RNA was extracted with hot acidic phenol, and Northern blots were carried out as described previously (2). The blots were hybridized to radiolabeled [α-^{32}P]dATP DNA probes prepared as described previously (2, 29). The probes used contained the following sequences: *ANB1*, +123 to +465; *HEM13*, −402 to +803; *ACT1*, 600-bp internal fragment, used as a loading control.

Crude protein extracts were prepared from mid-exponential-growth-phase cells boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) loading buffer containing β-mercaptoethanol for 5 min as previously described (2, 29). Proteins were fractionated by sodium dodecyl sulfate-PAGE and then electroblotted onto nitrocellulose membranes (2). The blots were probed with antibody against the HA epitope (F-7; Santa Cruz Biotechnology, Inc.) or c-*myc* epitope (9E10; Santa Cruz Biotechnology, Inc.) followed by horseradish peroxidase-conjugated anti-mouse antibody (Santa Cruz Biotechnology, Inc.). Bands were visualized with Western blotting luminol reagent (Santa Cruz Biotechnology, Inc.) in accordance with the manufacturer's recommendations. To ensure that equal amounts of extract were loaded in each lane, the blots were either stained with Ponceau S or, after the initial probing, stripped by a 5-min incubation in 0.2 N NaOH and then reprobed with rabbit polyclonal antibody against yeast eIF-5a (prepared by Alexander Kastaniotis). The immunoblots were quantitated by scanning the exposed X-ray film and using ImageQuant software (Molecular Dynamics).

The recruitment of HA epitope-tagged Mot3 to specific regions of DNA was measured by the immunoprecipitation of formaldehyde-cross-linked chromatin with antibody against the HA epitope and protein A-Sepharose resin (Amersham Biosciences) as described previously (2, 11, 25, 29). The relative efficiencies of immunoprecipitation were measured by quantitative radioactive PCR, performed as described previously (25, 29). The oligomer pairs amplified the following regions: *ANB1*, −505 to −231; *HEM13*, −617 to −388. PCR products were separated on 8% polyacrylamide gels, and quantitation was performed with a Molecular Dynamics Storm 860 PhosphorImager as described previously (25, 29). To establish that the amplification was in the linear range of incorporation of the labeled [α-^{32}P]dATP, samples were taken after various cycles, fractionated on polyacrylamide gels as described above, and quantitated.

RESULTS

Mot3 can repress *HEM13* but not *ANB1* in the absence of Rox1. In our previous analysis of the hypoxic repression of the expression of the *ANB1* gene, we determined that the Rox1 repressor was essential and that Mot3 enhanced repression by Rox1 severalfold. This contribution by Mot3 was effected through promotion of a synergistic interaction between the two closely spaced Rox1 sites in OpA (Fig. 1). In the absence of the Mot3 protein or the Mot3 site between them, these two sites acted independently or additively (20). To learn whether this activity of Mot3 is a general rule in hypoxic gene repression, we extended our studies to an analysis of the *HEM13* gene. To assess the relative contributions of the DNA binding Rox1 and Mot3 repressor proteins to repression of *HEM13*, the aerobic expression of an *HEM13-lacZ* fusion was compared in a wild-type strain and strains carrying deletions of *MOT3*, *ROX1*, and both genes. The relative repression, calculated as the ratio of β-galactosidase levels in the *rox1*Δ *mot3*Δ double deletion mutant to those in the wild type, represents the full extent of repression by the combination of the two repressor proteins. The ratio of enzyme levels in the double deletion mutant to the *mot3*Δ deletion mutant represents the extent of repression that Rox1 can achieve in the absence of Mot3, and that of the double deletion mutant to the *rox1*Δ deletion mutant represents the level of repression that Mot3 alone can effect.

Initially, we confirmed our findings concerning *ANB1* repression with an *ANB1-lacZ* fusion. As seen in Table 3, the levels of β-galactosidase activity expressed from an *ANB1-lacZ* fusion were similar in *rox1*Δ cells to those in *rox1*Δ *mot3*Δ cells,

indicating that Rox1 was essential for repression and that Mot3 could not significantly repress *ANB1-lacZ* in the absence of Rox1. In the absence of Mot3, on the other hand, Rox1 alone repressed *ANB1-lacZ* expression 54-fold (the β-galactosidase activity in *rox1*Δ *mot3*Δ cells divided by that in *mot3*Δ cells) compared to the 260-fold repression of *ANB1* with both proteins present (the ratio of β-galactosidase activity in *rox1*Δ *mot3*Δ cells to that in wild-type cells).

Surprisingly, the activity of Rox1 and Mot3 appeared to be different at *HEM13*; Mot3 appeared to be capable of repressing aerobic *HEM13* expression in the absence of Rox1. As seen from Table 3, a *HEM13-lacZ* fusion was repressed 44-fold by the combination of Rox1 plus Mot3 (the ratio of β-galactosidase activity in *rox1*Δ *mot3*Δ cells to that in wild-type cells), while Mot3 alone repressed expression 3-fold (the ratio of β-galactosidase activity in *rox1*Δ *mot3*Δ cells to that in *rox1*Δ cells) and Rox1 alone repressed expression 9-fold (the ratio of β-galactosidase activity in *rox1*Δ *mot3*Δ cells to that in *mot3*Δ cells). Combining the individual repression by each protein would give 60-fold repression, close to the 44-fold observed.

Mot3 binds to a subset of the putative Mot3 sites of *HEM13*. As seen above, there are two important differences between repression at *ANB1* and *HEM13*. The former is repressed to a much greater extent, and Mot3 can repress the latter in the absence of Rox1. We hypothesized that this difference may result from the arrangement of Rox1 and Mot3 sites in the two genes as illustrated in Fig. 1. Both genes have several Rox1 sites. For *ANB1*, we have grouped these sites into two operators, the coding sequence proximal OpB and the distal OpA, based upon the close proximity of the two Rox1 sites in each operator (20 and 30 bp, respectively). OpA of *ANB1* contains a Mot3 site between the two Rox1 sites, and these three sites have been shown to act synergistically to account for most of the strong repression of *ANB1* expression. *HEM13* also contains two closely positioned proximal Rox1 sites, designated R1 and R2 here, and two distal sites, R3 and R4. However, the two distal sites in *HEM13* are spaced very differently, with over 100 bp between them. R3 is closely spaced with three putative Mot3 sites; all four sites lie within about 70 bp of each other. There are two additional putative Mot3 sites, one far upstream of the most 5′ Rox1 site and the other within the beginning of the coding sequence. Thus, we addressed the question of how the arrangement of these sites affected repressor activity.

The Rox1 binding site has been well characterized, and we can accurately predict how well a given sequence will be bound by the protein (9). However, the published Mot3 binding site is small, 6 bp, and degenerate (C/A/T)AGG(T/C)A, such that a Mot3 site would be predicted to be found about once every 680 bp. It is unlikely that Mot3 binds so many sites in the genome and even more unlikely that it functions at so many sites, so initially, we determined which of the five putative Mot3 sites, designated sites M1 (most proximal) to M5, could bind Mot3 in vitro. A set of restriction fragments were generated from the upstream region of *HEM13* that contained individual, or in one case, two, putative Mot3 sites and used in a gel retardation assay with bacterially expressed Mot3. Relatively large fragments were used to maintain the larger contextual sequence of the consensus binding sites that may be important for binding. As shown in Fig. 2A, Mot3 bound to site M4 (lane 3), with the DNA protein complex indicated as M. A doublet was visible which correlated with the appearance of a doublet of the purified protein (data not shown); the reason for the doublet is not clear. The complex was specifically competed by an excess of nonlabeled specific OpA DNA (lane 4) containing the Mot3 binding site from the upstream region of *ANB1*, which was shown previously to both bind Mot3 protein in vitro and repress transcription in vivo (20). Since this competitor DNA had only the Mot3 site in common with the labeled fragment, it is likely that Mot3 was bound to its cognate site. An equivalent excess of unlabeled DNA lacking a Mot3 site did not affect binding (lane 5). Further evidence that the complex contained Mot3 was observed by a shift in the size of the complex when antibody against the HA tag of Mot3 was added (data not shown). The labeled restriction fragment also contained the R3 Rox1 site, and as a control, bacterially expressed and purified Rox1 was shown to bind to this fragment (lane 2) with the complex in this case designated with an R. Figure 2B demonstrates the specific binding of Mot3 to M2 and M3. Both sites were bound by the protein, as indicated by the appearance of two complexes (lane 3), indicated as a single protein molecule (M) and two molecules bound (2 M). Both complexes were successfully competed by excess specific (lane 4), but not nonspecific (lane 5), competitor. This fragment also contained a Rox1 site, R2, and it bound Rox1 (lane 2).

Surprisingly, the two putative sites 1 and 5 did not bind Mot3, even at high protein concentrations (data not shown), despite complete matches to the consensus sequence. A summary of contextual sequences 10 bp upstream and downstream of the Mot3 sites for all five *HEM13* sites, the *ANB1* site, and the fragment used to determine the Mot3 binding site in a previous study (15) are shown in Fig. 2C. Sites M3 and M5 have identical core binding sites yet very different binding activities. A comparison of the contextual sequences in sites that were bound by Mot3, M2, M3, M4, the *ANB1* site, and the fragment used to determine the Mot3 binding site with those of sites that did not bind, M1 and M5, provided no insight into the relationship between sequence and function.

The Rox1 site R3 is responsible for most Rox1-dependent repression. To assess the ability of the various Rox1 and Mot3 sites to function in repression in vivo, deletions of single sites and combinations of sites were generated in an *HEM13-lacZ* fusion construct. As described above, each construct was transformed into a series of strains, wild type, *rox1*Δ, *mot3*Δ, and *rox1*Δ *mot3*Δ, to assess the relative contribution of each site to repression by the two DNA binding proteins. For simplicity, only a subset of the results is presented here, where we felt the omitted data provided no additional insights.

A previous study had indicated that R3 was the largest contributor to repression by Rox1 and that deletion of R1 and

TABLE 3. Expression of the *HEM13* and *ANB1 lacZ* fusions in deletion strains

lacZ reporter	Result for genotype[a]:			
	Wild type	*mot3*Δ	*rox1*Δ	*rox1*Δ *mot3*Δ
HEM13	5.4 ± 0.7	28.5 ± 0.7	81 ± 3	239 ± 35
ANB1	1.1 ± 0.4	5.3 ± 0.2	205 ± 6	289 ± 13

[a] The enzyme assays were carried out in extracts from derivatives of RZ53-6. The numbers shown represent Miller units ± standard deviations.

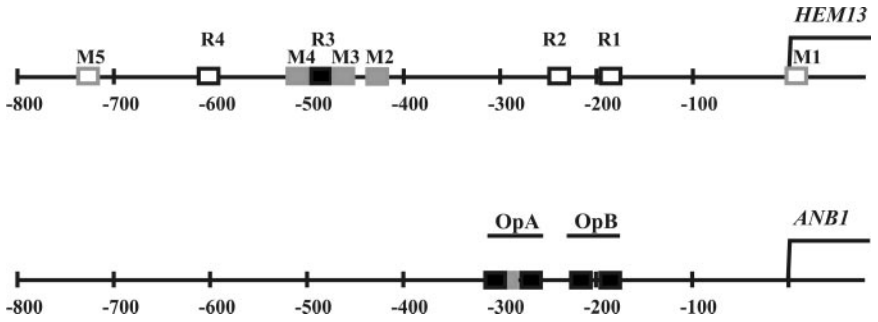

FIG. 1. Rox1 site mutations in the *HEM13* regulatory region. The upper diagram represents the wild-type promoter of *HEM13* with both Rox1 (R, black boxes) and Mot3 (M, gray boxes) consensus sites presented. The Mot3 sites that bound Mot3 protein and were active in repression are presented as filled boxes, while those that did not are presented as empty boxes. The Rox1 sites that proved to be less active in repression are shown as empty boxes. The arrow represents the beginning of the coding sequence, and the negative numbers indicate the distance (in base pairs) from the start of the coding sequence. The lower diagram shows the same for *ANB1*.

R2 alone had little effect (1). To confirm these findings and evaluate the possible influence of Mot3 on repression through these Rox1 sites, we deleted R1 and R2 individually and in combination and R3 and R4 in the R1R2 deletion background. The effect of these deletions is presented in Table 4 as the relative derepression, that is, how much repression was lost by deleting the site(s). This value was calculated as the enzyme activity in *rox1*Δ cells divided by the activity in wild-type cells for the deletion constructs normalized to the same ratio for cells carrying the wild-type *HEM13-lacZ* construct. Thus, a relative derepression of 1.0 represents no effect of the deletion on the ability of Rox1 to repress, and a high value for the relative derepression represents a large effect of the deletion. It is clear from this ratio that sites R1, R2, and R4 have a minimal effect on *HEM13-lacZ* repression, deletions of the R1 and R2 sites individually or in combination resulted in no significant derepression, and the deletion of R4 in the R1R2 deletion background had little effect. On the other hand, all constructs with a deletion of R3 resulted in maximal depression. Based upon these results, it appears that most of the Rox1 repression is through R3.

Two anomalies should be noted in the data. First, the deletion of all four Rox1 sites did not result in complete derepression; expression from this deletion was three times higher in *rox1*Δ cells than in wild-type cells. This observation suggests that either there are cryptic Rox1 sites, a possibility we reject due to the results of the gel retardation assays and the well-known sequence for Rox1 binding sites, or there is an indirect effect of the *rox1*Δ mutant on *HEM13* expression. Amillet et al. (1) mapped an upstream activation sequence that, when transferred to a heterologous gene, activated transcription three times greater anaerobically than aerobically. The activator has not been identified, but we suggest that it may be repressed aerobically by Rox1. In this case, a *rox1*Δ mutant would have two effects on the R1R2R3R4 deletion allele: elimination of repression through the *HEM13* Rox1 sites and enhanced aerobic expression due to loss of repression of the activator. Deletion of the Rox1 sites alone would result in less of an increase in aerobic repression because the activator would still be repressed. This is what we observed.

The second anomaly is that deletions that included R2 resulted in a 1.5- to 2-fold overall increase in *HEM13-lacZ* expression. We do not know the cause of this phenomenon, but it was clearly Rox1 independent, since the increase was apparent in the *rox1*Δ strain (Table 4) and the *rox1*Δ *mot3*Δ strain (data not shown). This anomaly points out the advantage of assaying the levels of gene expression of each plasmid in the various deletion strains; expression from each plasmid can be normalized to cells with and without the repressor.

Our confirmation of the previous finding that R3 contributed the major Rox1-dependent repression (1) can now be interpreted in the context that this site is surrounded by three Mot3 sites, and these results suggest that repression through this Rox1 site is potentiated by Mot3 binding. This conclusion is supported by the observation that the Mot3 effect on repression is weakened by the loss of the R3 site. The Mot3 effect can be visualized as the ratio of *HEM13-lacZ* expression in *mot3*Δ cells to that in wild-type cells. This ratio is 5.2 for the cells transformed with the wild-type plasmid and ranged between 5.0 and 7.9 for the deletions of R1, R2, and R4 individually or in combination. However, this ratio fell to between 2.8 and 3.5 for any combination that deleted R3. Consequently, we conclude that the bulk of Rox1 repression is achieved through R3 at least in part because of a weak interaction with Mot3. This interaction was not apparent from the analysis in Table 3 for the wild-type plasmid in the mutant transformants because of the small effect.

It should be noted that R2 bound Rox1 in vitro as shown in Fig. 2, yet its deletion had little effect on repression in vivo. In the case of R4, its distance from the TATA box and the transcriptional initiation region may account for its poor activity. However, for R2 and R1, the lack of repression activity is unclear. There may be some other protein that binds in this region that interferes with Rox1 binding in vivo. Perhaps this is the reason that all deletions, including R2, resulted in an overall 1.5- to 2-fold increase in the fusion expression in all strains.

The function of the Mot3 sites correlate with DNA binding, and Mot3 sites act additively. The five Mot3 sites were also deleted individually and, for M2 through M5, in combination, and the effect of these deletions on *HEM13-lacZ* expression are presented in Table 5. Sites M1 and M5, the two sites that did not bind Mot3, did not alter the level of repression in wild-type cells. In the case of M1, the original wild-type *HEM13-lacZ* fusion plasmid contained only the translational

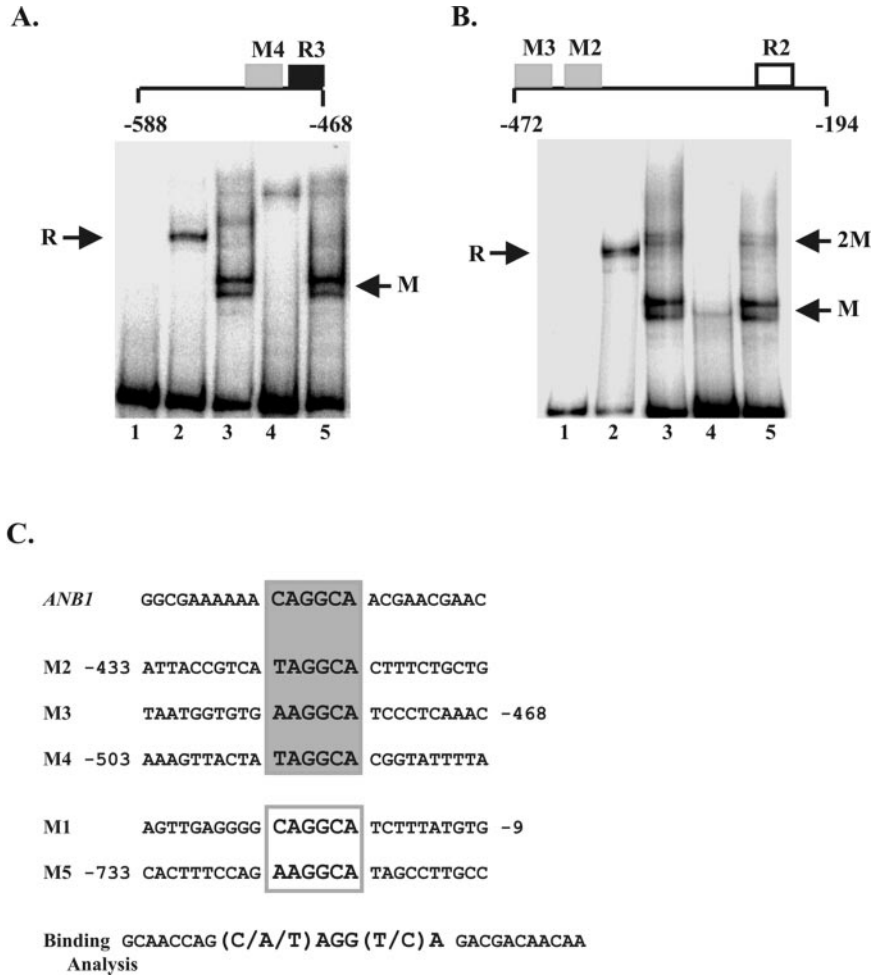

FIG. 2. Mot3 binds to the M2, M3, and M4 Mot3 sites in the regulatory region of *HEM13*. (A) The gel retardation assay was carried out as described in Materials and Methods with 25 ng of maltose binding protein-Rox1 (lane 2) or 100 ng of Mot3 (lanes 3 to 5) and 6.5 ng of [α-^{32}P]dATP-labeled SalI (−588)-MfeI (−468) restriction fragment prepared from YCp(33)*HEM13*ΔR4. This DNA fragment contained the sites R3 and M4 as shown above the gel. No protein was added to lane 1. Lane 4 contained a 20-fold excess of the specific competitor, a synthetic *ANB1* OpA DNA which contained a single, functional Mot3 binding site (underlined, 5′-TTTTTCCATTGTTCGTTCGTTGCCTGTTTTTTTGCCCT ATTGTTCTCA). Lane 5 contained a 20-fold excess of the nonspecific competitor (5′-AGCTTCCCCTTTCGTCCCCTTGTTTCCCCTTTTTT CCCCTTGAATTCCCCTTC), which lacks a Mot3 binding site. (B) The gel retardation was carried out as described above, except that an [α-^{32}P]dATP-labeled MfeI (−472)-HindIII (−194) restriction fragment was used. This fragment contained the sites R2, M2, and M3 as shown above the gel. (C) Alignment of Mot3 sites from *ANB1* OpA, M1 through 5 of *HEM13*, and the consensus sequence derived in the binding analysis of Grishin et al. (15). The core consensus sequence is highlighted with a gray background for those sequences that bound Mot3 and with a grey outline for those that did not.

initiation codon of the *HEM13* coding sequence and, therefore, did not contain M1. To test the function of M1, a new fusion was made that added five additional codons including the M1 site. This plasmid had the same levels of repression as the fusion without M1, and for the sake of clarity, the fusion lacking M1 will be referred to as wild type throughout. The deletion of site M2, M3, or M4 individually caused a modest 1.5- to 2.9-fold increase in *HEM13-lacZ* expression, as determined by the ratio of enzyme activities in wild-type cells. Thus, the sites that bound Mot3 in vitro caused some repression in vivo, while those that did not bind did not affect repression. It was also clear that the Mot3 sites functioned independently, as determined from the results with multiple deletions. For example, the deletion of M2 plus M3 resulted in a 3.3-fold increase in *HEM13-lacZ* expression compared to the 1.5- and 2.9-fold increases of the individual deletions. If these sites functioned independently, a 4.3-fold increase would be expected. Similarly, the deletion of M3 plus M4 resulted in a 4.1-fold increase in expression. The 2.9- and 2.6-fold increases caused by the individual deletions of M3 and M4 would be expected to give a 7.5-fold increase for an additive effect. The deletion of all three functional sites, M2, M3, and M4, resulted in a 4.7-fold increase in *HEM13-lacZ* expression compared to the 11-fold increase expected from independent function. Thus, there is no evidence that the Mot3 sites, despite their proximity, function synergistically. Also, we can conclude that M2, M3, and M4 account for all of the Mot3 repression activity at *HEM13* because the deletion of these three sites in combination resulted in the same level of expression in wild-type and

TABLE 4. Effect of Rox1 binding site deletions on expression of the *HEM13-lacZ* fusion

Site(s) deleted in YCp(33)*HEM13Z*[b]	Result for genotype[a]:			Fold derepression[c]	Mot3 effect[d]
	Wild type	*mot3*Δ	*rox1*Δ		
Wild type (none)	4.9 ± 0.8	25.4 ± 3.6	91 ± 11		5.2
R1	4.6 ± 0.1	32.0 ± 1.3	56 ± 0	1.5	7.0
R2	3.9 ± 0.3	30.9 ± 0.5	147 ± 9	0.5	7.8
R1, R2	18.9 ± 0.7	122 ± 26	253 ± 14	1.4	6.5
R3, R4	11.1 ± 0.6	39.2 ± 1.5	32 ± 4	6.3	3.6
R1, R2, R3	44.1 ± 2.7	127 ± 3	165 ± 7	4.9	2.9
R1, R2, R4	20.1 ± 0.5	100 ± 10	206 ± 20	1.8	4.8
R1, R2, R3, R4	46.6 ± 7.1	130 ± 19	148 ± 21	6.0	2.8

[a] The enzyme assays were carried out in extracts from derivatives of RZ53-6. The numbers shown represent Miller units ± standard deviations.
[b] Sites R1 through R4 are shown in Fig. 1.
[c] Relative derepression was calculated by taking the ratio of *rox1*Δ to the wild type for the mutant plasmid and dividing by the ratio of *rox1*Δ to the wild type for the wild-type (none) plasmid.
[d] The Mot3 effect is the ratio of expression of *mot3*Δ to that of the wild type.

*mot3*Δ cells (a 1.1 ratio), indicating that there are no cryptic Mot3 repression sites.

Also, as expected, the deletion of the nonbinding M5 in combination with any of the functional Mot3 sites did not increase derepression further, confirming that this site has no repression activity.

If Mot3 potentiates repression of Rox1 at site R3, then Rox1 should potentiate repression by Mot3. Again this effect can be visualized through the ratio of repression by Rox1 (enzyme activity in *rox1*Δ divided by that in wild-type cells) for the wild-type plasmid to that of the deletion plasmids. This ratio is 15-fold for the wild-type plasmid and only 6-fold for the plasmid containing the triple M2M3M4 deletion, suggesting that Rox1 repression is increased in the presence of Mot3. Furthermore, this ratio varies between 9 and 10 in the three double deletions involving these three sites, suggesting that all three sites must be present to achieve the full Rox1-Mot3 interaction. Thus, the results of these deletion analyses presented in Tables 4 and 5 strongly suggest that Mot3 and Rox1 function to help each other repress but that this interaction requires close proximity of the Rox1 and Mot3 binding sites. While this conclusion is similar to that reached in a deletion analysis of *ANB1* OpA (10, 27), in that case, Mot3 promoted a more dramatic synergy between two close Rox1 sites, where in this case, the effect involves a single Rox1 site.

Mot3 recruits Ssn6/Tup1 to enhance Rox1 repression at *ANB1*. Because Mot3 functioned to repress *HEM13* in the absence of Rox1, it seemed likely that Mot3 could recruit the Ssn6/Tup1 repression complex in the absence of Rox1. This conclusion agreed with the results of Sertil et al. (35) that overexpression of Mot3 could repress genes in the absence of Rox1 and that this repression depended on Ssn6 and Tup1. Also, ChIP experiments indicated that Mot3 could recruit Ssn6 to both *ANB1* and *HEM13* in *rox1*Δ cells at physiological concentrations, albeit at lower than wild-type levels, and of course, the complex was not functional in repression at *ANB1*. Is Mot3's recruitment of the repression complex also responsible for its ability to promote synergy between the closely spaced Rox1 sites of OpA in *ANB1* and the interaction between the Rox1 R3 site and Mot3 sites in *HEM13*? We tested this pos-

TABLE 5. Effect of Mot3 binding site deletions on expression of the *HEM13-lacZ* fusion

Site(s) deleted in YCp(33)*HEM13Z*[b]	Result for genotype[a]:			Fold derepression[c]	Rox1 effect[d]
	Wild type	*mot3*Δ	*rox1*Δ		
Wild type (none)	4.9 ± 0.8	25.4 ± 3.6	91 ± 11		15.2
M2	8.3 ± 0.6	29.5 ± 1.5	120 ± 12	1.5	14.4
M3	11.5 ± 1.6	21.0 ± 1.3	130 ± 6	2.9	11.4
M4	15.8 ± 1.0	30.9 ± 0.7	130 ± 10	2.6	8.3
M5	4.2 ± 0.5	18.8 ± 2.5	57 ± 6	0.7	13.4
M2, M3	22.1 ± 0.6	35.7 ± 1.0	230 ± 4	3.3	10.4
M2, M4	18.7 ± 0.5	35.4 ± 1.4	167 ± 8	2.7	9.0
M3, M4	23.1 ± 1.5	29.5 ± 1.0	218 ± 8	4.1	9.4
M3, M5	14.7 ± 0.8	30.5 ± 0.1	153 ± 6	2.6	10.4
M4, M5	11.5 ± 0.1	30.0 ± 1.1	110 ± 7	2.0	9.6
M2, M3, M4	31.1 ± 2.6	34.2 ± 1.7	185 ± 16	4.7	5.9
M2, M3, M5	17.8 ± 2.0	32.3 ± 0.9	178 ± 12	2.9	10.0
M3, M4, M5	15.9 ± 1.5	20.0 ± 1.6	159 ± 15	4.1	10.0
M2, M3, M4, M5	20.6 ± 0.3	26.4 ± 0.5	97 ± 2	4.1	4.7
+1	5.6 ± 0.4	23.3 ± 0.7	85 ± 4	1.2	15.1

[a] The enzyme assays were carried out in extracts from derivatives of RZ53-6. The numbers shown represent Miller units ± standard deviations.
[b] Sites M1 through M5 are shown in Fig. 1.
[c] Relative derepression was calculated by taking the ratio of *mot3*Δ to the wild type for the mutant plasmid and dividing by the ratio of *mot3*Δ to the wild type for the wild-type (none) plasmid.
[d] The Rox1 effect is the ratio of expression of *rox1*Δ to that of the wild type.

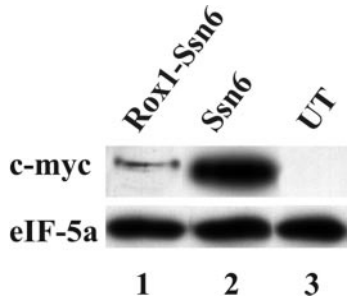

FIG. 3. The Rox1-Ssn6 fusion is expressed at a lower level than native Ssn6. Total cellular protein was prepared from aerobically grown RZ53-6Δ*rox1* cells transformed with YCp(33)*R1-S6e* expressing the c-*myc* epitope-tagged Rox1-Ssn6 fusion from the *ROX1* promoter (lane 1), YCp(22)*SSN6e* expressing c-*myc* epitope-tagged Ssn6 from the *SSN6* promoter (lane 2), or YCplac33 (UT, lane 3). The samples were subjected to an immunoblot and probed first with anti c-*myc* monoclonal antibody, stripped, and then reprobed with polyclonal antisera against eIF-5a.

sibility by creating a fusion between the coding sequences of the Rox1 DNA binding domain and Ssn6 tagged with the c-*myc* epitope. If Mot3 enhanced repression solely by helping Rox1 recruit the Ssn6/Tup1 complex, then this fusion protein would obviate the need for Mot3. However, if Mot3 performed an additional, Ssn6/Tup1 recruitment-independent function, then full repression would still require Mot3. It should be noted that the DNA binding domain of Rox1 that was present in this fusion has no repression activity without the remainder of the protein (9).

Initially, we determined the level of expression of the fusion protein compared to that of Ssn6. The fusion protein was expressed from the *ROX1* promoter and carried a c-*myc* epitope tag near the C terminus of Ssn6. Cells were transformed with this plasmid or a plasmid carrying an epitope-tagged *SSN6* expressed from its native promoter, and extracts were subjected to immunoblot analysis. The results, presented in Fig. 3, indicated that the fusion was expressed at one-seventh the level of the native Ssn6 protein.

The ability of the fusion to complement a *rox1*Δ mutant was tested. A plasmid carrying the fusion was transformed into *rox1*Δ, *rox1*Δ *mot3*Δ, and *rox1*Δ *tup1*Δ cells, each containing an integrated *ANB1-lacZ* fusion. As evident from the data presented in Table 6, the fusion repressed aerobic *ANB1-lacZ* expression as well as the wild-type Rox1 (comparing enzyme activities in the *rox1*Δ cells transformed with a wild-type *ROX1* or the fusion plasmid). Furthermore, this repression was dependent on Tup1 (comparing enzyme activities in the *rox1*Δ cells versus the *rox1*Δ *tup1*Δ cells).

TABLE 6. Repression by the Rox1-Ssn6 fusion

Genotype[a]	Result for:	
	ROX1[b]	*ROX1-SSN6*[c]
SSN6		
*rox1*Δ	3.3 ± 0.9	0.9 ± 0.1
*rox1*Δ *mot3*Δ	14 ± 1.4	4.3 ± 0.3
*rox1*Δ *tup1*Δ	67 ± 10	143 ± 28
*ssn6*Δ		
*rox1*Δ *ssn6*Δ	103 ± 5	4.7 ± 0.2
*rox1*Δ *mot3*Δ *ssn6*Δ	128 ± 12	5.9 ± 0.9

[a] The enzyme assays were carried out in extracts from cells containing an integrated *ANB1-lacZ* fusion in derivatives of MZ22-4 for *SSN6* or KZ211 for *ssn6*Δ. The numbers shown represent Miller units ± standard deviations.
[b] *ROX1* denotes transformants expressing a wild-type *ROX1* plasmid.
[c] *ROX1-SSN6* denotes transformants expressing the *ROX1-SSN6* fusion.

Repression by the fusion plasmid was still enhanced by Mot3, as evident from the 4.8-fold decrease in repression in the *rox1*Δ *mot3*Δ transformants compared to the *rox1*Δ transformants. This effect is similar to the 4.3-fold loss of repression in the *rox1*Δ *mot3*Δ cells compared to the *rox1*Δ cells transformed with the wild-type *ROX1* plasmid. However, since these cells contained a genomic wild-type *SSN6* allele, it was possible that the Mot3 effect resulted from its recruitment of an additional Ssn6 to the DNA. To determine whether the fusion construct could support full repression in the absence of the possibility for additional Ssn6 recruitment by Mot3, it was transformed into *rox1*Δ *ssn6*Δ and *rox1*Δ *ssn6*Δ *mot3*Δ cells. As can be seen in Table 6, repression was somewhat weaker in the *ssn6*Δ transformants than in those carrying a wild-type *SSN6* gene but was similar to that of the *SSN6* cells carrying the *mot3*Δ allele. Furthermore, deletion of *MOT3* in the *ssn6*Δ background did not further weaken repression. The difference between the effect of Mot3 on the fusion with or without native Ssn6 was probably a result of the sevenfold-lower expression of the fusion; Mot3 could not recruit the Rox1-Ssn6 fusion at the lower level of Ssn6 in the cell. Thus, when unable to recruit Ssn6, Mot3 could not enhance repression. Consequently, we believe that the role of Mot3 is to help Rox1 recruit Ssn6 and help stabilize the Rox1-Ssn6 complex. The same strains transformed with the wild-type *ROX1* plasmid were, of course, completely derepressed for *ANB1-lacZ* expression due to the absence of free Ssn6 from the cell.

Rox1 stabilizes Mot3 binding to DNA. The ability of Mot3 to repress *HEM13-lacZ* expression in the absence of Rox1 raised a question concerning the rapid induction of *HEM13* upon anaerobiosis (29). We previously reported that the kinetics of *ANB1* and *HEM13* induction correlated with the disappearance of Rox1 from the cell, but while *MOT3* expression is regulated by oxygen availability at the level of transcription, Mot3 dissociated from *ANB1* and *HEM13* well before its protein disappeared from the cell during induction (29, 35). This observation suggested that there may be an active mechanism that promotes the release of Mot3 from the hypoxic repression complex prior to the drop in cellular Mot3 protein levels. We explored this possibility by fusing the *MOT3* coding region followed by four copies of the HA epitope to the *TUP1* promoter. *TUP1* is expressed constitutively, so this construct expressed Mot3-HA independent of oxygen availability but without severe overexpression. The constitutive expression of this pT1-M3-HA was confirmed by Western analysis. RZ53-6Δ*mot3* cells carrying YCp(22)pT1-M3-HA or YCp(23) *MOT3-HA* were grown aerobically and anaerobically for 1, 2, and 4 h to mid-exponential phase. An untagged control, consisting of cells carrying YCp(22)*MOT3*, was grown aerobically. Crude extracts were prepared and subjected to immunoblot analysis. As seen in Fig. 4A, Mot3-HA was expressed at lower levels aerobically from its native promoter than that from the *TUP1* promoter (lanes 1 and 5, respectively), but the difference was not excessive. As reported previously (29), when under the control of its native promoter, the amount of Mot3-HA

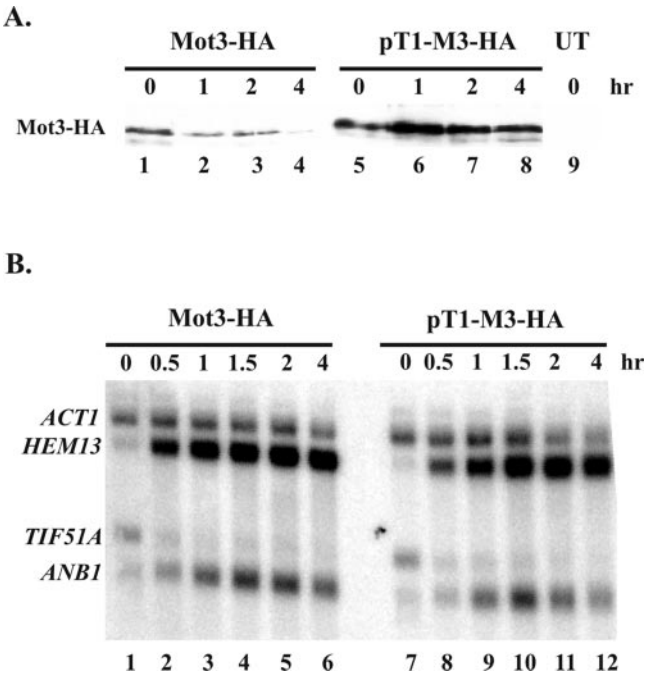

FIG. 4. Constitutively expressed Mot3 does not affect the rate of hypoxic gene induction. (A) RZ53-6Δ*mot3* cells containing Mot3-HA expressed from its native promoter (Mot3-HA, lanes 1 to 4) or constitutively expressed Mot3-HA (pT1-M3-HA, lanes 5 to 8) were grown aerobically (0) or anaerobically for the number of hours indicated (1, 2, and 4), and RZ53-6Δ*mot3* cells containing untagged Mot3 (UT, lane 9) were grown aerobically. Crude protein extracts were prepared and subjected to an immunoblot with monoclonal antibody against the HA epitope. Equal loading of the samples was determined by Ponceau S staining (data not shown). (B) Total cellular RNA was prepared from RZ53-6Δ*mot3* cells containing Mot3 expressed from its native promoter (Mot3-HA, lanes 1 to 6) or constitutive Mot3 (pT1-M3-HA, lanes 7 to 12) grown to mid-exponential phase aerobically (0) or anaerobically for the number of hours indicated (0.5, 1, 1.5, 2, and 4). RNA was hybridized with ^{32}P-labeled probes to *ANB1*, *HEM13*, and *ACT1* (as a loading control). The positions of the specific RNAs are indicated to the left of the blot. While a probe to *TIF51A* was not used, its high degree of sequence similarity to *ANB1* resulted in cross-hybridization.

present in the cell decreased slowly during anaerobiosis to nearly undetectable levels after 4 h (Fig. 4A, lanes 1 to 4). On the other hand, constitutively expressed Mot3-HA was relatively unaffected by hypoxia; protein levels remained at or above the aerobic level throughout anaerobic growth (lanes 5 to 8).

Despite the constitutive, somewhat higher expression, Mot3-HA did not greatly alter the rate of induction of *ANB1* or *HEM13*. RZ53-6Δ*mot3* cells carrying YCp(22)pT1-M3-HA or YCp(23)*MOT3-HA* were grown aerobically or anaerobically for 0.5, 1, 1.5, 2, and 4 h to mid-exponential phase, and total RNA was prepared and subjected to Northern analyses. The blot was probed for the hypoxic RNAs of *HEM13* and *ANB1* and for that of unregulated *ACT1* as a loading control. Due to the high sequence similarity shared between *ANB1* and its aerobically expressed paralog, *TIF51A*, cross-hybridization of the *ANB1* probe was observed and served as an internal control for hypoxia. The rates of induction for *ANB1* were nearly indistinguishable in cells expressing Mot3-HA from its own promoter (Fig. 4B, lanes 1 to 6) compared to cells expressing Mot3-HA constitutively (Fig. 4B, lanes 7 to 12). *HEM13* in the same lanes showed only a minor delay in induction.

To determine whether the constitutively expressed Mot3-HA dissociated from the hypoxic genes during induction, we performed ChIP analysis. RZ53-6Δ*mot3* cells carrying YCp(22)pT1-M3-HA were grown aerobically and anaerobically for 1, 2, and 4 h; cells carrying YCp(22)*MOT3* were grown aerobically as a negative, untagged control. These cells were harvested for ChIP, and the upstream repression sequences of *ANB1* and *HEM13* were amplified by PCR. The results presented in Fig. 5 show that the levels of Mot3-HA bound to *ANB1* and *HEM13* in cells grown aerobically (hour zero) were significantly higher than those of the negative untagged Mot3 control, approximately 5-fold and 10-fold, respectively (Fig. 5A, compare time zero and UT). The levels of immunoprecipitated *ANB1* decreased steadily during induction, as shown in Fig. 5B, despite the constant levels of Mot3-HA in the cells. Interestingly, Mot3-HA did not appear to dissociate from *HEM13* (Fig. 5C). We believe that this apparent discrepancy between the two genes reflects the larger number of Mot3 sites at *HEM13*, one site in *ANB1*, and three functional sites in *HEM13*. Since only one bound molecule is sufficient for immunoprecipitation by ChIP, dissociation could occur to a significant degree at a gene with three sites but not be easily detected. This would explain the lack of an effect on *HEM13* induction. One Mot3 bound may be sufficient for immunoprecipitation, but it is not sufficient for repression.

It is possible that there is a specific mechanism that removes Mot3 from DNA during hypoxia or that Mot3 binding at *ANB1* requires Rox1, which disappears from the cell very rapidly upon the onset of hypoxia (8, 29). We distinguished between these two possibilities by comparing the immunoprecipitation of *ANB1* DNA with Mot3-HA in aerobically grown wild-type versus *rox1*Δ cells. As seen in Fig. 6, Mot3-HA was stably associated with the *ANB1* regulatory region in wild-type cells but not in the *rox1*Δ cells. Quantitation indicated that four times more *ANB1* immunoprecipitated with the HA antibody in the wild-type versus mutant cells. The same samples showed only a twofold difference in the amount of *HEM13* immunoprecipitated, once again demonstrating the persistence of Mot3 at this gene. These results indicate that the dissociation of Mot3 from *ANB1* and, to a lesser extent from *HEM13*, correlated with the dissociation of Rox1 and indicates that Rox1 stabilizes Mot3 binding.

DISCUSSION

A comparison of the similarities and differences in the arrangement of the Rox1 and Mot3 sites in the *ANB1* and *HEM13* genes provides some insight into how these sites interact and how they may function. First, the strongest repression in both genes is mediated by Rox1 and Mot3 sites in close proximity. From a previous study, we found that OpA of *ANB1*, which consists of two Rox1 sites 31 bp apart with a Mot3 site in between, was responsible for 76-fold repression, while OpB, which consists of two Rox1 sites 21 bp apart without a Mot3 site effects only an eightfold repression (10). Deletion of or point mutations in the Mot3 site of OpA reduced repression to about fivefold. Elimination of one of the Rox1 sites of OpA reduced repression to eightfold. Thus, the Mot3

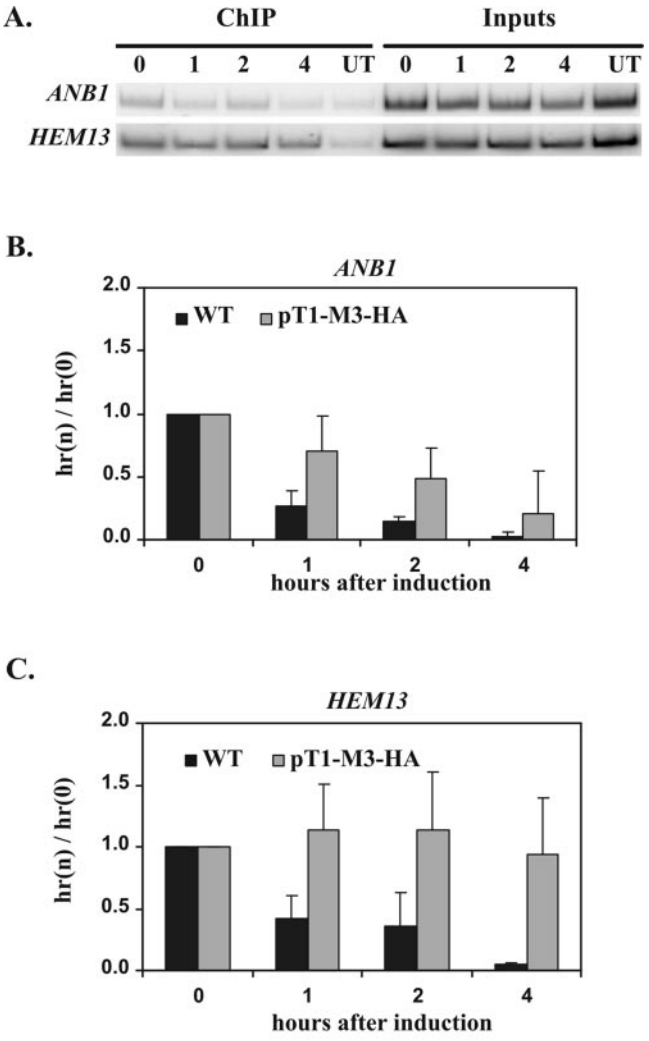

FIG. 5. Constitutively expressed Mot3 dissociates from *ANB1*, but not *HEM13*, during hypoxia. (A) ChIP assays with monoclonal antibody against the HA epitope were performed with RZ53-6Δ*mot3* cells containing YCp(23)pT1-M3-HA expressing Mot3-HA constitutively or YCp(22)*MOT3* expressing untagged Mot3 (UT). pT1-M3-HA cells were grown aerobically (0) and anaerobically for the number of hours indicated (1, 2, and 4). DNA samples isolated from the immunoprecipitation (ChIP) and prior to immunoprecipitation (Input) were amplified by PCR with oligonucleotide primers to the *ANB1* and *HEM13* regulatory regions. [α-^{32}P]dATP was added to the PCR mixtures, and the labeled products were fractionated by PAGE. The bands were visualized and quantitated by using a STORM PhosphorImager and the ImageQuant software package. (B and C) The radioactivity in the ChIP samples for *ANB1* (B) and *HEM13* (C) was normalized as follows. First, the input samples were normalized by dividing the radioactivity in each input band by that in the hour 0 (aerobic) input sample. Then, each ChIP sample was normalized to its input by dividing the ChIP sample by the normalized input sample. The untagged sample was subtracted from each of the samples with epitope-tagged protein, and finally, the ChIP samples were normalized to the hour 0 sample by dividing each normalized ChIP sample by the normalized hour 0 sample. These normalized ChIP values were plotted as histograms (pT1-M3-HA, gray bars) and represent an average of the results from at least four independent experiments. The data presented for the wild-type (WT, black bars) represent Mot3-HA expressed from its native promoter throughout the hypoxic induction and have been reproduced from Mennella et al. for the sake of comparison (29).

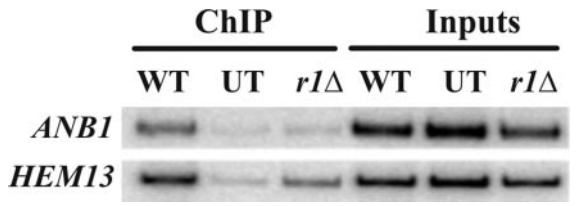

FIG. 6. Rox1 is required for Mot3 binding to the *ANB1* gene and, to a lesser extent, to the *HEM13* gene. ChIP assays with monoclonal antibody against the HA epitope were performed with RZ53-6Δ*mot3* transformed with YCp(23)*MOT3-HA* (WT) or YCp(23)*MOT3* (untagged, UT) and with RZ53-6Δ*rox1*Δ*mot3* cells transformed with YCp(23)*MOT3-HA* (*rox1*Δ). Cells were grown aerobically to mid-exponential phase. DNA samples were prepared before (Input) and after (ChIP) immunoprecipitation. The radioactivity in the bands was quantitated and normalized as described in the legend to Fig. 5.

site acted synergistically with the two Rox1 sites to give the strong repression of *ANB1*. This present study demonstrated that the strongest Rox1 site in *HEM13* was R3, which is flanked by three functional Mot3 sites, all located within 70 bp. The three Mot3 sites acted additively to increase repression by R3. Second, the naturally occurring *ANB1* OpA combination of two Rox1 sites plus a single Mot3 site represses much more strongly than the *HEM13* combination of one Rox1 site and three Mot3 sites. Based upon in vitro binding studies, there appears to be no large difference in the strength of these different Rox1 or Mot3 sites in these genes, and therefore, we believe that the difference in repression is due to the different combinations according to the following rules. Multiple Mot3 sites or multiple Rox1 sites function additively, as in the case of the Mot3 sites in *HEM13* in a *rox1*Δ strain and the Rox1 sites in *ANB1* OpB or OpA with the Mot3 site deleted or the wild-type OpA in a *mot3*Δ strain. The sites in combination function more than additively, but the overall synergy depends on the combination of sites. Multiple Mot3 sites plus a single Rox1 site are much weaker than multiple Rox1 sites plus a single Mot3 site. The different combinations of sites in *ANB1* and *HEM13* achieve the purpose of differential repression, with much greater repression of *ANB1* than of *HEM13*.

We demonstrated here that Mot3 functions to potentiate Rox1 repression by helping in the recruitment of the Ssn6/Tup1 general repression complex. The ability of Mot3 to recruit the Ssn6/Tup1 complex has been demonstrated before. Great overexpression of Mot3 resulted in *ANB1* repression even in the absence of Rox1 (35), and ChIP experiments with antibody against Ssn6 can bring down the *ANB1* gene in the absence of Rox1 but not in the absence of both Rox1 and Mot3 (29). Our present study confirms these findings and extends them to demonstrate that Mot3 function is not required for some additional step in repression. A fusion of Ssn6 to the DNA binding domain of Rox1 eliminated the Mot3 effect on repression. Previous in vitro studies had shown that Rox1 and Mot3 did not bind cooperatively to DNA; in gel retardation assays with purified Rox1 and Mot3, there was no increased binding when the proteins were added together versus separately. Thus, Mot3 does not function by increasing Rox1 binding directly (20). Our present finding reinforces this conclusion; if Mot3 functioned through a cooperative interaction with DNA and Rox1 that increased Rox1 recruitment to the *ANB1*

gene in vivo, Mot3 should still have increased recruitment of the Rox1-Ssn6 fusion. Similarly, if Mot3 functioned to enhance some step post-Ssn6/Tup1 recruitment, it still should have increased repression of the fusion construct.

The general repression complex is comprised of four Tup1 molecules and one Ssn6 molecule (39, 40). Rox1 interacts most strongly with Ssn6 (38), and ChIP experiments indicated that Ssn6 mediated the recruitment of the general repression complex to the hypoxic genes in vivo; Ssn6 can be recruited to *ANB1* and *HEM13* in the absence of Tup1, but Tup1 cannot be recruited in the absence of Ssn6. Thus, it appears that both Rox1 and Mot3 interact with Ssn6. The Ssn6 protein contains 10 TPR repeats that comprise the functional domain of the protein and have been implicated in the interaction with Tup1 and the various regulon-specific DNA binding repressors that recruit the general repression complex (26, 38). Each protein interacts with a specific subset of TPR repeats, and Rox1 is proposed to interact with repeats four through seven (38). It is not known with which repeats Mot3 interacts. Also, we do not know the stoichiometry of the complex at the hypoxic genes in terms of the number of general repression complexes recruited by each Rox1 molecule and each Mot3 molecule and whether the increased repression results from increased numbers of Ssn6/Tup1 complexes recruited, increased stability of a fixed number of complexes, or both. However, we believe that the experiment with the Rox1-Ssn6 fusion provides some insight to these questions. Mot3 enhanced repression of the fusion protein in *SSN6* when it could recruit Ssn6 but not in an *ssn6*Δ background when it could not. Consequently, it seems likely that Mot3 functions to recruit an additional Ssn6. Nonetheless, there must be some level of interaction between the repression complexes recruited by Rox1 and Mot3 for the following reasons. First, Mot3 promotes synergy with Rox1 sites, and second, Rox1 helps in Mot3 binding. It is unclear at this point whether the higher-order interaction of these independently recruited Ssn6 molecules results from an Ssn6-Ssn6 interaction, Tup1 interactions, or a change in the stoichiometry of the Ssn6-Tup1 interaction.

Finally, we show here that Mot3 dissociated from *ANB1* under anaerobiosis even when constitutively expressed. We believe that this dissociation resulted from the loss of Rox1; ChIP analysis showed that less Mot3 bound to the *ANB1* regulatory region in *rox1*Δ cells than in wild-type cells. Based upon these data, we propose that Mot3 binding to DNA is weak and is stabilized through the interactions of Rox1 and Mot3 with the general repression complex. In the same experiments, constitutively expressed Mot3 persisted at *HEM13*, and Mot3 binding to *HEM13* was reduced only twofold in the absence of Rox1, presumably due to the multiple Mot3 sites in this region. The Mot3 occupancy at *HEM13* in the *rox1*Δ mutant also explains the Rox1-independent repression observed at this gene but not at *ANB1*. Finally, it should be noted that in the absence of Mot3, the hypoxic genes were induced at a faster rate, again suggesting that Mot3 stabilizes the Rox1-Ssn6/Tup1 complex (29).

From the conclusions presented above, we propose the following model for the interactions between Rox1 and Mot3. The combination of Rox1 and Mot3 bound close together within a regulatory region results in synergy due to interactions through the general repression complex. Multiple Rox1 molecules bound without Mot3 provide only additive repression, perhaps due to an inability to promote interactions between independently bound repression complexes. Similarly, multiple Mot3 molecules bound without Rox1 can only function additively. Also, we propose that Rox1 binds more stably to DNA than Mot3, so that Mot3 dissociates from the DNA rapidly upon the onset of hypoxia due to the rapid dissociation of Rox1. Even if Mot3 were to persist in the cell, as in the case of the constitutively expressed Mot3, Rox1 dissociation would promote Mot3 dissociation. Consequently, Rox1 alone is a stronger repressor than Mot3 alone, and the combination of Rox1 and Mot3 is stronger still. Thus, the cell has an array of combinatorial repressor sites that it can use to effect differential repression of the hypoxic genes.

Interestingly, we have also found that Mot3 binding requires more than the degenerate 6-bp consensus sequence. Sites M1 and M5, which conform to this sequence, did not bind Mot3 in vitro. What the additional requirements are were not apparent from a comparison of the sequence surrounding the 6-bp sites.

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