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Roles of Transcription Factor Mot3 and Chromatin in Repression of the Hypoxic Gene *ANB1* in Yeast

ALEXANDER J. KASTANIOTIS, THOMAS A. MENNELLA, CHRISTIAN KONRAD, ANA M. RODRIGUEZ TORRES,† AND RICHARD S. ZITOMER*

Department of Biological Sciences, University at Albany/SUNY, Albany, New York 12222

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The hypoxic genes of *Saccharomyces cerevisiae* are repressed by a complex consisting of the aerobically expressed, sequence-specific DNA-binding protein Rox1 and the Tup1-Ssn6 general repressors. The regulatory region of one well-studied hypoxic gene, *ANB1*, is comprised of two operators, OpA and OpB, each of which has two strong Rox1 binding sites, yet OpA represses transcription almost 10 times more effectively than OpB. We show here that this difference is due to the presence of a Mot3 binding site in OpA. Mutations in this site reduced OpA repression to OpB levels, and the addition of a Mot3 binding site to OpB enhanced repression. Deletion of the *mot3* gene also resulted in reduced repression of *ANB1*. Repression of two other hypoxic genes in which Mot3 sites were associated with Rox1 sites was reduced in the deletion strain, but other hypoxic genes were unaffected. In addition, the *mot3*Δ mutation caused a partial derepression of the Mig1–Tup1-Ssn6-repressed *SUC2* gene, but not the α2–Mcm1–Tup1-Ssn6-repressed *STE2* gene. The Mot3 protein was demonstrated to bind to the *ANB1* OpA in vitro. Competition experiments indicated that there was no interaction between Rox1 and Mot3, indicating that Mot3 functions either in Tup1-Ssn6 recruitment or directly in repression. A great deal of evidence has accumulated suggesting that the Tup1-Ssn6 complex represses transcription through both nucleosome positioning and a direct interaction with the basal transcriptional machinery. We demonstrate here that under repressed conditions a nucleosome is positioned over the TATA box in the wild-type *ANB1* promoter. This nucleosome was absent in cells carrying a *rox1*, *tup1*, or *mot3* deletion, all of which cause some degree of derepression. Interestingly, however, this positioned nucleosome was also lost in a cell carrying a deletion of the N-terminal coding region of histone H4, yet *ANB1* expression remained fully repressed. A similar deletion in the gene for histone H3, which had no effect on repression, had only a minor effect on the positioned nucleosome. These results indicate that the nucleosome phasing on the *ANB1* promoter caused by the Rox1–Mot3–Tup1-Ssn6 complex is either completely redundant with a chromatin-independent repression mechanism or, less likely, plays no role in repression at all.

Transcriptional repression in eukaryotic cells often involves the assemblage of large complexes that repress through active mechanisms such as direct interactions with the basal transcriptional machinery and organization of chromatin into repressive structures (16, 18, 34). The repression of the hypoxic genes in baker's yeast provides an example of such a complex involving the DNA-binding protein Rox1 and the general repression complex Tup1-Ssn6 (20, 46, 47). Our studies have focused on a number of aspects of hypoxic gene regulation, including how differential levels of repression of the hypoxic genes are achieved, how the repression complex forms on the DNA, and how the complex inhibits transcription.

The hypoxic genes encode oxygen-related functions in respiration, heme, and membrane biosynthesis that are required at higher levels when molecular oxygen is limiting (46, 47). The expression of these genes is repressed under aerobic conditions by Rox1 binding to their regulatory regions (2, 5, 7). To achieve this oxygen-dependent repression, the *ROX1* gene is transcriptionally induced aerobically and repressed anaerobically (2, 6). The level of Rox1-dependent repression of different hypoxic genes is variable, and we have divided these genes into two classes in terms of the strength of repression. The first includes unique genes that encode functions required under aerobic conditions, such as *HEM13*, *OLE1*, *ERG11*, and the autorepressed *ROX1* itself. Because they are required aerobically, these genes can only be partially repressed. The second includes genes that have an aerobic homologue, such as *HMG1-2*, *COX5A-5B*, *AAC1-2-3*, and *TIF51A-ANB1* (where the first gene is the aerobic and the last is the hypoxic homologue). These genes can be completely repressed. Variations in the quality and number of the Rox1 binding sites in the regulatory regions of the hypoxic genes contribute to this differential repression, but this is not the complete explanation (7). Our extensive analysis of one strongly repressed hypoxic gene, *ANB1*, revealed that there are two operators upstream of this gene, each consisting of two Rox1 sites in close proximity (7, 24). All four sites bind Rox1 with similar affinities, but the upstream operator, OpA, represses almost 10 times more effectively than does OpB, which is closer to the TATA box. This difference is not due to the location of these sites, but is a function of some intrinsic property of their sequences. We report here that this difference is due to the presence of a binding site for the protein Mot3 in OpA. This site is present in some but not all Rox1-repressed genes. Furthermore, we provide evidence here that Mot3 functions by either aiding in the recruitment of a general repression complex to the *ANB1* promoter or helping the general repression complex function.

Rox1-dependent repression also requires the general repression complex Tup1-Ssn6 (2, 45). This complex has no DNA-binding activity, but rather interacts with a variety of regulon-specific DNA-binding proteins to target specific genes for

* Corresponding author. Mailing address: Department of Biological Sciences, University at Albany/SUNY, Albany, NY 12222. Phone: (518) 442-4385. Fax: (518) 442-4767. E-mail: rz144@csc.albany.edu.

† Permanent address: Department de Biologia Celular y Molecular, Univ. de la Coruna, Campus de La Zapateira sln, 15071 La Coruna, Spain.

repression. These regulons include, in addition to the hypoxic genes, the **a** mating type and haploid-specific genes, the glucose-repressed genes, DNA damage-inducible genes, flocculence genes, and others (10, 12, 21, 26, 30, 32, 41, 42). Two alternate mechanisms for Tup1-Ssn6-dependent repression have been proposed. There is ample evidence for the ability of this complex to organize chromatin (4, 8, 9, 23, 27, 36, 37, 39). Nucleosomes are phased by Tup1-Ssn6 in some repressed genes. This phasing is probably achieved through the ability of Tup1 to interact with hypoacetylated histones H3 and H4. The importance of this phasing has been demonstrated by the observation that deletions of the N-terminal coding region of either of these two histones caused a partial derepression of some Tup1-Ssn6-repressed genes. Finally, the TATA-binding protein (TBP) is excluded from binding to the TATA box by the Tup1-Ssn6 complex, consistent with a model of a positioned nucleosome blocking TBP access. On the other hand, there is evidence that Tup1-Ssn6 interacts directly with the basal transcriptional machinery (22, 33, 35, 40, 44). Anchoring either Tup1 or Ssn6 to DNA can inhibit transcription of chromatin-free DNA in vitro. Mutations have been isolated in the RNA polymerase II mediator complex that cause derepression of some Tup1-Ssn6-repressed genes, indicating a genetic interaction. While it may be possible that these two alternate repression mechanisms have some common components, at this point the link is not obvious, and we assume that they represent alternate and, for some genes, redundant mechanisms. In this study, we provide evidence for this view for *ANB1*. Nucleosomes show Tup1-, Rox1-, Mot3-, and histone H4-dependent phasing on the *ANB1* regulatory region, but while deletion of *TUP1*, *ROX1*, or *MOT3* results in at least partial loss of repression, deletion of the N-terminal coding sequence of H4 does not.

MATERIALS AND METHODS

Strains and growth conditions. The strain RZ53-6 (*MAT*α *trp1-289 leu2-3,112 ura3-52 ade1-100*) and the RZ53-6Δ*rox1* and RZ53-6Δ*tup1* derivatives have been described (5, 45). RZ53-6Δ*mot3* and RZ53-6Δ*rlm3* were derived from the wild type and Δ*rox1* strains, respectively, by displacement of the *MOT3* gene with the *mot3*::*kanMX* construct described below. The strain P1/I8 contained deletions of both the *HHT1-HHF1* and *HHT2-HHF2* loci, which encode the isoforms of histones H3 and H4 (31). The cells were maintained carrying plasmids encoding the wild-type *HHT1-HHF1* genes or the N-terminal deletions of histone H3 (*hht1-2-HHF1*) or H4 (*HHT1-hhf1-8*). An *srb10*Δ allele was transformed into this strain using the p*srb10*::*LYS2* plasmid described below.

Cells were grown at 30°C (with vigorous shaking for liquid cultures) on either rich YPD medium or SC medium lacking the appropriate nutrient when selection for plasmid maintenance was required (19). Yeast transformants were carried out as described (19). When cells were transformed with a *kanMX*-containing fragment, they were initially plated on YPD and incubated at 30°C for 6 h. Then a 5-ml overlay of YPD (0.7% agar) containing 8 mg of geneticin was applied.

Plasmids. All plasmid constructions were carried out using standard techniques (1). Enzymes were purchased from New England Biolabs and used as recommended by the vendor. PCRs were carried out with *Taq* polymerase (Perkin-Elmer) as recommended by the vendor. Genomic DNA for PCRs was prepared from RZ53-6 as described (19). Genomic sequences were obtained from the *Saccharomyces* Genome Database maintained at Stanford University. The sequence for a given gene is numbered with the first A in the ATG initiation codon as +1; bases 5′-wards are numbered negatively, and those 3′-wards are numbered positively.

YEp(112)*ANB1* and YEp(195)*ANB1* contained the 2.4-kb *Bam*HI-*Hin*dIII fragment carrying the *ANB1-CYC1* genes (28) cloned into the *Hin*dIII and *Bam*HI sites of YEplac112 and YEplac195, respectively (13). The *STE2-lacZ* plasmid has been described (17).

The p*mot3*::*kanMX* plasmid used to generate *mot3*Δ yeast strains was constructed as follows. A genomic fragment of the *MOT3* coding sequence plus 775 bp upstream and 490 bp downstream was generated by PCR with *Hin*dIII and *Bam*HI restriction sites at either end. This fragment was cloned into the *Hin*dIII and *Bam*HI sites of pBSM13 (Stratagene, Inc.), creating pBS*MOT3*. This plasmid was digested with *Stu*I (−160 of *MOT3*) and *Sac*II (140 bp preceding the termination codon of the *MOT3* gene), and the *MOT3* coding sequence released was replaced with a 1.5-kb *Sma*I-*Sac*II fragment containing the *kanMX* gene obtained by PCR from pFA6a*kanMX4* (43). A *Sna*BI-*Bam*HI fragment was used to transform yeast cells to generate the *mot3*Δ strains. The deletions were confirmed by PCR.

The plasmid p*srb10*::*LYS2* was constructed as follows. The 4-kb *Pst*I-*Bgl*II *SRB10* gene fragment was subcloned into the *Pst*I and *Bam*HI sites of pUC9 (1). A 5.7-kb *Sph*I-*Sma*I fragment of *LYS2* was inserted into the *Eco*RV and *Sph*I sites of this plasmid, replacing the *SRB10* sequences from −582 to +1301. A *Pst*I-*Sma*I fragment was used to transform yeast cells to generate the *srb10*Δ strains.

The *URA3* centromeric *ANB1-lacZ* fusion plasmid YCpAZ33 and its derivatives YCp(33)AZΔA, YCp(33)AZΔB, and YCp(33)AZΔAΔB, carrying deletions of OpA, OpB, and both OpA and OpB, respectively, have been described (7). The various mutations in OpA were constructed by PCR as follows. OpA is contained within a 400-bp *Xho*I-*Hin*dIII fragment that extends from the 3′ end of OpA (an *Xho*I site at −242) upstream to the *Hin*dIII site. To generate mutations in OpA, PCR primers were synthesized that contained the *Xho*I site at the 5′ end and extended into OpA with the desired mutations. A PCR was then carried out with a second primer for synthesis from the *Hin*dIII site, and the product was digested with *Hin*dIII and *Xho*I and ligated into YCp(33)AZΔB similarly digested. All constructs were confirmed by sequence analysis.

The insertion of the Mot3 binding site into OpB was achieved as follows. A PCR primer was synthesized that introduced 10 bp, including a Mot3 site, into OpB. This DNA, along with a second synthetic DNA that primed synthesis from the *Xho*I site bordering OpA, was used in a PCR that generated an 80-bp product. This product was in turn used as a primer in conjunction with a synthetic DNA that primed from a *Sac*I site within the *lacZ* coding sequence. The 2-kb product was digested with *Xho*I and *Sac*I and ligated into *Sac*I-*Xho*I-digested YCp(33)AZΔOpA to generate YCp(33)AZΔAOpB7(+10). The correct construct was confirmed by sequence analysis. YCp(33)AZΔAOpB8(+10) was described previously (7).

The *ROX1-lacZ*, *HEM13-lacZ*, *AAC3-lacZ*, and *COX5B-lacZ* fusion plasmids in the YCplac33 vector have all been described (6, 7).

The plasmid pET-MBP/Rox1, encoding a maltose-binding protein (MBP)-Rox1 fusion that was used for expression of Rox1 in *Escherichia coli*, was constructed as follows. The *MAL-ROX1* fusion from p*MAL-ROX1* (2) was amplified by PCR using primers that added an *Nde*I site to the beginning of the MBP coding sequence and a *Hin*dIII site 800 bp downstream from the *ROX1* coding sequence. This fragment was cloned into the *Nde*I and *Hin*dIII sites of pET-24a (Novagen).

The glutathione-*S*-transferase (GST)-Mot3 fusion plasmid pET-*GST*/*MOT3* that was used for expression of Mot3 in *E. coli* was constructed as follows. The *GST* coding sequence was PCR amplified from pACG2T (PharMingen) with primers that placed an *Nde*I site at the ATG initiation codon and a *Bam*HI site immediately after the thrombin protease site 3′ to the *GST* sequence. This fragment was digested with *Nde*I and *Bam*HI and ligated into *Nde*I-*Bam*HI-digested pET24a. The *MOT3* coding sequence was PCR amplified from pBS*MOT3* using primers that placed a *Bam*HI site at the beginning of the coding sequence and a c-*myc* epitope, termination codon, and a *Sal*I site at the 3′ end. The fragment was digested with *Bam*HI and *Sal*I and cloned into the *Bam*HI and *Sal*I sites of the pET-*GST* construct.

Enzyme and RNA assays. β-Galactosidase assays were carried out as described (19). All assays were performed multiple times with multiple independent transformants for each plasmid in each strain. Invertase assays were carried out as described (3, 14). Cells were grown repressed in SC containing 4% glucose or derepressed in SC containing 2% raffinose.

RNA was prepared (48) and blots were carried out as described (1). Cells were grown on SC medium either aerobically with vigorous shaking or anaerobically by bubbling nitrogen through the cultures for 2 h before harvesting.

Protein purification. The MBP-Rox1 fusion was expressed in BL21-Codon Plus (DE3)-RIL cells (Stratagene). One liter of cells was grown in L-broth plus kanamycin (34 μg/ml) and chloramphenicol (20 μg/ml). The fusion was induced with 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Cells were harvested and broken in a French press. The fusion protein was purified using amylose beads as described (2).

The His-tagged Mot3 protein was expressed and purified as described (25). The GST-Mot3 fusion protein was expressed and purified in a similar manner, except glutathione beads were used for the purification. Where indicated, 10 μg of the fusion was cleaved with 1 U of thrombin under the conditions recommended by the vendor (Pharmacia).

Gel retardation assays. The gel retardation assays have been described (2). The synthetic DNAs used are indicated in the appropriate figures. The radioactivity in the gel bands was quantitated using a Storm 860 PhosphorImager (Molecular Dynamics).

Micrococcal nuclease sensitivity assays. Cells used for the sensitivity assays were transformed with the multicopy plasmid YEp(112)*ANB1* for the RZ53-6 derivatives and YEp(195)*ANB1* for the P1/I8 strains. They were grown to mid-exponential phase in 400 ml of SC with tryptophan or uracil at 30°C with vigorous aeration. Chromatin preparations were carried out as described (1) with modification. The *tup1*Δ cells were quite flocculent, and it was difficult to obtain efficient spheroplast formation with enzyme treatment alone. Therefore, a short, vigorous mixing with glass beads (0.5 mm) followed the zymolyase treatment for all strains to maintain uniformity.

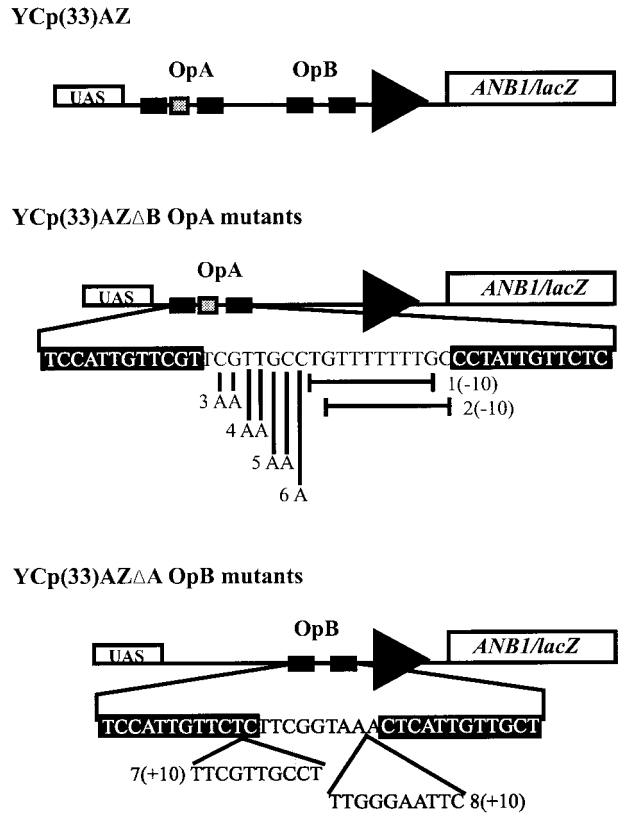

FIG. 1. Mutations in the *ANB1* regulatory region. The top diagram represents the wild-type *ANB1-lacZ* fusion gene in the plasmid YCp(33)AZ. The diagram includes the UAS (open box on left), the two operators with the Rox1 binding sites (solid boxes), and the Mot3 binding site in OpA (grey box), the TATA box (the triangle), and the coding sequence (open box on right). The middle diagram presents the OpA mutations constructed in an OpB deletion plasmid, YCp(33)AZΔOpB. The sequence of OpA is shown with the Rox1 binding sites indicated in solid boxes. The lower diagram presents the OpB mutations constructed in an OpA deletion plasmid, YCp(33)AZΔOpA. The sequence of OpB is shown with the Rox1 binding sites indicated in solid boxes.

The analysis of the micrococcal nuclease sensitivity was carried out using Southern blots of 1.2% agarose gels (1). The ^{32}P probe was the 340-bp *Sal*I-*Bgl*II fragment from +125 to +464 of the *ANB1* coding sequence.

RESULTS

A sequence in *ANB1* OpA is involved in Rox1-mediated repression. The repression region of the *ANB1* gene consists of four Rox1 sites that we have divided into two operators, A and B, as illustrated in Fig. 1. The level of repression effected by OpA is nearly 10 times greater than that by OpB despite equivalent levels of noncooperative Rox1 binding to the sites within these operators (7). The two Rox1 sites act synergistically in repression from OpA, while the two Rox1 sites in OpB act additively. This difference did not appear to be due to a difference in spacing of the Rox1 sites in the operators or to the position of the operator relative to the TATA box. Rather, the sequence between the Rox1 sites of OpA appeared to contain information that rendered it a better repression site. This prompted us to examine the operator sequences more carefully. Comparison with the regulatory regions of other Rox1-regulated genes revealed a conserved sequence closely associated with the 5′ OpA Rox1 binding site, TCGTTGCCT. This sequence has been noted before (24, 29), and a point mutation in it that affected *ANB1* repression has been described (29), but its importance was overshadowed by the extensive studies of the Rox1 binding sites. This sequence was also affected by a previously described 10-bp deletion in OpA which changed the last T residue of the sequence to a C residue and caused severe loss of repression (7).

We further defined this sequence and investigated its role in OpA-mediated repression by constructing a series of mutations in the *ANB1-lacZ* reporter plasmid. All the constructs also carried a deletion of OpB to increase the sensitivity to changes in OpA activity. The expression from each mutant construct was assayed in both wild-type and *rox1*Δ cells; *ANB1* expression is completely derepressed in the *rox1*Δ background, allowing a calculation of the fold repression caused by each mutation. The results are shown in Table 1. Initially we recreated the 10-bp deletion between the Rox1 sites, but shifted 1 bp 3′-wards to leave the last T residue in the sequence intact [OpA 2(−10)]. This deletion had little effect on repression, indicating that the effect of the previously described OpA 1(−10) deletion was due to the C-to-T transition rather than the 10-bp deletion. To further analyze this sequence, three double-base-pair substitutions were generated through the first 6 bp of the sequence, and the seventh pair was changed from a CG to an AT, recreating the Mehta and Smith allele (29). The results indicated that the first two base pairs were not essential for function, and the site could be reduced to the sequence TTGCCT. All these deletions caused only partial derepression compared to the complete derepression seen in a *rox1* deletion strain (Table 1).

If this site were solely responsible for the relative strength of OpA compared to OpB, inserting it into OpB should increase its repression activity. We previously found that a 10-bp insertion of a random sequence in OpB weakened repression [OpB 8(+10)] (7). However, insertion of the repression-enhancing sequence from OpA into OpB [OpB 7(+10)] resulted in a 2.3-fold increase in repression compared to that for the wild-type OpB and a 5-fold increase compared to the other 10-bp insertion (Table 1). While this effect was not as dramatic as that seen in the native OpA, it demonstrates that this sequence can enhance repression at other Rox1 sites.

The Mot3 DNA-binding protein acts through this OpA sequence. At this point in our investigations, we were alerted to a factor possibly working through this sequence. Sertil et al. (38) had identified the *DAN1* gene, which was induced during anaerobiosis and regulated by a Rox1-independent regulatory system. Subsequently, they isolated a mutation that de-

TABLE 1. Effect of operator mutations on *ANB1-lacZ* expression[a]

Fusion	Other operator	Mean β-galactosidase activity (Miller units) ± SD		Fold repression (*rox1*Δ/wild type)
		Wild type	*rox1*Δ	
ΔOpB	OpA-wild type	1.3 ± 0.7	97 ± 34	75
	OpA 1(−10)	22 ± 5	116 ± 14	5
	OpA 2(−10)	1.6 ± 0.7	88 ± 12	55
	OpA 3	1.1 ± 0.2	93 ± 25	84
	OpA 4	10 ± 3	101 ± 38	10
	OpA 5	15 ± 4	102 ± 19	7
	OpA 6	4.4 ± 3	107 ± 31	24
ΔOpA	OpB-wild type	12 ± 8	83 ± 16	7
	OpB 7(+10)	5.6 ± 2	91 ± 23	16
	OpB 8(+10)	31 ± 2	96 ± 9	3

[a] The *ANB1-lacZ* fusions were constructed in either YCp(33)AZΔOpB (ΔOpB) or YCp(33)AZΔOpA (ΔOpA), as indicated in the table. The mutations are shown in Fig. 1. The enzyme assays were carried out in extracts of RZ53-6 (wild type) or RZ53-6Δ*rox1* (Δ*rox1*).

TABLE 2. Effect of a *mot3* deletion on repression of hypoxic genes[a]

lacZ fusions	Mean β-galactosidase activity (Miller units) ± SD (fold repression relative to Δ*rox1*)		
	Wild type	*mot3*Δ	*rox1*Δ
ANB1	0.7 ± 0.5 (121)	4.8 ± 1 (17)	85 ± 13
*ANB1*ΔOpB	1.3 ± 0.7 (75)	9.5 ± 3 (10)	97 ± 34
*ANB1*ΔOpB OpA 1(−10)	22 ± 5 (5)	27 ± 4 (4)	116 ± 14
*ANB1*ΔOpB OpA 2(−10)	1.6 ± 0.7 (55)	15 ± 4 (6)	88 ± 12
AAC3	0.2 ± 0.1 (6)	0.8 ± 0.2 (1.5)	1.2 ± 0.1
HEM13	1.7 ± 0.9 (13)	4.5 ± 0.9 (5)	23 ± 2.4
COX5B	0.4 ± 0.2 (5)	0.5 ± 0.2 (4)	2.1 ± 0.7
ROX1	15 ± 2 (13)	17 ± 5 (12)	199 ± 51

[a] The *lacZ* fusion plasmids were maintained in cells on derivatives of YCplac33. The *ANB1-lacZ* alleles included the wild type with both OpA and OpB intact (*ANB1*) and the indicated mutant alleles. Enzyme assays were carried out in RZ53-6 (wild type), RZ53-6Δ*mot3* (*mot3*Δ), or RZ53-6Δ*rox1* (*rox1*Δ).

repressed *DAN1* and, surprisingly, *ANB1*. This mutation was complemented by the *MOT3* gene (Charles V. Lowry, personal communication). Mot3 is a DNA-binding protein that contains two zinc fingers and appears to be involved in the regulation of a variety of genes (15, 25). It binds to the consensus sequence T(G/A)CCT(A/T/G), which matches the sequence found in OpA.

To determine whether Mot3 enhanced repression through OpA, we created a *mot3* deletion allele and determined its effect on the expression of OpA wild-type and mutant derivatives of the *ANB1-lacZ* reporter. Repression of the wild-type fusion or the construct containing only the OpA site (ΔOpB) was decreased about sevenfold in *mot3*Δ relative to the full repression in wild-type cells versus full derepression in *rox1*Δ cells (Table 2). This decrease is comparable to the 7- to 15-fold-decreased repression caused by the more severe mutations in the TTGCCT element described above. If Mot3 acts through this sequence, the combination of a mutation in this sequence with *mot3*Δ should not show increased derepression compared to that observed with a wild-type sequence in a *mot3*Δ strain. Such was the case, as seen in Table 2, where the OpA 1(−10) mutation showed the same level of repression in the *mot3*Δ strain as did the wild-type OpA or the neutral OpA 2(−10) mutant.

The effect of the *mot3* deletion on *ANB1* mRNA accumulation was determined by RNA blot analysis (Fig. 2). RNA was prepared from cells grown either aerobically (repressed) or anaerobically (derepressed). As is evident from the blot, *ANB1* RNA levels were partially derepressed in the *mot3*Δ strain compared to the repressed wild type and the completely derepressed *rox1*Δ or nearly completely derepressed *tup1*Δ strains. Quantitation of the blot indicated that the *mot3*Δ mutant accumulated 46-fold less RNA than the *rox1*Δ mutant aerobically. A comparison to the wild type was impossible to calculate due to the inability to detect any *ANB1* RNA in the repressed wild type. These findings confirm the general effects seen with the *lacZ* fusion.

Mot3 functions as part of the Rox1-dependent repression complex. Mot3 could act to augment repression through the Rox1–Tup1-Ssn6 repression complex, or it could repress *ANB1* expression independently. If the former were the case, the combination of loss of Mot3 repression plus loss of Rox1 repression should give no further increase in *ANB1* repression beyond that observed with the loss of Rox1 repression. On the other hand, if the latter were the case, we would expect that *ANB1* expression would be higher when both repression mechanisms were lost. An inspection of Table 1 indicates that mutations in the Mot3 site that cause partial loss of repression in a wild-type strain, OpA 1(−10), 4, 5, and 6, do not cause any additional loss of repression in a *rox1*Δ strain, comparing 97 Miller units of activity for the plasmid with the wild-type Mot3 site compared to 101 to 116 Miller units for the mutant plasmids.

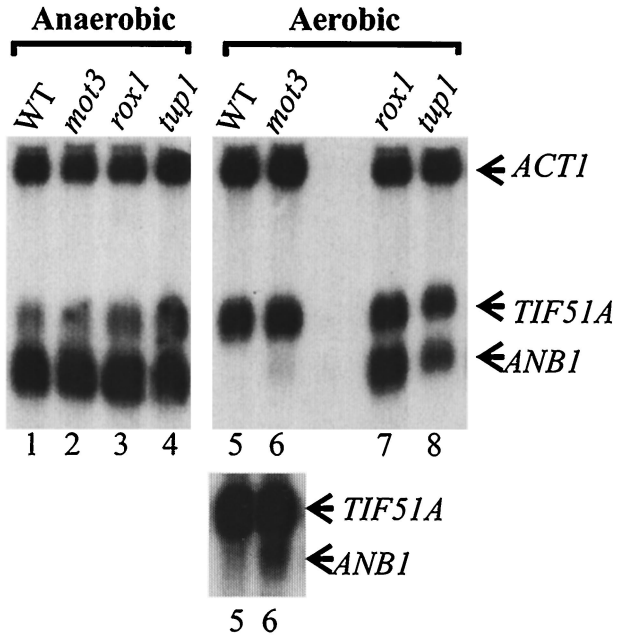

FIG. 2. *ANB1* RNA levels are partially derepressed by *mot3*Δ. RNA was prepared from cells grown derepressed (anaerobically, lanes 1 to 4) or repressed (aerobically, lanes 5 to 8) from RZ53-6 (wild type [WT], lanes 1 and 5), RZ53-6*mot3*Δ (lanes 2 and 6), RZ56-6*rox1*Δ (lanes 3 and 7), and RZ53-6*tup1*Δ (lanes 4 and 8). The blot was hybridized to ^{32}P-labeled DNA probes prepared from the *ACT1* and *ANB1* genes. The *ANB1* probe cross-hybridizes to the aerobically expressed *TIF51A* RNA. An overexposed segment of lanes 5 and 6 is presented below the main autoradiograph for better visualization of the *ANB1* band in the *mot3*Δ strain.

To confirm this observation, we also compared wild-type *ANB1-lacZ* expression in cells containing a *rox1 mot3* double deletion to that in strains carrying either deletion alone. Extracts from *rox1*Δ *mot3*Δ cells contained 113 ± 26 Miller units of enzyme activity, compared to 85 ± 13 units from extracts of *rox1*Δ and 4.8 ± 1 units from extracts of *mot3*Δ cells. These results clearly indicate that there was only a slight increase in expression of *ANB1* in the double deletion compared to the

TABLE 3. Effect of a *mot3* deletion on repression of other Tup1-Ssn6-repressed genes[a]

Strain	Mean *SUC2* expression (μmol of glucose/min/100 mg) ± SD		Mean *STE2-lacZ* expression ± SD
	Repressed	Derepressed	
Wild type	8.0 ± 1.5	113 ± 11	0.13 ± 0.1
*mot3*Δ	15.6 ± 3	109 ± 21	0.21 ± 0.1
*tup1*Δ	203 ± 17	264 ± 64	61 ± 4.7

[a] The strains used were RZ53-6 (wild type), RZ53-6Δ*mot3* (*mot3*Δ), and RZ53-6Δ*tup1* (*tup1*Δ). *SUC2* expression was measured by invertase assays. The activity is expressed as micromoles of glucose released per minute per 100 mg (dry weight) of cells. Cells were grown on SC medium with either 4% glucose (repressed) or 2% raffinose (derepressed). *STE2-lacZ* expression was determined by β-galactosidase assays carried out in cells transformed with the pSL1169 plasmid. Activity is expressed in Miller units.

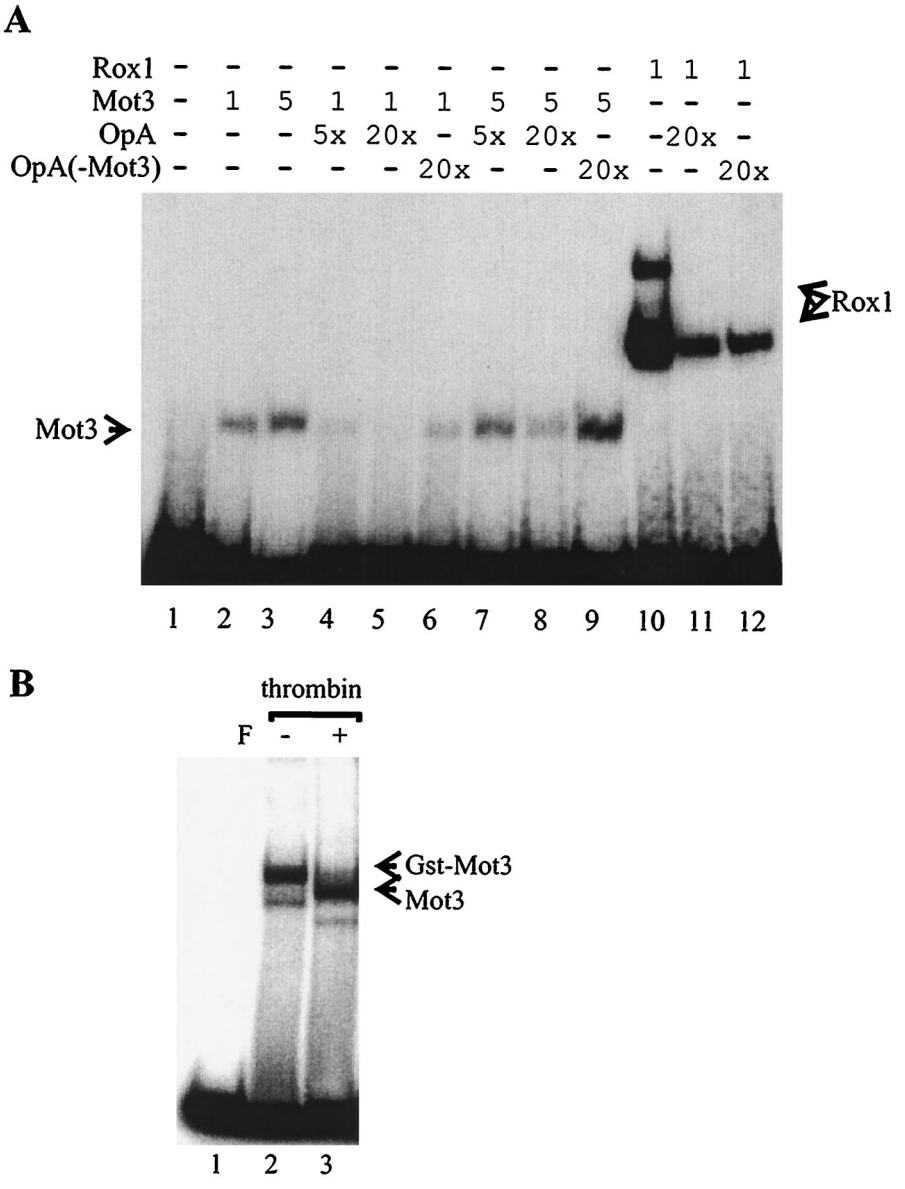

FIG. 3. Mot3 binds to OpA. (A) Gel retardation was carried out with ^{32}P-labeled synthetic double-stranded DNA containing the OpA sequences (5′-TTTTCGT TTTTCCATTGTTCGTTCGT**TGCCT**CCTATTGTTCTCGAGCCTAAAA). The Rox1 sites are underlined, and the Mot3 site is in boldface. The DNA was synthesized so that the annealed molecules had single-stranded 5′ ends that could be filled in for labeling (1). DNA used for competition either contained (as above) or lacked (5′-TTTTCGTTTTTCCATTGTTCGTTTTTTTTGCCCTATTGTTCTCGAGCCTAAAA) the putative Mot3 binding site. Competitor was added at 5 or 10 times the concentration of labeled DNA. The His-tagged Mot3 protein was prepared as described, and either 1 or 5 μl was added per binding reaction where indicated. MBP-Rox1 was prepared as described, and 1 ng was added per binding reaction where indicated. (B) Gel retardation was carried out with ^{32}P-labeled synthetic double-stranded DNA (labeled as above) containing the OpA sequence lacking the two Rox1 binding sites (Mot3-DNA), 5′-TTTTTCC------CGTTCGT**TGCCT GT**TTTTTTTGCCCT------CTCAAAA. The sequences underlined represent the Rox1 binding sites, with the dashes indicating deleted bases. The sequence in boldface is the Mot3 binding site. GST-Mot3 fusion (10 ng) was added in lane 2, and 10 ng of the cleaved Mot3 (plus free GST) was added to lane 3. No protein was added to lane F (free DNA).

rox1 deletion strain. Therefore, we conclude that Mot3 functions primarily through the same pathway as Rox1.

Some, but not all, Tup1-Ssn6-repressed genes are partially derepressed in the *mot3*Δ strain. There are putative Mot3 binding sites in close proximity to Rox1 binding sites in the regulatory regions of the hypoxic genes *ACC3*, *COX5B*, and *HEM13* but not *ROX1*, which is autorepressed. To determine whether Mot3 plays a role in their repression, we examined the effect of the *mot3* deletion on the expression of *lacZ* reporter genes, comparing the level of derepression to that observed in a *rox1* deletion strain (Table 2). As expected, the *AAC3* and *HEM13* fusions were partially derepressed, while the *ROX1* fusion was not. Surprisingly, the *COX5B* was unaffected by the *mot3* deletion, but this gene was not regulated strongly under these conditions, perhaps minimizing any Mot3 effect. Overall, these results suggest that Mot3 plays a general role in enhancing Rox1-dependent repression of a number of, but not all, hypoxic genes.

To determine if Mot3 was involved in the repression of other Tup1-Ssn6 genes, we measured the expression of the Mig1–Tup1-Ssn6-repressed *SUC2* gene (42) through invertase activity in wild-type, *mot3*Δ, and *tup1*Δ strains (Table 3). There was a small but significant twofold derepression observed in the absence of Mot3. This derepression was only a fraction of the

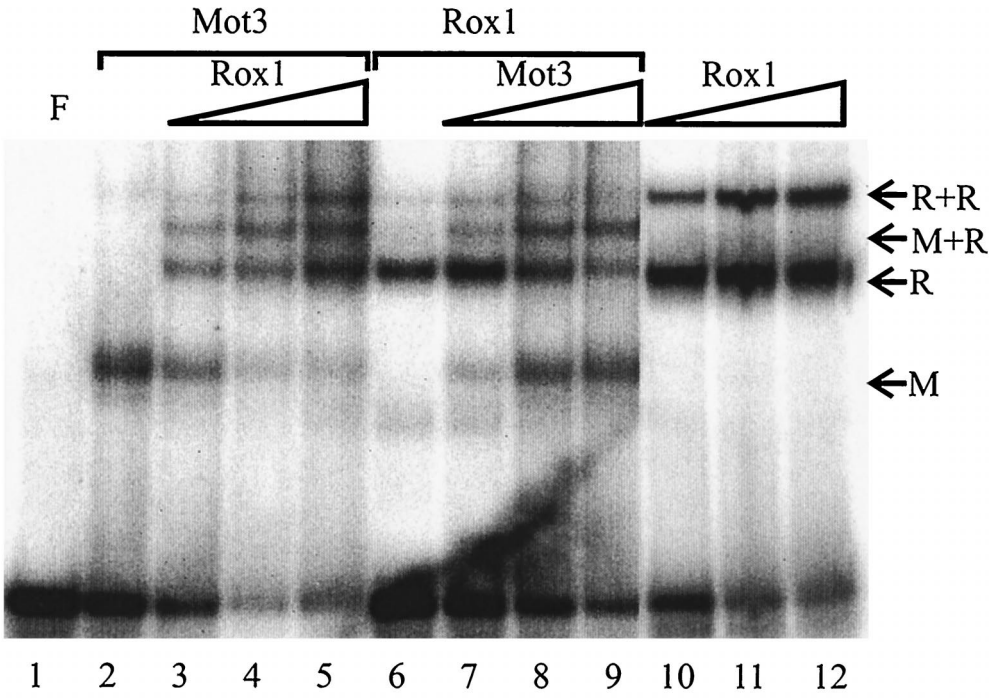

FIG. 4. Mot3 and Rox1 bind to OpA independently. Gel retardation was carried out with a synthetic ^{32}P-labeled DNA (labeled as in Fig. 3) containing the two Rox1 and one Mot3 sites. The sequence was 5′-TTTTTCCATTGTTCGTTCGT**TGCCTG**TTTTTTTGCCCTATTGTTCTCAAAA, where the underlined sequences are the Rox1 binding sites and the sequence in bold is the Mot3 binding site. Protein was added to the indicated lanes as follows: none to lane 1; 10 ng of Mot3 and 0, 5, 20, and 50 ng of MBP-Rox1 to lanes 2, 3, 4, and 5, respectively; 10 ng of MBP-Rox1 and 0, 2.5, 10, and 40 ng of Mot3 to lanes 6, 7, 8, and 9, respectively; and 25, 50, and 100 ng of MBP-Rox1 to lanes 10, 11, and 12, respectively. The Mot3 used in this experiment was the GST-Mot3 fusion cleaved with thrombin. The deduced complexes are indicated to the right, where M represents Mot3 and R represents MBP-Rox1.

25-fold derepression observed in the *tup1*Δ strain. There is a Mot3 site adjacent to the two Mig1 binding sites in the *SUC2* regulatory region, and Mot3 has been shown to bind to the *SUC2* regulatory region (15). It should be noted that we did not observe the small decrease in derepressed levels of *SUC2* expression in the *mot3*Δ reported by Grishin et al. (15). Perhaps this difference is due to the different strains or growth conditions.

We also determined the effect of the *mot3*Δ on the α2–Mcm1–Tup1-Ssn6-repressed *STE2/lacZ* fusion (17). In this case we observed no Mot3 effect.

Mot3 binds specifically to OpA in vitro. To demonstrate that the putative Mot3 site in the *ANB1* OpA can bind the Mot3 protein, we performed in vitro binding studies using a six-histidine-tagged version of Mot3 expressed in and partially purified from *E. coli* cells (25). Gel retardation studies were performed using a radiolabeled synthetic DNA containing OpA, and as can be seen in Fig. 3, a slower migrating band was visible in the presence of Mot3 (lane 2 and 3). To demonstrate that this band represented specific binding to the Mot3 sequence, competitor DNA was added that contained either the Mot3 site (lanes 4, 5, 7, and 8) or a deletion of the Mot3 site (lanes 6 and 9). Only the DNA containing the Mot3 site competed effectively to reduce the levels of the retarded band. OpA contains two Rox1 binding sites, and the labeled DNA bound two molecules of Rox1 (lane 10). Both competitors contained the two Rox1 sites also, and both competed equally well to reduce Rox1 binding (lanes 11 and 12).

Expression and purification of this fusion were inefficient, so we generated a new plasmid encoding a GST-Mot3 fusion expressed from a T7 promoter. The resulting fusion protein was expressed at high levels, was more easily purified, and bound to the Mot3 site of OpA (Fig. 3B, lane 2). This GST-Mot3 fusion protein also contained a site for the thrombin protease between GST and Mot3, and treatment of the purified fusion protein with this protease resulted in a faster migrating band (Fig. 3A, compare lanes 2 and 3). These results leave little doubt that Mot3 can bind the putative Mot3 site in the *ANB1* OpA and, combined with the genetic evidence above, conclusively demonstrate that Mot3 enhances the activity of Rox1-dependent operators.

It should be noted that for both the fusion and free Mot3, a minor, faster migrating complex was visible. Since the change in size of this complex upon thrombin cleavage is about the same as that for the major complex, we believe that it represents a minor, alternate conformation of Mot3. Both forms behaved identically in all subsequent experiments, but the amount of the minor band varied.

Mot3 and Rox1 do not bind cooperatively to DNA. We envision three models whereby Mot3 can enhance Rox1-dependent repression: (i) Mot3 could interact with Rox1 to enhance its binding to DNA; (ii) Mot3 could bind independently to DNA and help recruit the Tup1/Ssn6 repression complex; or (iii) Mot3 could help the repression complex function, for example, by interacting with nucleosomes or the basal transcriptional machinery. To distinguish between the first and latter two possibilities, we tested for cooperative interactions between Rox1 and Mot3 binding to DNA in vitro. It is difficult to assess cooperative interactions directly by gel retardation because the pattern of bands is complex. There are two Rox1 binding sites plus one Mot3 site in OpA, resulting in five different DNA complexes that can form. This banding pattern can be seen in Fig. 4, where two sets of titrations were carried out: one in which increasing Rox1 was added to a constant amount of Mot3 (lanes 2 to 5), and the second in which increasing amounts of Mot3 were added to a constant amount of

Rox1 (lanes 6 to 9). We also added increasing amounts of Rox1 in the absence of Mot3 (lanes 10 to 12) to help in identification of the complexes containing one or two molecules of Rox1. For this experiment, the GST-Mot3 fusion was digested with thrombin to release the Mot3 protein, ensuring that the GST moiety did not interfere with a potential Mot3-Rox1 interaction. The Rox1 protein used in these experiments was fused to the MBP, but this fusion repressed *ANB1* expression in yeast cells as well as the wild-type Rox1 (data not shown), and therefore, if Rox1 interacts with Mot3, the fusion would do so, too. All the expected bands were visualized except the fully loaded DNA, which would contain two Rox1 and one Mot3 molecule. If Rox1 and Mot3 interacted cooperatively, we would expect that the amount of complex containing Mot3 (Fig. 4) would increase with increasing Rox1. This was not the case; the fraction of DNA to which Mot3 bound remained constant at about 0.3 in lanes 2 to 5. Similarly, the fraction of DNA in Rox1-containing complexes increased only about twofold from lanes 6 to 9. These effects were reproducible, but the absolute numbers varied from experiment to experiment. The results indicate that Rox1 and Mot3 do not interact directly.

We wished to confirm the above conclusion using a more sensitive assay that was not dependent on the proper identification of a complex pattern of bands. To this end, we carried out competition assays using a radiolabeled DNA containing the OpA Mot3 site alone (Mot3-DNA) and unlabeled competitor containing the Mot3 site plus both Rox1 sites (OpA-DNA). We reasoned that if Rox1 and Mot3 interacted cooperatively, the level of competition would be greater in the presence of Rox1 than in its absence, and this greater competition could easily be followed by the disappearance of the single band representing the labeled Mot3-DNA complex. The results of this experiment are shown in Fig. 5A; the presence of Rox1 did not increase the competitiveness of the DNA containing (lanes 8, 9, and 10) compared to DNA lacking (lanes 5, 6, and 7) Rox1 sites. Quantitation of the radioactivity in the bands indicated that the levels of competition with both DNAs were almost exactly the predicted values for the dilution of the labeled DNA with nonlabeled DNA. This experiment was repeated a number of times with various Rox1 concentrations and a range of competitor DNA concentrations, and in every case, the results resembled those in Fig. 5A. As a control, to ensure that Rox1 bound specifically, Rox1 binding to radiolabeled Mot3-DNA (lanes 2 and 3) or radiolabeled OpA-DNA (lanes 6 to 8) was determined (Fig. 5B). Clearly, within the concentration range used, Rox1 binding was specific. Thus, at least in vitro, Rox1 and Mot3 do not enhance each other's binding to DNA, suggesting that the role of Mot3 is to aid in the recruitment or function of the Tup1-Ssn6 complex.

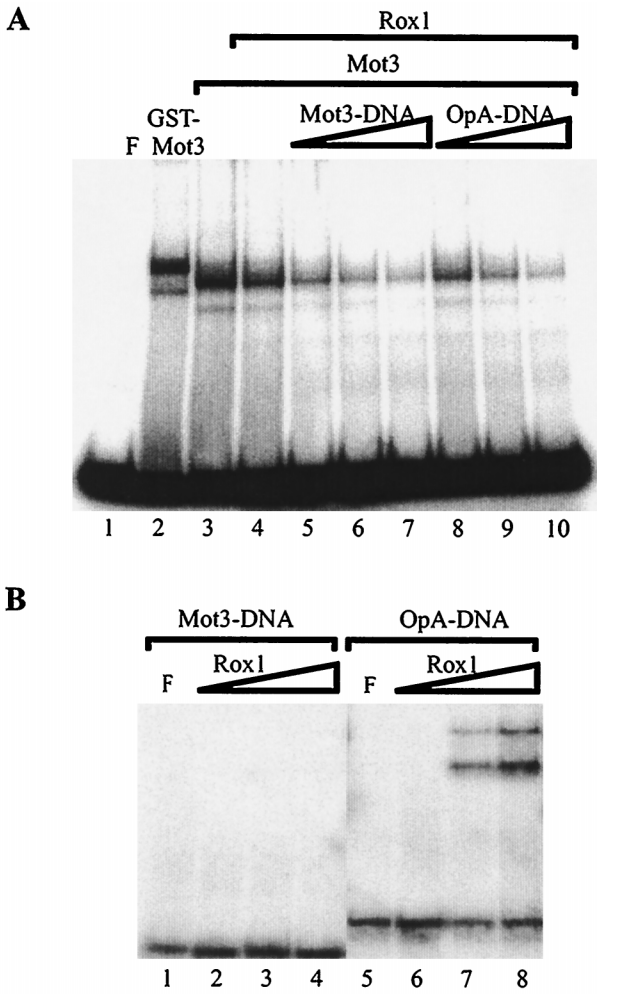

FIG. 5. Mot3 and Rox1 do not bind cooperatively to DNA. (A) Gel retardation was carried out with the ^{32}P-labeled Mot3-DNA shown in Fig. 3B. The same DNA was used as an unlabeled competitor in 1, 2.5, and 5 times the labeled DNA in lanes 5, 6, and 7, respectively. A DNA fragment containing the Mot3 and Rox1 sites (shown in Fig. 4) was also used as unlabeled competitor at 1, 2.5, and 5 times the labeled DNA in lanes 8, 9, and 10, respectively. GST-Mot3 fusion (10 ng) was added in lane 2, and 10 ng of the cleaved Mot3 (plus free GST) was added to each of lanes 3 to 10; 20 ng of MBP-Rox1 was added to each of lanes 4 to 10. (B) Gel retardation was carried out with either the ^{32}P-labeled Mot3-DNA (lanes 1 to 4) or ^{32}P-labeled OpA-DNA (lanes 5 to 8). MBP-Rox1 was added at 5 ng (lanes 2 and 6), 20 ng (lanes 3 and 7), and 100 ng (lanes 4 and 8).

Effect of Mot3 on chromatin arrangement at *ANB1*. To determine how Mot3 contributes to the assembly of the repression complex on the *ANB1* regulatory region, we used the previously established ability of this complex to alter chromatin structure in other regulons as a marker for its presence at the *ANB1* locus in vivo. Micrococcal nuclease sensitivity assays were used to probe chromatin structure, and as shown in Fig. 6, we observed three reproducible differences among the patterns obtained with chromatin-bound DNA from wild-type and mutant cells or deproteinated (naked) DNA. Two sensitive sites (solid arrows) were observed in deproteinated DNA that were protected in repressed wild-type cells but not in derepressed *tup1*Δ or *rox1*Δ cells. These two sites map around the TATA box (Fig. 6), suggesting that the repression complex blocks access to the TBP. The protected region in wild-type cells is approximately 170 bp, in the size range expected for a nucleosome, as drawn in the diagram. This observation agrees with the finding that TBP binding to the *ANB1* TATA box was significantly greater in *tup1*Δ than in wild-type cells (23). Interestingly, in the partially derepressed *mot3*Δ cells, these bands appeared less intense, suggesting that the partial depression results from either the incomplete assembly or partial function of the repression complex.

A third site (open arrow) was nuclease sensitive in wild-type, *mot3*Δ, and *tup1*Δ cells but not in *rox1*Δ cells or in deproteinated DNA (Fig. 6). This site maps close to OpA (Fig. 6), and we believe that it reflects increased sensitivity caused by Rox1 bending of DNA, making it an indicator of Rox1 binding. Since this sensitive site was present in the *tup1*Δ and *mot3*Δ cells, we believe that Rox1 was bound to the DNA in these cells independently of complex formation, supporting the model that Mot3 helps recruit the repression complex or aids in repression rather than aiding in Rox1 binding.

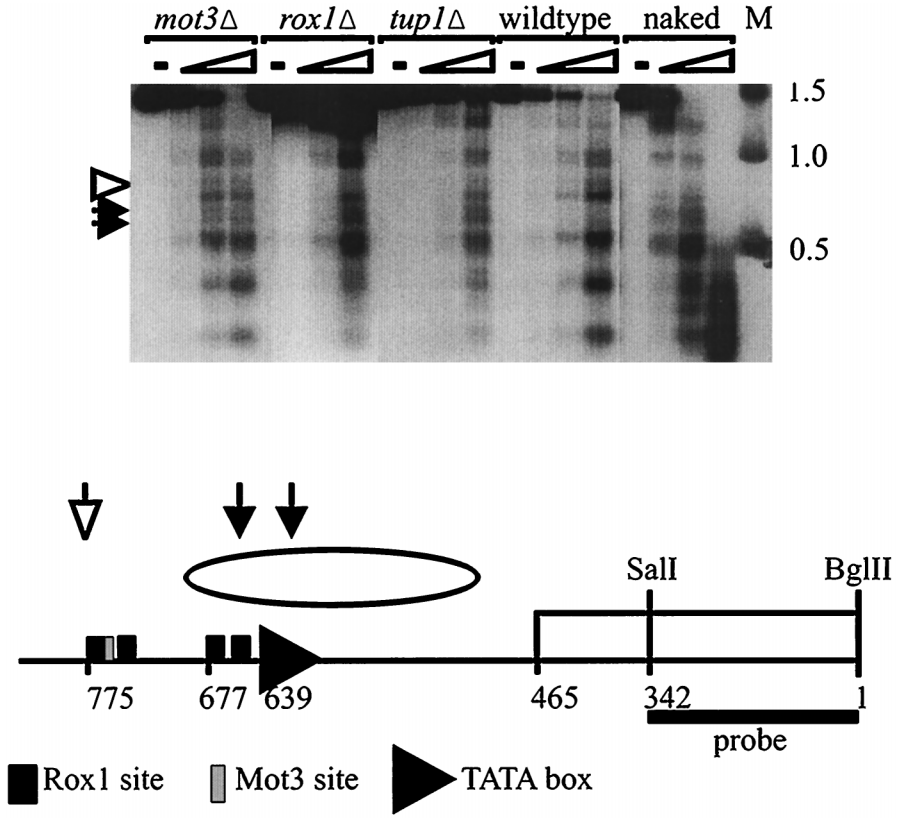

FIG. 6. Repression complex alters chromatin structure at *ANB1*. (A) The autoradiograph represents a Southern blot of micrococcal nuclease-digested chromatin carried out as described in Materials and Methods. Chromatin was prepared from RZ53-6 (wild type) cells and its derivatives transformed with YEp(112)*ANB1*, as indicated. Micrococcal nuclease was added to final concentrations of 0 (−), 1, 4, or 12 U/0.4 ml, and digestion was carried out for 10 min at 37°C. The samples in the lanes marked naked were prepared from RZ53-6 and deproteinated before the addition of 0, 1, 3, or 9 U of micrococcal nuclease per 0.4 ml for 10 min at 37°C. All samples were digested with *Bgl*II plus *Eco*RI after deproteination. This digestion generated a 1.4-kb fragment in the absence of nuclease. A 1-kb ladder size standard (New England Biolabs) was loaded in lane M; due to its high concentration, this DNA cross-hybridized to the probe, and the sizes (in kilobases) are indicated to the right. The open arrow represents the Rox1-dependent sensitive site, and the solid arrows represent the repression complex-dependent resistant sites. (B) Diagram of the *ANB1* regulatory region. The Rox1 binding sites are represented as black boxes, the Mot3 site as a grey box, the TATA box as a triangle, and the coding sequence as an open box. The numbers designate base pairs starting from the *Bgl*II site. The *Bgl*II-*Sal*I hybridization probe is indicated. The open and solid arrows are described above, and the ellipse represents the proposed positioned nucleosome.

Nuclease-protected sites result from an interaction between the repression complex and nucleosomes. Tup1 interacts with histones H3 and H4 (8), and although deletions in the N-terminal regions of either of these two proteins causes at least partial depression of some Tup1-Ssn6-repressed regulons, *ANB1* repression is not affected (7). Nonetheless, given the differences in the nuclease sensitivity between wild-type cells and derepressed mutants, we investigated the nuclease sensitivity of *ANB1* in these histone deletions. As shown in Fig. 7, in cells carrying the H4 N-terminal deletion, the two nuclease-sensitive sites protected in the wild-type cells (arrows) were not protected, giving the same pattern as that for *rox1*Δ and *tup1*Δ cells and deproteinated DNA. In cells carrying the H3 N-terminal deletion, these sites were only slightly sensitive, indicating a less dramatic effect of this allele.

These results demonstrate that histone H4 plays a role in the protection of the TATA box in repressed wild-type cells. Given this finding and the nucleosome-size length of DNA protected, we believe that access to the TATA box is blocked by a repression complex-recruited positioned nucleosome, as suggested in the diagram in Fig. 6. Interestingly, these results also demonstrate that the positioning of this nucleosome is not required for repression, since the H4 mutant is not derepressed (7), and confirmed below for the low-copy *ANB1-lacZ* fusion and confirmed for the high-copy plasmid used for this chromatin analysis by RNA blots (data not shown). While it is formally possible that the positioned nucleosome plays no role in repression, there is a growing body of evidence that the Tup1-Ssn6 complex can repress through both nucleosome-dependent and nucleosome-independent mechanisms, and we believe that these data add to it.

Srb10 does not play a role in *ANB1* repression. Mutations in the *SRB10* gene were isolated in screens for derepression of both the glucose-repressed genes and **a** mating type genes, regulons which are repressed by the Tup1-Ssn6 complex (22, 40, 44). Srb10 is a protein kinase member of the mediator complex, a component of the RNA polymerase II holoenzyme (11). Although it is unclear what role Srb10 plays in Tup1-Ssn6 repression, it seemed to be a good candidate to test for a role in the nucleosome-independent pathway. We constructed an *srb10* deletion and *srb10*Δ *hhf1-8* and *srb10*Δ *hht1-2* double mutants. Neither the single deletion nor the double deletions affected repression significantly (Table 4). While there was a less than twofold increase in the *srb10* deletion strain, there was no further increase in combination with the histone mutations. Fully derepressed expression was well above 150 Miller units in all cases. Clearly Srb10 does not play a major role in the nucleosome-independent pathway for repression of *ANB1*. This finding raises the intriguing possibility that the general

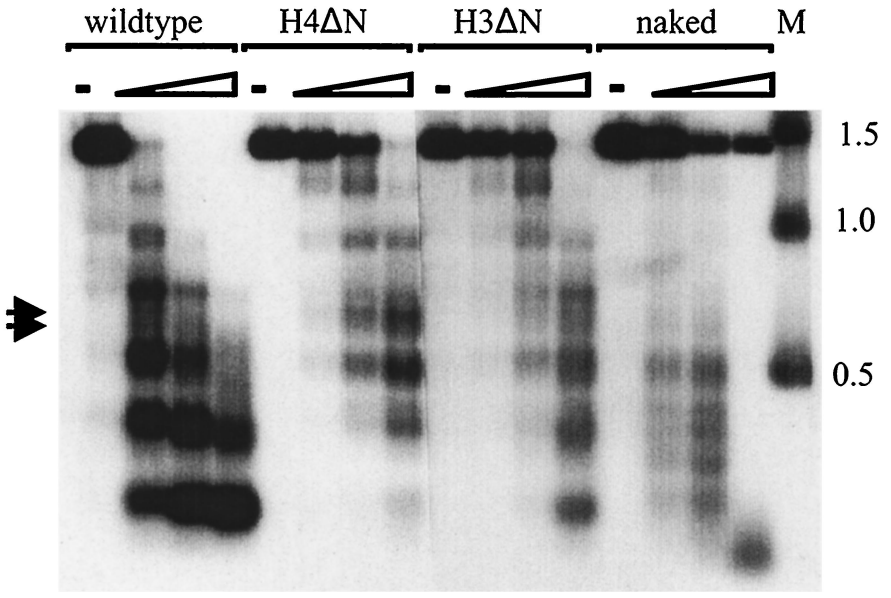

FIG. 7. Mutations in the N-terminal domain of histone H4 alters chromatin structure at *ANB1*. The autoradiograph represents a Southern blot of micrococcal nuclease-digested chromatin carried out as described in Materials and Methods and the legend to Fig. 4. Chromatin was prepared from P1/I8 cells carrying YEp(195)*ANB1* and plasmids with the *HHT1-HHF1* alleles (wild type), the *HHT1-hhf1-8* alleles (H4ΔN), or the *hht1-2-HHF1* alleles. Micrococcal nuclease was added to final concentrations of 0 (−), 2, 8, or 24 U/0.4 ml for the wild-type and H4ΔN samples; 0, 0.25, 1, or 8 U/0.4 ml for the H3ΔN samples; and 0, 1, 3, or 9 U for the naked DNA samples (prepared from the wild-type cells). Lane M, size markers (in kilobases). The arrows indicate bands representing the sites protected in the wild-type samples.

repression complex interacts with the basal transcriptional machinery in different ways when anchored to different genes.

DISCUSSION

Role of Mot3 in hypoxic gene repression. We report here that the transcription factor Mot3 enhances repression by the Rox1–Tup1-Ssn6 complex. Mot3 was originally identified in two separate genetic screens, one for suppressors of adaptation to pheromone and the other for high-copy suppressors of the synthetic lethality of the *mot1-24 spt3*Δ double mutation (encoding general transcription factors) (15, 25). Using *MOT3* overexpression and a *mot3* deletion, Grishin et al. (15) showed that Mot3 negatively regulated (either directly or indirectly) a set of pheromone-inducible genes and positively regulated an eclectic set of other genes. They also demonstrated that a LexA-Mot3 fusion could act as an activator in an otherwise upstream activation sequence (UAS)-less reporter gene, but did not act as a repressor when a UAS was present. A *mot3* deletion conveys no dramatic phenotype under a variety of growth and stress conditions except for a mild increase in UV sensitivity. Both studies suggested that Mot3 is a global transcription factor; it affects the expression of a variety of genes but may not be essential for the expression of any given gene. No insights were gained from these previous studies as to how it might function in the repression of hypoxic genes.

We demonstrated here that a *mot3* deletion results in partial derepression of the hypoxic gene *ANB1* and some but not all of the other hypoxic genes tested. Mot3 acts by binding directly to the *ANB1* OpA, as indicated by the ability of Mot3 to bind to OpA in vitro and by the loss of Mot3-dependent repression caused by mutations in the Mot3 OpA binding site. A number of lines of evidence strongly suggest that Mot3 acts in conjunction with the Rox1–Tup1-Ssn6 complex rather than independently. First, Mot3 sites are always closely associated with Rox1 sites in those genes regulated by both. Second, a *rox1 mot3* double deletion caused only a slight increase in *ANB1* expression beyond that resulting from the *rox1* deletion alone, indicating that Mot3 has no significant repression activity in the absence of Rox1. Third, the effect of a *mot3* deletion on chromatin structure is similar to that of a *rox1* or *tup1* deletion only less severe, as might be expected if the three proteins function through the same pathway.

We also presented two lines of evidence that Mot3 functions by either helping Rox1 recruit the Tup1-Ssn6 complex or by helping the complex repress transcription rather than by helping Rox1 bind to DNA. First, we showed by in vitro competition experiments that Mot3 bound equally well to OpA without bound Rox1 as to OpA containing bound Rox1, indicating no cooperative interactions between the two proteins. Second, in vivo micrococcal nuclease sensitivity experiments revealed a Rox1-induced sensitive site that was present in both wild-type and *mot3*Δ cells, indicating that Rox1 binds to DNA independently of Mot3. In addition, Mot3 appeared to contribute weakly to the repression of the Rox1-independent, Tup1-Ssn6-repressed *SUC2* gene, further suggesting a general role for Mot3 in repression rather than a Rox1-specific function. Thus,

TABLE 4. Effect of histone N-terminal deletion mutations and *srb10* deletion on *ANB1-lacZ* expression[a]

Strain	Mean β-galactosidase activity (Miller units) ± SD
Wild type	1.1 ± 0.2
hht1-2 (H3ΔN)	2.0 ± 0.7
hhf1-8 (H4ΔN)	1.0 ± 0.2
*srb10*Δ	2.8 ± 1
*hht1-2 srb10*Δ	3.8 ± 1
*hhf1-8 srb10*Δ	2.5 ± 1.7

[a] The strain P1/I8 and its derivatives were used.

we believe that Mot3 is a supplementary factor for repression of the hypoxic genes. It enhances Rox1-dependent repression for strongly repressed genes like *ANB1*. We envision that it helps Rox1 recruit the Tup1-Ssn6 complex through a direct interaction with Tup1-Ssn6 or somehow aids in the repression function directly. In the former case, the interaction cannot be strong, since Rox1 must be present to achieve repression. If Mot3 functions in repression directly, perhaps it does so through a weak interaction with nucleosomes or the basal transcriptional machinery to potentiate Tup1-Ssn6 function. Alternatively, Mot3 may act through altering the topology of DNA to enhance repression; the results of Mot3 DNase I protection experiments led Madison et al. (25) to suggest that Mot3 alters DNA topology. These latter mechanisms are attractive because they can accommodate the opposing effects of Mot3 on different, unrelated genes. If Mot3 interacts with nucleosomes and/or the transcriptional machinery or alters DNA topology, it could serve to promote either repression or activation depending upon what other DNA-binding proteins are involved in regulating the target gene. These effects would not require a specific interaction between Mot3 and a large variety of different gene-specific proteins.

Role of chromatin in repression of *ANB1*. There is ample evidence that Tup1-Ssn6 can organize chromatin, and our findings suggesting that the complex positions a nucleosome over the TATA box are not surprising. It agrees with the report of Kuras and Struhl (23) that TBP cannot bind to the *ANB1* regulatory region under repressed conditions. What is surprising is that the loss of this positioned nucleosome has no effect on repression. We found that in wild-type cells, the region around the TATA box was protected from micrococcal nuclease digestion and this protection was lost in cells lacking Rox1, Tup1, or the N-terminal region of histone H4. However, we previously reported (7) and confirmed here (Table 4) that this histone mutation does not cause derepression. Either nucleosome phasing plays no role in *ANB1* repression, or there are redundant mechanisms, one nucleosome dependent and the other nucleosome independent. Clearly the evidence favors the second possibility. Mutations in histone H3 or H4 cause derepression in other systems, and we believe that our proposed positioned nucleosome is responsible for the inability of TBP to bind to the *ANB1* TATA box under repressed conditions. The elimination of the positioned nucleosome without loss of repression in the H4 mutant provides us with the opportunity to genetically dissect the nucleosome-independent pathway, which we hope will ultimately the provide tools to study how both pathways operate in *ANB1* repression.

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 2000. Current protocols in molecular biology. John Wiley and Sons, Inc., New York, N.Y.
2. **Balasubramanian, B., C. V. Lowry, and R. S. Zitomer.** 1993. The Rox1 repressor of the *Saccharomyces cerevisiae* hypoxic genes is a specific DNA-binding protein with a high-mobility-group motif. Mol. Cell. Biol. **13:**6071–6078.
3. **Carrico, P. M., and R. S. Zitomer.** 1998. Mutational analysis of the Tup1 general repressor of yeast. Genetics **148:**637–644.
4. **Cooper, J. P., S. Y. Roth, and R. T. Simpson.** 1994. The global transcriptional regulators, SSN6 and TUP1, play distinct roles in the establishment of a repressive chromatin structure. Genes Dev. **8:**1400–1410.
5. **Deckert, J., R. A. Khalaf, S. M. Hwang, and R. S. Zitomer.** 1999. Characterization of the DNA binding and bending HMG domain of the yeast hypoxic repressor Rox1. Nucleic Acids Res. **27:**3518–3526.
6. **Deckert, J., R. Perini, B. Balasubramanian, and R. S. Zitomer.** 1995. Multiple elements and auto-repression regulate Rox1, a repressor of hypoxic genes in *Saccharomyces cerevisiae*. Genetics **139:**1149–1158.
7. **Deckert, J., A. M. Torres, S. M. Hwang, A. J. Kastaniotis, and R. S. Zitomer.** 1998. The anatomy of a hypoxic operator in *Saccharomyces cerevisiae*. Genetics **150:**1429–1441.
8. **Edmondson, D. G., M. M. Smith, and S. Y. Roth.** 1996. Repression domain of the yeast global repressor Tup1 interacts directly with histones H3 and H4. Genes Dev. **10:**1247–1259.
9. **Edmondson, D. G., W. Zhang, A. Watson, W. Xu, J. R. Bone, Y. Yu, D. Stillman, and S. Y. Roth.** 1998. In vivo functions of histone acetylation/deacetylation in Tup1p repression and Gcn5p activation. Cold Spring Harbor Symp. Quant. Biol. **63:**459–468.
10. **Elledge, S. J., Z. Zhou, J. B. Allen, and T. A. Navas.** 1993. DNA damage and cell cycle regulation of ribonucleotide reductase. Bioessays **15:**333–339.
11. **Feaver, W. J., O. Gileadi, Y. Li, and R. D. Kornberg.** 1991. CTD kinase associated with yeast RNA polymerase II initiation factor b. Cell **67:**1223–1230.
12. **Friesen, H., S. R. Hepworth, and J. Segall.** 1997. An Ssn6-Tup1-dependent negative regulatory element controls sporulation-specific expression of *DIT1* and *DIT2* in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **17:**123–134.
13. **Gietz, R. D., and A. Sugino.** 1988. New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene **74:**527–534.
14. **Goldstein, A., and J. O. Lampen.** 1975. Beta-D-fructofuranoside fructohydrolase from yeast. Methods Enzymol. **42:**504–511.
15. **Grishin, A. V., M. Rothenberg, M. A. Downs, and K. J. Blumer.** 1998. Mot3, a Zn finger transcription factor that modulates gene expression and attenuates mating pheromone signaling in *Saccharomyces cerevisiae*. Genetics **149:**879–892.
16. **Hampsey, M.** 1998. Molecular genetics of the RNA polymerase II general transcriptional machinery. Microbiol. Mol. Biol. Rev. **62:**465–503.
17. **Hwang-Shum, J. J., D. C. Hagen, E. E. Jarvis, C. A. Westby, and G. F. Sprague, Jr.** 1991. Relative contributions of MCM1 and STE12 to transcriptional activation. Mol. Gen. Genet. **227:**197–204.
18. **Johnson, A. D.** 1995. The price of repression. Cell **81:**655–658.
19. **Kaiser, C., S. Michaelis, and A. Mitchell.** 1994. Methods in yeast genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
20. **Kastaniotis, A. J., and R. S. Zitomer.** 2000. Rox1 mediated repression: oxygen dependent repression in yeast. Adv. Exp. Med. Biol. **475:**185–195.
21. **Keleher, C. A., M. J. Redd, J. Schultz, M. Carlson, and A. D. Johnson.** 1992. Ssn6-Tup1 is a general repressor of transcription in yeast. Cell **68:**709–719.
22. **Kuchin, S., and M. Carlson.** 1998. Functional relationships of Srb10-Srb11 kinase, carboxy-terminal domain kinase CTDK-I, and transcriptional corepressor Ssn6-Tup1. Mol. Cell. Biol. **18:**1163–1171.
23. **Kuras, L., and K. Struhl.** 1999. Binding of TBP to promoters in vivo is stimulated by activators and requires Pol II holoenzyme. Nature **399:**609–613.
24. **Lowry, C. V., M. E. Cerdan, and R. S. Zitomer.** 1990. A hypoxic consensus operator and a constitutive activation region regulate the *ANB1* gene of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:**5921–5926.
25. **Madison, J. M., A. M. Dudley, and F. Winston.** 1998. Identification and analysis of Mot3, a zinc finger protein that binds to the retrotransposon Ty long terminal repeat (delta) in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **18:**1879–1890.
26. **Marquez, J. A., A. Pascual-Ahuir, M. Proft, and R. Serrano.** 1998. The Ssn6-Tup1 repressor complex of *Saccharomyces cerevisiae* is involved in the osmotic induction of HOG-dependent and -independent genes. EMBO J. **17:**2543–2553.
27. **Matallana, E., L. Franco, and J. E. Perez-Ortin.** 1992. Chromatin structure of the yeast *SUC2* promoter in regulatory mutants. Mol. Gen. Genet. **231:**395–400.
28. **Mehta, K. D., D. Leung, L. Lefebvre, and M. Smith.** 1990. The *ANB1* locus of *Saccharomyces cerevisiae* encodes the protein synthesis initiation factor eIF-4D. J. Biol. Chem. **265:**8802–8807.
29. **Mehta, K. D., and M. Smith.** 1989. Identification of an upstream repressor site controlling the expression of an anaerobic gene (*ANB1*) in *Saccharomyces cerevisiae*. J. Biol. Chem. **264:**8670–8675.
30. **Mizuno, T., N. Nakazawa, P. Remgsamrarn, T. Kunoh, Y. Oshima, and S. Harashima.** 1998. The Tup1-Ssn6 general repressor is involved in repression of *IME1* encoding a transcriptional activator of meiosis in *Saccharomyces cerevisiae*. Curr. Genet. **33:**239–247.
31. **Morgan, B. A., B. A. Mittman, and M. M. Smith.** 1991. The highly conserved N-terminal domains of histones H3 and H4 are required for normal cell cycle progression. Mol. Cell. Biol. **11:**4111–4120.
32. **Mukai, Y., S. Harashima, and Y. Oshima.** 1991. AAR1/TUP1 protein, with a structure similar to that of the beta subunit of G proteins, is required for a1-alpha 2 and alpha 2 repression in cell type control of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11:**3773–3779.

33. **Papamichos-Chronakis, M., R. S. Conlan, N. Gounalaki, T. Copf, and D. Tzamarias.** 2000. Hrs1/Med3 is a Cyc8-Tup1 corepressor target in the RNA polymerase II holoenzyme. J. Biol. Chem. **275:**8397–8403.
34. **Pazin, M. J., and J. T. Kadonaga.** 1997. What's up and down with histone deacetylation and transcription? Cell **89:**325–328.
35. **Redd, M. J., M. B. Arnaud, and A. D. Johnson.** 1997. A complex composed of tup1 and ssn6 represses transcription *in vitro*. J. Biol. Chem. **272:**11193–11197.
36. **Roth, S. Y., A. Dean, and R. T. Simpson.** 1990. Yeast alpha 2 repressor positions nucleosomes in *TRP1/ARS1* chromatin. Mol. Cell. Biol. **10:**2247–2260.
37. **Roth, S. Y., M. Shimizu, L. Johnson, M. Grunstein, and R. T. Simpson.** 1992. Stable nucleosome positioning and complete repression by the yeast alpha 2 repressor are disrupted by amino-terminal mutations in histone H4. Genes Dev. **6:**411–425.
38. **Sertil, O., B. D. Cohen, K. J. Davies, and C. V. Lowry.** 1997. The *DAN1* gene of *S. cerevisiae* is regulated in parallel with the hypoxic genes, but by a different mechanism. Gene **192:**199–205.
39. **Shimizu, M., S. Y. Roth, C. Szent-Gyorgyi, and R. T. Simpson.** 1991. Nucleosomes are positioned with base pair precision adjacent to the alpha 2 operator in *Saccharomyces cerevisiae*. EMBO J. **10:**3033–3041.
40. **Song, W., I. Treich, N. Qian, S. Kuchin, and M. Carlson.** 1996. *SSN* genes that affect transcriptional repression in *Saccharomyces cerevisiae* encode SIN4, ROX3, and SRB proteins associated with RNA polymerase II. Mol. Cell. Biol. **16:**115–120.
41. **Teunissen, A. W., J. A. van den Berg, and H. Y. Steensma.** 1995. Transcriptional regulation of flocculation genes in *Saccharomyces cerevisiae*. Yeast **11:**435–446.
42. **Treitel, M. A., and M. Carlson.** 1995. Repression by SSN6-TUP1 is directed by MIG1, a repressor/activator protein. Proc. Natl. Acad. Sci. USA **92:**3132–3136.
43. **Wach, A., A. Brachat, C. Alberti-Segui, C. Rebischung, and P. Philippsen.** 1997. Heterologous *HIS3* marker and GFP reporter modules for PCR-targeting in *Saccharomyces cerevisiae*. Yeast **13:**1065–1075.
44. **Wahi, M., and A. D. Johnson.** 1995. Identification of genes required for alpha 2 repression in *Saccharomyces cerevisiae*. Genetics **140:**79–90.
45. **Zhang, M., L. S. Rosenblum-Vos, C. V. Lowry, K. A. Boakye, and R. S. Zitomer.** 1991. A yeast protein with homology to the beta-subunit of G proteins is involved in control of heme-regulated and catabolite-repressed genes. Gene **97:**153–161.
46. **Zitomer, R. S., P. Carrico, and J. Deckert.** 1997. Regulation of hypoxic gene expression in yeast. Kidney Int. **51:**507–513.
47. **Zitomer, R. S., and C. V. Lowry.** 1992. Regulation of gene expression by oxygen in *Saccharomyces cerevisiae*. Microbiol. Rev. **56:**1–11.
48. **Zitomer, R. S., J. W. Sellers, D. W. McCarter, G. A. Hastings, P. Wick, and C. V. Lowry.** 1987. Elements involved in oxygen regulation of the *Saccharomyces cerevisiae CYC7* gene. Mol. Cell Biol. **7:**2212–2220.