The Anatomy of a Hypoxic Operator in *Saccharomyces cerevisiae*

Jutta Deckert,[1] Ana Maria Rodriguez Torres,[2] Soo Myung Hwang,[3] Alexander J. Kastaniotis and Richard S. Zitomer

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

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ABSTRACT

Aerobic repression of the hypoxic genes of *Saccharomyces cerevisiae* is mediated by the DNA-binding protein Rox1 and the Tup1/Ssn6 general repression complex. To determine the DNA sequence requirements for repression, we carried out a mutational analysis of the consensus Rox1-binding site and an analysis of the arrangement of the Rox1 sites into operators in the hypoxic *ANB1* gene. We found that single base pair substitutions in the consensus sequence resulted in lower affinities for Rox1, and the decreased affinity of Rox1 for mutant sites correlated with the ability of these sites to repress expression of the hypoxic *ANB1* gene. In addition, there was a general but not complete correlation between the strength of repression of a given hypoxic gene and the compliance of the Rox1 sites in that gene to the consensus sequence. An analysis of the *ANB1* operators revealed that the two Rox1 sites within an operator acted synergistically *in vivo*, but that Rox1 did not bind cooperatively *in vitro*, suggesting the presence of a higher order repression complex in the cell. In addition, the spacing or helical phasing of the Rox1 sites was not important in repression. The differential repression by the two operators of the *ANB1* gene was found to be due partly to the location of the operators and partly to the sequences between the two Rox1-binding sites in each. Finally, while Rox1 repression requires the Tup1/Ssn6 general repression complex and this complex has been proposed to require the aminoterminal regions of histones H3 and H4 for full repression of a number of genes, we found that these regions were dispensable for *ANB1* repression and the repression of two other hypoxic genes.

BAKER'S yeast contains a set of hypoxic genes that provide a well-studied example of transcriptional repression (for review, see Zitomer and Lowry 1992; Zitomer *et al.* 1997a). This repression is mediated by an aerobically expressed repressor protein Rox1 and the general repression complex Tup1/Ssn6. Rox1 is a DNA-binding protein with an HMG motif that binds to the hypoxic consensus sequence YYYATTGTTCTC (Balasubramanian *et al.* 1993); copies of this sequence are found upstream of all the hypoxic genes studied to date (Table 1). As with other HMG proteins that have been termed architectural factors due to their ability to induce topological changes in DNA (Grosschedl *et al.* 1994; Wolffe 1994), Rox1 bends DNA 90° when it binds (Deckert *et al.* 1995b).

An NMR-derived structure of the HMG motif of the SRY protein indicated extensive contacts between the protein and its DNA site (Werner *et al.* 1995). Interestingly, many of the site-specific HMG proteins bind to similar sequences, sharing the ATTGTT internal sequence of the Rox1 consensus sequence (Grosschedl *et al.* 1994). This conservation of the DNA-binding site may reflect a combination of the requirements for the extensive protein DNA contacts and for the bending of the DNA. A detailed analysis of the DNA sequence requirements for Rox1 binding might provide insight into the protein-DNA interactions for the HMG class.

The hypoxic genes vary in the extent to which they are expressed aerobically (Zitomer and Lowry 1992; Zitomer *et al.* 1997a). While most of the hypoxic gene products are required for aerobic as well as hypoxic growth, some are encoded by single copy genes, such as *HEM13*, *OLE1*, and *ERG11*, and others are encoded by gene pairs with aerobically expressed homologues, such as *COX5B*, *AAC3*, *HMG2*, and *ANB1*. The unique genes are not as tightly repressed as the hypoxic members of gene pairs. This differential aerobic expression could be achieved through either varying levels of Rox1 repression or different mechanisms for transcriptional activation. Because the hypoxic genes vary both in the extent to which their Rox1-binding sites conform to the consensus sequence and in their arrangement of these sites (Table 1), analyses of the importance of these factors might provide some insight into how differential repression is achieved.

Corresponding author: Richard Zitomer, Department of Biological Sciences, University at Albany/State University of New York, Albany, NY 12222. E-mail: rz144@cnsvax.albany.edu

[1] *Present address:* Department of Biochemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115.

[2] *Present address:* Department de Biologia Celular y Molecular, Univ. de La Coruna, Campus de La Zapateira sln, 15071 La Coruna, Spain.

[3] *Present address:* Department of Clinical Pathology, Jisan Junior College, Pugok-dong, Kumjung-Ku, Pusan, Korea.

Rox1 also contains a repression domain that can mediate Ssn6/Tup1-dependent repression when tethered to the DNA through a heterologous DNA-binding domain (Balasubramanian *et al.* 1993; Deckert *et al.* 1995b; Zitomer *et al.* 1997b). The corepressor proteins Tup1 and Ssn6 have been termed general repressors as they are involved in mediating repression of a growing number of regulons, including the **a**-specific genes in *MAT*α cells and haploid-specific genes in diploid cells (Mukai *et al.* 1991; Keleher *et al.* 1992); catabolite repressed genes (Schultz and Carlson 1987; Trumbly 1988; Williams and Trumbly 1990); flocculence genes (Fujita *et al.* 1990; Teunissen *et al.* 1995); DNA damage-inducible genes (Zhou and Elledge 1992); and meiosis-specific genes (Friesen *et al.* 1997). Regulatory regions are targeted for repression by the interaction between the Tup1/Ssn6 complex and sequence-specific DNA-binding proteins, such as Rox1, Mig1, α2/MCM1, and α2/a1 (Komachi *et al.* 1994; Treitel and Carlson 1995; Tzamaris and Struhl 1995; Zitomer *et al.* 1997b).

There are two proposed mechanisms for Tup1/Ssn6 function, and with substantial evidence supporting both, it is likely that both mechanisms function. For the first, the repression complex directly interacts with and inhibits the general transcription machinery. Mutations that relieve Tup1/Ssn6-dependent repression have been isolated in a number of the components of the RNA polymerase II holoenzyme (Wahi and Johnson 1995; Song *et al.* 1997), and repression has been demonstrated *in vitro* on naked DNA (Redd *et al.* 1997). The second proposal involves the formation of a repressive chromatin structure. Derepression of the catabolite-repressed gene *SUC2* in the absence of glucose is accompanied by the loss of two positioned nucleosomes from the upstream region (Mattallana *et al.* 1992). An *ssn6* mutant displays an open chromatin structure resembling the derepressed state regardless of the presence of glucose. Similarly, binding of the α2 repressor upstream of the **a**-specific gene *STE6* positions two nucleosomes adjacent to the α2/MCM1 operator (Roth *et al.* 1990; Shimizu *et al.* 1991). This positioning effect is lost in a *tup1* or *ssn6* mutant strain (Cooper *et al.* 1994). Deletion of the amino terminus of histone H4 also causes both a disruption of the nucleosomal pattern and partial derepression of the **a**-specific genes (Roth *et al.* 1992). Furthermore, Tup1 was shown to interact with the aminoterminal tails of histones H3 and H4, and mutations that affect this interaction lead to derepression of **a**-specific genes and DNA damage-inducible genes (Edmundson *et al.* 1996).

To further understand repression of the hypoxic genes, we explored a number of features of the Rox1 DNA-binding site and the organization of these sites in the hypoxic *ANB1* gene. We report here the sequence requirements for Rox1 binding to a single site, the interaction of multiple Rox1-binding sites that comprise an operator, and the lack of effect of histone aminoterminal deletions on repression.

MATERIALS AND METHODS

Strains, cell growth, and transformations: The *Saccharomyces cerevisiae* strain RZ53-6 (Balasubramanian *et al.* 1993) and its congenic derivative RZ53-6Δ*rox1* (Deckert *et al.* 1995a) were described. The strain P1/I8 has deletions of both loci encoding histones H3 and H4 (*HHT1-HHF1* and *HHT2-HHF2*), and three transformants were used in this study, each containing one of the following plasmids: YCp(*LEU2*)*HHT1-HHF2*, wild type for both genes (WT); YCp(*LEU2*)*hht1-2-HHF1*, carrying a deletion of amino acids 1–28 in the coding region of the H3 gene (H3ΔN); and YCp(*LEU2*)*HHT1-hhf1-8*, carrying a deletion of amino acids 2–26 in the coding region of the H4 gene (H4ΔN) (Morgan *et al.* 1991).

Yeast cells were grown at 30° in either rich media (YPD) or synthetic media (SC) lacking specific growth requirements (Rose *et al.* 1990). Yeast transformations were carried out as described (Chen *et al.* 1992).

The *Escherichia coli* strain HB101, used for plasmid constructions, was maintained and transformed as described (Ausubel *et al.* 1994). The protease-deficient *E. coli* strain PR745, used for Rox1 expression, was grown as recommended by the vendor (New England Biolabs, Beverly, MA).

Rox1-binding site plasmids: The plasmids pCY4-R1OpWT (Deckert *et al.* 1995b) and mutant derivatives were constructed by insertion of the oligonucleotides 5′-ATTGTTCTC and 5′-GAGAACAAT into the *Sma*I site of pCY4 where the CCC of the *Sma*I site plus the oligonucleotide inserts generated the Rox1 consensus site (YYYATTGTTCTC). Mutant variants of this site were created by inserting double-stranded oligonucleotides deviating by a single base in the core sequence ATTGTT (positions four through nine). The orientation and sequence of all insertions were verified by sequence analysis.

***ANB1* operator plasmids:** *YCp(33)AZ:* This was constructed by subcloning the *Sma*I fragment containing the *ANB1/lacZ* fusion from YCpAZ6 (Lowry *et al.* 1990) into the *Sma*I site of YCplac33 (Gietz and Sugino 1988).

YCp(33)AZΔOpB: The *Sac*I site in the multiple cloning site of YCp(33)AZ was destroyed by inserting the oligonucleotides 5′-CCTGCAGCG and 5′-CATGGGACGTCGCTTAA between the *Eco*RI and *Kpn*I sites. This plasmid was used for all subsequent constructions. A PCR was performed using the primers 5′-TATCTGAGTTCCGACCATGGAATAGGAAACTTTGAAC and the reverse primer 5′-CAGGAAACAGCTATGACCATG and YCp(33)AZ as a template, resulting in a deletion of operator B from −187 to −213. The 0.4-kb PCR product was used as a megaprimer together with the primer 5′-CTTACCATC CAGCGCCACCATCC in a second PCR. The final 3-kb product, containing sequences from the *ANB1* upstream region through *Sac*I site in the *lacZ* gene, was digested with *Hin*dIII and *Sac*I and ligated into YCp(33)AZ with the unique *Sac*I site.

YCp(33)AZΔOpAΔOpB: A PCR was performed using the reverse primer, the primer 5′-CCGCTCGAGGAAAAACGAA AAAAAAAAAAACACAGA, and YCp(33)AZ as a template. The 0.3-kb product was digested with *Hin*dIII and *Xho*I and inserted into YCp(33)AZΔOpB digested with the same enzymes. The resulting plasmid contained a deletion of both operator A (−277 to −315) and operator B (−187 to −213).

The remaining plasmids containing operator A variations were constructed in an identical manner using the following primers in combination with the reverse primer:

YCp(33)AZΔ5′OpAΔOpB: A 30-bp deletion from −286 to

−315 (resulting in a deletion of the 5′ Rox1-binding site of operator A), 5′-AGGCTCGAGAACAATAGGAAAAACGA AAAAAAAAAAAACACAG;

YCp(33)AZΔ3′OpAΔOpB: A 8-bp deletion from −277 to −284 (resulting in a deletion of the 3′ Rox1-binding site of operator A), 5′-CCGCTCGAGGGCGAAAAAACAGGCAACGAA CG;

YCp(33)AZ+5bpOpAΔOpB: A 5-bp AAAAT insertion at −277/−287, 5′-AGGCTCGAGAACAATAGGGATTTTCGA AAAAACAGGCAACGAACG;

YCp(33)AZ-5bpOpAΔOpB: A 5-bp deletion from −289 to −293, 5′-AGGCTCGAGAACAATAGGGCGACAGGCAAC GAACGAAC;

YCp(33)AZ+10bpOpAΔOpB: A 10-bp AAAATGAATT insertion at −277/−287, 5′-CCGCTCGAGAACAATAGGGAAT TCATTTTCGAAAAAACAGGCAACG;

YCp(33)AZ-10bpOpAΔOpB: A 10-bp deletion from −287 to −295, 5′-AGGCTCGAGAACAATAGGGCGCAACGAACG AACAATGG;

YCp(33)AZOpA(A_3)ΔOpB: A T/A to A/T base pair substitution at position 3 of the 3′ Rox1-binding site of operator A, 5′-TTAGGCTCGAGAACAATTGGGCAAAAAAAACAGG;

YCp(33)AZOpA(G_3)ΔOpB: A T/A to G/A base pair substitution at position 3 of the 3′ Rox1-binding site of operator A, 5′-TTAGGCTCGAGAACAATCGGGCAAAAAAAACAGG;

YCp(33)AZOpA(A_6)ΔOpB: A T/A to A/T base pair substitution at position 9 of the 3′ Rox1-binding site of operator A, 5′-AGGCTCGAGTACAATTGGGCAAAAAAAACAG;

YCp(33)AZOpA($2A_3$)ΔOpB: An A/T base pair substitutions at positions 3 of both Rox1-binding sites of operator A, 5′-AGGCTCGAGAACAATTGGGCAAAAAAAACAGGCAACGA ACGAACAATTGAAAAACGAAAAA.

YCp(33)AZΔOpA: A 2.7-kb *Xho*I-*Sac*I fragment from YCp(33)AZ carrying operator B was inserted into YCp(33)AZΔOpAΔOpB digested with *Xho*I and *Sac*I.

YCp(33)AZΔOpA+10bpOpB: A PCR was performed using the reverse primer, primer 5′-CCGAGCAACAATGAGTGAATTC CCAATTACCGAAGAGAACAATGG, and YCp(33)AZ as a template, inserting 10-bp into operator B. The resulting 0.4-kb product was used as a megaprimer in a second PCR together with primer 5′-CTTACCATCCAGCGCCACCATCC on the same template. The final PCR product was digested with *Xho*I and *Sac*I and ligated into YCp(33)AZΔOpAΔOpB digested likewise to replace the operator B deletion. The resulting plasmid carries a 10-bp TTGGGAATTC insertion between base pairs −199 and −198.

YCp(33)AZΔOpB(BinA): A synthetic double-stranded DNA with appropriate single-stranded ends and containing the sequence 5′-GGCCGTCCATTGTTCTCTTCGGTAAACTCATT GTTGC was ligated into the *Eag*I-*Xho*I sites of YCp(33)AZΔOpAΔOpB (containing an *Eag*I-*Xho*I linker in the XhoI site). The resulting plasmid contained a replacement of the operator A sequence with that of operator B.

YCp(33)AZΔOpA(AinB): A PCR was performed using the reverse primer, primer 5′-CGGCCATGGAGAACAATAGGGC GAAAAAACAGGCAACGAACGAACAATGGAATAGGAAACT TTGAACG, and YCp(33)AZΔOpAΔOpB as a template. The product was digested with *Nco*I and *Hin*dIII and inserted into the corresponding sites of YCp(33)AZΔOpAΔOpB. The resulting plasmid contained a replacement of the operator B sequence with that of operator A.

YCp(33)COX5B/Z: A PCR was carried out with the primers 5′-CGCAAGCTTCATCGGTCCGTTGGCATA and 5′-GAGCT GCAGCATCTTTACAATGAATATGTGGC and genomic DNA prepared from RZ53-6 as a template. The product was digested with *Hin*dIII and *Pst*I and ligated into the same sites of YCp(33)*ROX1/lacZ* (Deckert *et al.* 1995a). The resulting plasmid contained a replacement of the *ROX1* upstream sequences and ATG translational initiation codon with the upstream *COX5B* sequences −380 through the initiation codon, creating a *COX5B/lacZ* fusion.

YCp(33)AAC3/Z: The *AAC3/lacZ* fusion was generated in the same manner as that for *COX5B*, except the primers used in the initial PCR were 5′-TGGCTGCAGCATTGTTCTCAAG GCACAGT and 5′-CGCAAGCTTGGAGTTCTTAATCAAC.

β-Galactosidase and invertase assays: For β-galactosidase assays, cells were grown to mid-log phase in synthetic media lacking the appropriate nutrient(s) to maintain selection for the plasmid(s) carried by the cells. For aerobic growth, cells were grown overnight and then diluted and grown to mid-log phase with vigorous shaking for at least 5 hr. For anaerobic growth, the overnight cultures were diluted into media supplemented with 2 μg/ml ergosterol and 0.2% Tween 80 and grown to mid-log phase overnight with gentle shaking in chambers containing a BBL anaerobic GasPak. For each construct, β-galactosidase assays were carried out at least six times and with at least two independent transformants as described (Rose *et al.* 1990).

For invertase assays, cells were grown to mid-log phase on YP media with 4% glucose or 2% raffinose as the energy source. The assays were carried out as described (Goldstein and Lampen 1975).

Rox1 purification and gel retardation: The maltose-binding protein MBP-Rox1(HMG) fusion was purified to over 90% as described (Balasubramanian *et al.* 1993). Full-length Rox1 containing an aminoterminal addition of six histidines was expressed in a baculovirus expression system and purified as described (Zitomer *et al.* 1997b). Gel retardation experiments were performed as described (Balasubramanian *et al.* 1993).

RESULTS

Mutagenesis of the Rox1 consensus site: The Rox1-consensus binding site YYYATTGTTCTC has been established by a number of lines of experimentation, including the following: deletion of this sequence from a number of hypoxic regulatory regions caused derepression (see Table 1); a synthetic version of this site was capable of substituting for the deleted *ANB1* operator region to reestablish repression (Lowry *et al.* 1990); and *in vitro* DNA-binding and DNase protection experiments demonstrated that Rox1 bound to this sequence (Balasubramanian *et al.* 1993; Zitomer *et al.* 1997b). However, as seen in Table 1, a survey of hypoxic gene regulatory regions indicates that many Rox1 sites deviate from this consensus at one or more bases. The six internal base pairs, ATTGTT, are more highly conserved; of the 27 sequences shown, 18 contain an exact match, 4 have a T at the first position, and 5 vary at the last position. These 6 bp also comprise part of the binding sites for other HMG proteins such as SRY, LEF-1, TCF-1, and STE11 (for review, see Grosschedl *et al.* 1994). For these reasons, we designated these 6 bp as the core sequence. The variants from the core sequence may be low-affinity sites *in vivo* that would be only partially occupied at cellular repressor concentrations. In this scenario, the strengths of the binding sites present in a particular regulatory region would determine the level of repression of the gene. We believe that differential

TABLE 1

Rox1-binding sites in hypoxic regulatory regions

Gene	Operator[a]	Evidence[b]	References
HEM13	−584 CGT ATTCTT CAA −595	Deletion analysis	Amillet *et al.* (1995)
	−475 TCA ATTGTT TAG −464		
	−238 TGC **T**TTGTT CAA −249		
	−185 CCC ATTGTT CTC −174		
ERG11	−429 CTT ATTGT**C** TTC −440	Consensus	Turi and Loper (1982)
	−358 CCT ATTGT**G** CAT −247		
HMG2	−282 CGC ATTGTT TTG − 271	Consensus	Thorsness *et al.* (1989); Yeast Genome Database
CPR1	−95 TCA TTTGTT CCT −84	Consensus	Turi and Loper (1992)
SUT1	−361 CGT ATTGTT GAA −372	Consensus	Bouret and Karst (1995)
	−342 AGC **T**TTGTT CTT −331		
	−243 GTT **T**TTGTT CTT −232		
	−115 GTT ATTGTT TCA −104		
OLE1	−272 CCT ATTGTT ACG −261	Consensus	Stukey *et al.* (1990)
	−212 TAT ATTGTT AAC −223		
COX5b	−367 TGA ATTGTT TTA − 378	Consensus	Hodge *et al.* (1990)
	−228 TGT ATTGTT CGA −217		
CYC7	−499 GTA ATTGT**C** TCC −488	Consensus	Zitomer *et al.* (1987)
	−333 CCT ATTGT**A** TTA −322		
AAC3	−197 TTC ATTGTT TGG −186	Deletion analysis	Sabova *et al.* (1993)
	−145 TCC ATTGTT CTT −134		
ANB1	−316 TCC ATTGTT CGT −305	Deletion analysis gel retardation	Lowry *et al.* (1990); Balasubramanian *et al.* (1993)
	−285 CCT ATTGTT CTC −274		
	−218 TCC ATTGTT CTC −207		
	−197 CTC ATTGTT GCT −186		
ROX1	−397 CCT ATTGTT GCT −386	Deletion analysis, gel retardation	Deckert *et al.* (1995a)
	−364 CGT ATTGT**C** TTG −353		
Consensus	**YYY ATTGTT CTC**[c]		

The table was adapted from Zitomer *et al.* (1997a). A search for Rox1-binding sites in the upstream regions of hypoxic genes yielded the sequences presented in the table. The significance of several of the putative Rox1 operators has been established by deletion analysis or gel retardation experiments as indicated. Comparison of the sequences resulted in the consensus sequence identified previously (Lowry *et al.* 1990).

[a] Sequences are numbered with the first base in the coding strand immediately 5′ to the ATG initiation codon as −1.

[b] Consensus indicates the operators were identified solely by sequence homology.

[c] Y indicates pyrimidine.

degrees of repression for different hypoxic genes are an important aspect in their regulation. Many of the hypoxic genes encode oxygen-dependent functions that are required at low levels during aerobic growth and at higher levels under limiting oxygen. While some have aerobic homologues and can be completely repressed aerobically, others, such as *HEM13*, *OLE1*, and *ERG11*, have no aerobic counterparts (Zitomer and Lowry 1992), and therefore complete repression of these genes would inhibit growth in the presence of oxygen.

To establish whether Rox1 would interact with putative DNA target sites that deviated from the consensus sequence, we examined the Rox1 binding to sites containing all 18 possible single base pair substitutions in the core sequence. Complementary oligonucleotides carrying each altered Rox1 site were ligated into a vector creating plasmids pCY4-R1OpWT and derivatives (see materials and methods) and excised as part of a 420-bp fragment. The relative dissociation constants (K_D) were measured in gel retardation assays in which the MBP-Rox1(HMG) fusion protein purified from *E. coli* cells was titrated against a low, constant concentration of each DNA fragment. The results are summarized in Table 2, and sample gel retardation experiments using operator mutants at positions 6 and 8 are shown in Figure 1. (The experiments used to determine the relative K_D values involved more extensive titrations.)

Rox1 binding was dramatically affected by changes in the center of the binding site at T/A-5 and T/A-6. Any substitutions at these residues resulted in a 13- to 29-fold decrease in affinity. Structural analysis of a protein-DNA complex formed by the homologous HMG protein SRY revealed that an isoleucine partially intercalates between these two base pairs (Werner *et al.* 1995). This interaction is crucial for the distortion of the DNA site for the protein-induced bend. The core DNA-binding site and this isoleucine residue are conserved between SRY and Rox1, and mutations that alter this isoleucine

TABLE 2

Relative K_D for Rox1 binding to mutant sites

	Original base in Rox1 consensus sequence						
Change	C_3	A_4	T_5	T_6	G_7	T_8	T_9
A	190 (4.7)	**20 (1)**	290 (15)	250 (13)	150 (7.5)	160 (8)	100 (5)
C	**40 (1)**	250 (13)	580 (29)	360 (18)	180 (9)	170 (8.5)	70 (3.5)
G	490 (12)	160 (8)	480 (24)	260 (13)	**20 (1)**	180 (9)	150 (7.5)
T	25 (0.6)	75 (3.8)	**20 (1)**	**20 (1)**	270 (14)	**20 (1)**	**20 (1)**

The K_D values are presented in nanomolars. The bases in the Rox1 consensus sequence used in this experiment are numbered as follows: $C_1C_2C_3A_4T_5T_6G_7T_8T_9C_{10}T_{11}C_{12}$. The single base pair mutants of the core sequence ATTGTT (positions 4 to 9) were cloned into pCY4 (see materials and methods), and the fragments used in gel retardation experiments were 420-bp *Hin*dIII fragments. The mutations in C_3 were generated using 32-bp synthetic DNA (see materials and methods). Each binding reaction contained 15,000 cpm of DNA (equivalent to 1 ng) and 5–800 ng of MPB-Rox1(HMG) protein. The numbers in parentheses are the fold increase in the K_D values (fold weakening of the binding) compared to the consensus sequence. The values for the consensus sequence are presented in boldface type. The K_D for the consensus sequence of the 32-bp synthetic DNA was twofold higher than for the 420-bp fragment as seen in the C_3 column.

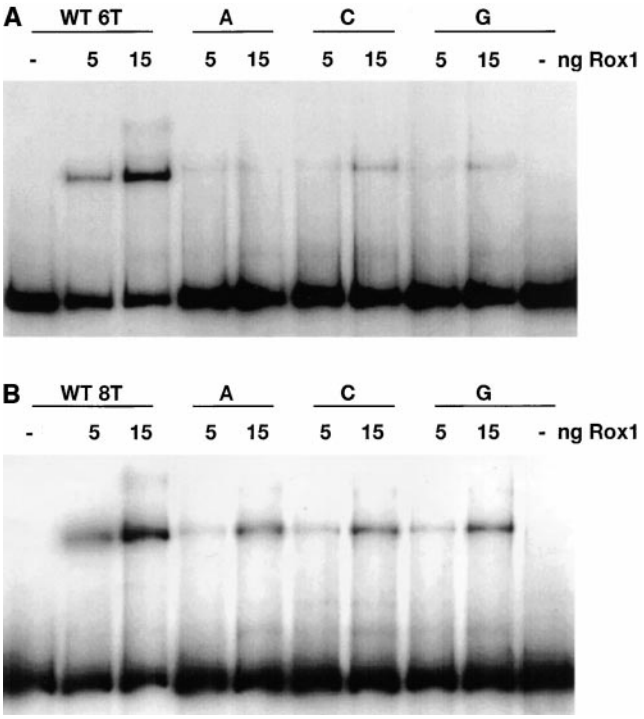


Figure 1.—Rox1 binding to mutant sites. The autoradiographs represent gel retardation experiments. (A) The gel retardation was carried out with the varying amounts of MBP-Rox1(HMG) protein and 420-bp DNA fragments containing a single Rox1 site with either the consensus sequence (lanes WT 6T) or single base pair variants (lanes A, C, and G). The base pairs of the 12-bp consensus sequence are numbered as in Table 2. The DNA used is a *Hin*dIII restriction fragment derived from pCY-R1OpWT or the mutant derivatives pCY-R1Op6A, pCY-R1Op6C, pCY-R1Op6G. The amount of protein used in the assay is indicated above each lane. (B) A gel retardation experiment was carried out as described in A, but with DNA derived from pCY-R1OpWT or the mutant derivatives pCY-R1Op8A, pCY-R1Op8C, pCY-R1Op8G.

in Rox1 have been isolated as loss-of-function mutations (Deckert 1997). Consequently, it is likely that an analogous intercalation is involved in Rox1-DNA interaction, and alterations of these base pairs would interfere with this interaction.

The changes best tolerated by Rox1 were located at the first and last position of the core sequence. Substituting a T/A for the first A/T resulted in only a fourfold decrease in the binding affinity. Interestingly, the sequence TTTGTT is a high-affinity site for the HMG proteins LEF-1, TCF-1, and STE11 (Giese *et al.* 1992; van der Wetering and Clevers 1992). This sequence also appears in the regulatory region of four hypoxic genes (see Table 1), and our results suggest that these sites should mediate some repression.

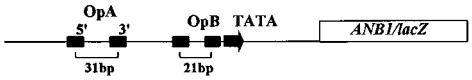
Similarly, changing the last T/A to a C/G or T/A only reduced the affinity of Rox1 by four- or fivefold, respectively. These nonconsensus core sites can be found in the regulatory regions of the Rox1 repressed genes *CYC7*, *ERG11*, and *ROX1* itself (Table 1) and are probably involved in mediating Rox1 repression. The genes carrying these sequences in their upstream region are not very tightly repressed by Rox1, presumably because of the presence of these lower affinity-binding sites.

The structural analysis of the SRY-DNA complex indicated that specific contacts were also made between the protein and the noncore base pair three of the DNA. This base pair is less well conserved than the core base pairs; of the 27 sites listed in Table 1, 14 contain a T/A, 9 contain a C/G, and 4 contain an A/T. To determine the importance of this base pair, we tested Rox1 binding to the four possible base pairs at this position. These could not be generated as the core variants were because the subcloning procedure used a *Sma*I site that placed a C/G at the third position. Therefore, we tested binding of the MBP-Rox1(HMG) protein to a set of four

32-bp synthetic DNA molecules, each identical in sequence except at position three of the Rox1 consensus sequence. The results of this experiment, shown in Table 2, indicated that a pyrimidine/purine base pair at this residue bound DNA well, but substitution of either an A/T or a G/C caused a 5- and 12-fold reduction in binding affinity, respectively. As in the case for similar reductions in affinity within the core sequence, there are naturally occurring sites that contain an A/T base pair at the third position; presumably these sites cause less, but still significant, repression. However, there is no site listed in Table 1 that contains a G/C base pair at position three: a 14-fold reduction in binding affinity may not be tolerable.

Effect of point mutations on *in vivo* repression: To determine whether base pair substitutions in the Rox1-binding site affected repression *in vivo*, a number of substitutions were generated in the *ANB1* regulatory region. *ANB1* is repressed over 200-fold by *ROX1*, and this repression is mediated through four Rox1 sites arranged in two pairs in the upstream region (see Figure 2). Previous studies revealed that the upstream-most pair of sites, termed operator A, was responsible for most of the Rox1-mediated repression, while the 3′ pair of sites, termed operator B, did not play a strong role (Lowry *et al.* 1990). Consequently, a deletion of operator B was constructed, and base pair substitutions were generated in the Rox1-binding sites of operator A. The mutations were generated in a centromeric *ANB1*/*lacZ* fusion plasmid to allow quantitation of repression levels. Three single point mutations were created in the 3′ Rox1-binding site of operator A: a G/C or A/T substitution for the T/A base pair at position 3 and an A/T substitution for the T/A at position 9. In addition, a double mutation was created in operator A in which an A/T base pair was substituted for the C/G base pair at position 3 of the 5′ Rox1-binding site of operator A plus an A/T base pair was substituted for the T/A in position 3 of the 3′ site. The effects of these mutations on repression of *ANB1*/*lacZ* expression were determined through β-galactosidase assays in both *ROX1* wild-type and Δ*rox1* strains, and the fold repression for each mutant was calculated as the ratio of the two (Δ*rox1*/wild type).

The results of these experiments are presented in Table 3. The *in vitro* binding studies indicated that a T/A to A/T substitution at position 3 resulted in a 7.6-fold reduction in Rox1-binding affinity (Table 2). As seen in Table 3, the same mutation in one of the two Rox1-binding sites reduced repression 6.3-fold (from 76-fold repression for the wild-type operator A to 12-fold repression for the A_3 mutant). A G/C substitution at the same position resulted in a more severe 20-fold reduction in Rox1 affinity (Table 2) and caused a 17-fold reduction in the level of repression as seen for the G_3 compared to the wild-type operator A. The A/T substitution at position 9 of the 3′ Rox1-binding site, A_9, resulted in only a 2.3-fold reduction in the level of repression compared to the wild-type operator A, which corresponds to the 5-fold reduction in the Rox1-binding affinity that results from this mutation. Thus, in all three cases, there is an excellent correlation between the effect of a mutation on Rox1-binding affinity



ANB1-Promoter	β-galactosidase Activity		Fold Repression
	Wildtype Strain	*Δrox1* Strain	(*Δrox1*/Wildtype)
WT	0.43	114	265
ΔOpB	1.5	114	76
ΔOpA ΔOpB	66	89	1.3
Δ3'OpA ΔOpB	30	83	2.8
Δ5'OpA ΔOpB	13	73	5.6
+5bp OpA ΔOpB	2.2	87	40
-5bp OpA ΔOpB	2.8	83	30
+10bp OpA ΔOpB	2.2	89	41
-10bp OpA ΔOpB	16	130	8.3
ΔOpA	13	81	6.2
ΔOpA +10bp OpB	31	96	3.1
ΔOpA(AinB)	0. 42	64	152
ΔOpB(BinA)	24	102	4.2

Figure 2.—Repression mediated by *ANB1* operator variants. The level of β-galactosidase activity was measured in cells from the wild-type yeast strain RZ53-6 (column labeled wildtype) and the isogenic *ROX1* deletion strain RZ53-6Δ*rox1* (column labeled Δ*rox1*), grown aerobically in SC-uracil medium and transformed with the indicated plasmids carrying an *ANB1*/*lacZ* reporter gene with mutations in the *ANB1* upstream region. YCp(33)AZ (row WT) contains a 630-bp portion of the *ANB1* upstream region, which directs Rox1 regulation of *ANB1*; YCp(33)AZΔOpB (row ΔOpB) contains a deletion of the two Rox1 sites in operator B. The following plasmids contain a deletion of operator B and additional modifications: YCp(33)AZΔOpAΔOpB (row ΔOpAΔOpB) contains an additional deletion of operator A; YCp(33)AZΔ3′OpAΔOpB (row Δ3′OpAΔOpB) and YCp(33)AZΔ5′OpAΔOpB (row Δ5′OpAΔOpB) contain additional deletions of either the 3′ or the 5′ Rox1 site in operator A, respectively; YCp(33)AZ + 5-bp OpAΔOpB (row + 5-bp OpAΔOpB) and YCp(33)AZ − 5-bp OpAΔOpB (row − 5-bp OpAΔOpB) contain a 5-bp insertion or deletion between the Rox1 sites in operator A, respectively; YCp(33)AZ + 10-bp OpAΔOpB (row + 10-bp OpAΔOpB) and YCp(33)AZ − 10-bp OpAΔOpB (row − 10-bp OpAΔOpB) contain a 10-bp insertion or deletion between the sites in operator A, respectively. The plasmid YCp(33)AZΔOpA (row ΔOpA) contains a deletion of the two Rox1 sites in operator A, and YCp(33)AZΔOpA + 10-bp OpB (row ΔOpA + 10-bp OpB) contains an insertion of 10 bp between the Rox1 sites in operator B, in addition to the deletion of operator A. The plasmid YCp(33)AZΔOpA(AinB) [row ΔOpA(Ain B)] contains a deletion of operator A at its normal position and the substitution of operator A for operator B. The plasmid YCp(33)AZΔOpB(BinA) [row ΔOpB(BinA)] contains a deletion of operator B at its normal position and the substitution of operator B for operator A. Fold repression was determined for each reporter plasmid by dividing the β-galactosidase activity measured in the *ROX1* deletion strain by that measured in the wild-type strain.

TABLE 3

Effect of Rox1-bindging site mutations on repression of *ANB1* expression

ANB1 mutation	β-Galactosidase activity: Wild type	β-Galactosidase activity: Δ*rox1*	Fold repression (Δ*rox1*/wild type)
ΔOpB	1.5	114	76
OpA(A_3)ΔOpB	10	121	12
OpA(G_3)ΔOpB	21	94	4.5
OpA(A_9)ΔOpB	3.6	118	33
OpA($2A_3$)ΔOpB	28	80	2.8

β-Galactosidase assays were carried out in permeabilized cells of strain RZ53-6 (wild type) or RZ53-6Δ*rox1* transformed with the indicated plasmids. Cells were grown aerobically in SC-uracil medium to select for the plasmid.

and *in vivo* repression. Finally, the mutant containing A/T substitutions at position 3 in both the 5′ and 3′ Rox1-binding sites of operator A resulted in an even further reduction in repression than the single mutation, as was expected. These results clearly indicate that the level of repression is determined by the strength of Rox1 binding.

***ANB1* operator deletion and insertion analysis:** Although Rox1 binds as a monomer to a single site, Rox1 sites are present in multiple copies upstream of almost all known hypoxic genes (see Table 1). To determine how many sites are required for repression and whether multiple sites act additively or synergistically, an analysis of the *ANB1* regulatory region was carried out using, as above, the *ANB1*/*lacZ* fusion plasmid to construct operator mutations. The results are presented in Figure 2. Initially we confirmed the relative importance of the two operators. Expression of the *ANB1*/*lacZ* fusion containing four intact Rox1 sites in the upstream region was repressed 265-fold as determined by comparing the β-galactosidase activities in a wild-type *vs.* a Δ*rox1* strain. Deletion of operator B resulted in a slight derepression; the upstream operator A alone was still able to direct a 76-fold repression. In contrast, operator B alone mediated only a 6-fold repression of β-galactosidase expression. These results agreed with the previous observations (Lowry *et al.* 1990). They also suggested that the degree of resemblance of Rox1 sites to the consensus sequence is not the sole determinant of the strength of a Rox1 operator; all four sites in these operators have an intact core sequence and a pyrimidine at position 3, and each contains a site that perfectly matches the consensus sequence. Deletion of all four Rox1 sites reduced repression to an insignificant level, suggesting that operators A and B together account for the full degree of Rox1-mediated repression.

To investigate whether multiple sites facilitate repression, either the 5′ or the 3′ site of operator A was deleted in the *ANB1* promoter lacking operator B. Repression directed by these single sites was inefficient. The 3′ site, which matches the consensus sequence perfectly, resulted in a 5.6-fold repression, while the 5′ site mediated only a 2.8-fold repression. The 76-fold repression mediated by two Rox1 sites in the intact operator A exceeded the combined 16-fold repression predicted, were the two sites to function independently. Thus, the two sites act synergistically. (The additive effect of two sites acting independently is defined here by the multiplication of the fold repressions rather than their addition because we envision repression as controlling the fraction of time that a promoter is available for transcription. Therefore, transcription can only occur during the fraction of time when the two independent repression sites are free.)

The Rox1-binding sites in both operators A and B are positioned on the same face of the DNA helix and are separated by three and two helical turns, respectively. To investigate if this spacing were required for efficient Rox1 repression, possibly by allowing cooperative Rox1 binding, the Rox1 sites in a promoter containing only operator A were positioned on opposite sides of the helix by inserting or removing 5 bp between the sites. As a test for a possible distance requirement, 10 bp were added or deleted, resulting in the addition or deletion of a full helical turn and thus maintaining the position of the Rox1 sites on the same side of the helix.

Insertion or deletion of 5 bp resulted in only small reductions in repression by operator A alone, to 40-fold and 30-fold repression, respectively. These small effects indicate that shifting the protein-binding sites to opposite faces of the DNA helix did not eliminate the synergy between them. Addition of 10 bp between the operator A sites had a similarly small effect, and the repression of the *ANB1*/*lacZ* gene was still 41-fold. Interestingly, reducing the distance between the operator A site by 10 bp to the spacing found in the weaker operator B decreased repression from 76- to 8.3-fold. This result suggested that the 21-bp spacing in operator B may not be optimal and might contribute to the low level of repression by this operator. To test this hypothesis, we increased the distance between Rox1-binding sites in an *ANB1* promoter containing only operator B to 31 bp and assayed the expression of the *ANB1*/*lacZ* fusion. Surprisingly, changing operator B to resemble the stronger operator A did not increase the level of repression, but rather decreased the repression from 6.2- to 3.1-fold.

The above results indicate that some other feature of operator B besides the distances between the Rox1-binding sites limits the level of repression compared to operator A. It is possible that there may be some sequence requirement for the DNA between the two sites, perhaps reflecting a topological requirement for the synergy between sites. Alternatively, the positioning of a Rox1 operator relative to the TATA box or activation

sequences may be the crucial difference between the effectiveness of the A and B operators. To distinguish between these possibilities, we constructed two separate insertions in the ΔOpAΔOpB plasmid: in the first plasmid, the operator A sequence was placed into the B position [ΔOpA(AinB)], and in the second, the operator B sequence was placed in the A position [ΔOpB (BinA)]. As seen in Figure 2, operator A repressed expression of the *ANB1/lacZ* fusion 152-fold from the B position as compared to 76-fold from its native position. This increase in repression was more than 24-fold greater than that obtained with operator B in the B position, indicating that operator A is a better operator. Operator B in the A position resulted in only a 4.2-fold repression, about the same as that observed for operator B in its native position and much lower than the 76-fold repression by operator A in the A position. These data indicate that operator A is inherently stronger than operator B, presumably due to the sequences between the Rox1-binding sites, because that is the only difference between the ΔOpB and ΔOpB(BinA) or between ΔOpB and ΔOpA(AinB). These results also indicate that the location of the operators matters since operator A gave stronger repression from the B position than from the A position.

Rox1 binding to the *ANB1* operators: The above results demonstrated that Rox1-mediated repression of *ANB1* is enhanced by multiple Rox1 sites. To determine if this were due to cooperative binding of Rox1 to its recognition sites, gel retardation experiments were performed to assess the *in vitro* affinity of purified full-length Rox1 protein to DNA fragments derived from the wild-type and mutant *ANB1* upstream regions. Full-length protein was used in these experiments to ensure that if cooperativity occurred through interactions outside the HMG domain, they would not be missed.

The results of a typical experiment are shown in Figure 3. The wild-type operator A (WT OpA) and the operator A mutant deleted for 10 bp between the two Rox1 sites (−10-bp OpA) showed two complexes at higher Rox1 concentrations, while the operator A containing only the 3′ (Δ5′OpA) or 5′ (Δ3′OpA) sites showed only one, as expected. (The faint larger complex seen in all samples at the highest protein concentration represents nonspecific aggregation. This artifact can be easily distinguished from specific binding because the aggregation complexes migrate at a different rate from the specific complexes.) For the single site fragments the relative K_D's for the Rox1-DNA complexes were 35 nm for the 3′ site and 70 nm for the 5′ site, which correlated well with the higher repression mediated by the 3′ site alone and the fact that this site matches the consensus sequence perfectly.

The possible interaction of Rox1 molecules bound to the two wild-type operator A sites was determined by comparing the occupancy of the sites in operators containing only a single site with that of the operator containing two sites. If Rox1 binding to the 5′ and 3′ site were independent, then, at a given Rox1 concentration, the fraction of wild-type DNA bound (migrating as both monomers and dimers) should be equal to the sum of the fractions of Δ5′ and Δ3′ DNA bound in separate reactions. This prediction results in a theoretical curve for Rox1 binding to the wild-type operator DNA, as can be seen in Figure 4. The amount of Rox1-DNA complex formed with the wild-type operator A did not exceed the predicted values for independent binding to the two single sites. This suggests that the binding of Rox1 to both sites in operator A is not cooperative.

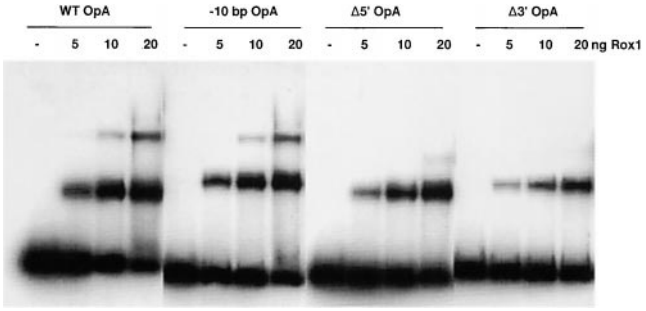


Figure 3.—Rox1 binding to *ANB1* operator mutants. The autoradiograph represents a gel retardation experiment carried out with varying amounts of full-length Rox1 protein and 15,000 cpm (1 ng) of the following *Hin*dIII-*Nco*I DNA fragments containing the *ANB1* operator A or mutant derivatives: a 387-bp fragment from YCp(33)AZΔOpB (lanes WT OpA), a 377-bp fragment from YCp(33)AZ − 10-bp OpAΔOpB (lanes −10 OpA), a 357-bp fragment from YCp(33)AZΔ5′OpAΔOpB (lanes Δ5′OpA), and a 378-bp fragment from YCp(33)AZΔ3′OpAΔOpB (lanes Δ3′OpA). The amount of Rox1 protein used is indicated above each lane. (−) indicates no added protein.

As another measure for cooperativity, the Hill coefficient was determined for Rox1 binding to either a single site alone and to two sites present on one DNA fragment. In all cases the coefficient was 1.5, indicating that the binding of Rox1 to multiple sites is not cooperative *in vitro*. The coefficient of 1.5, rather than the expected 1.0 for the single and the two independent sites, probably reflected some inactive protein present in the Rox1 preparations; this would result in an overestimate of the effective concentration of protein added to the binding reaction. Similar results were obtained in experiments using the MBP-Rox1(HMG) protein purified from *E. coli*, which contained only the HMG domain of Rox1 (data not shown).

Deletion of 10 bp between Rox1 sites in operator A decreased *ANB1/lacZ* repression ninefold, but, as can be seen in Figure 3, the affinity of Rox1 for those sites was only slightly reduced. Clearly, as is the case for the synergy between two sites, the effect of this altered spacing on *in vivo* repression cannot be explained by changes in repressor binding.

Finally, a comparison of Rox1 binding to the wild-type operators A and B revealed that the sites in operator

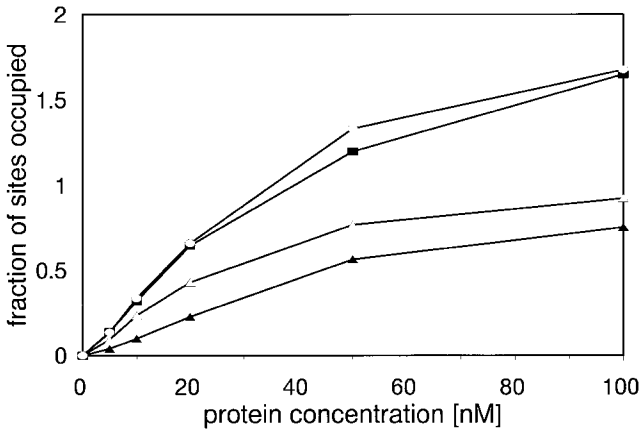


Figure 4.—Rox1-binding curves indicate no cooperativity between Rox1 sites. The data from the gel retardation experiment in Figure 3, carried out with varying amounts of full-length Rox1 protein and WT OpA, Δ5′ OpA, and Δ3′OpA, were analyzed as follows. The fraction of sites occupied per DNA molecule (fraction of sites occupied) was determined for each protein concentration by dividing the amount of DNA complexed with Rox1 by the total amount of DNA present in each binding reaction. Because the wild-type operator A fragment contains two Rox1 sites and shows an additional retarded band (see Figure 3), the amount of upper complex was multiplied by two and added to the lower complex. To determine a theoretical binding curve for independent binding to the two sites in operator A, the resulting values for the single site operators were added for each protein concentration. The calculated fraction of sites occupied was plotted as a function of the protein concentration. The data points are shown as solid squares for the wild-type operator A (WT OpA), open triangles for Δ5′ OpA, solid triangles for Δ3′ OpA, and open circles for the theoretical curve.

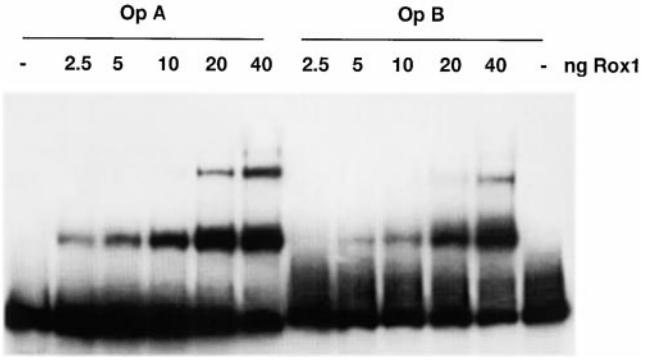


Figure 5.—Rox1 binding to the *ANB1* operators A and B. The autoradiograph represents a gel retardation experiment performed with the full-length Rox1 protein and 15,000 cpm (1 ng) of DNA fragments containing the two Rox1-binding sites of either the *ANB1* operator A or B. The *ANB1* operator A (lanes OpA) was excised as a 387-bp *Hin*dIII-*Nco*I fragment from plasmid YCp(33)AZΔOpB, and operator B (lanes OpB) was excised as a 360-bp *Xho*I-*Bam*HI fragment from plasmid YCp(33)AZ.

B were only slightly weaker targets for Rox1. A gel retardation experiment comparing Rox1 binding to the two operators is presented in Figure 5. The overall binding to the B sites was ~2-fold lower than to the A sites. In contrast, the level of repression mediated by operator B was 3.5-fold less than that mediated by operator A when each was in the B position and 18-fold less when each was in the A position.

In summary, while multiple Rox1 sites can act synergistically *in vivo* to achieve a high level of repression, no cooperativity was observed for Rox1 binding *in vitro*. These results suggest that the *in vivo* synergy results from a higher-order complex involving other factors that form on the operators. In addition, the differences between repression mediated by the A and B operators cannot be explained by their relative affinities for Rox1 or the spacing between their Rox1 sites.

Role of histones H3 and H4 in *ANB1* repression: Rox1 repression is mediated through the Tup1/Ssn6 general repression complex (Zhang *et al.* 1991; Balasubramanian *et al.* 1993; Tzamaris and Struhl 1995). A role for nucleosomes in Tup1/Ssn6-mediated repression of **a**-mating type and glucose-repressed genes has been suggested by both nucleosome phasing experiments and the ability of mutations in the genes encoding histones H3 and H4 to derepress the above regulons (Mattallana *et al.* 1992; Cooper *et al.* 1994; Edmundson *et al.* 1996). Therefore, we investigated the effects of amino-terminal deletions of H3 and H4 on the expression of the *ANB1* gene. The histone mutant and corresponding wild-type strains were transformed with a *ANB1*/*lacZ* fusion, and β-galactosidase assays were performed on permeablized cells grown under both repressed (aerobic) and derepressed (anaerobic) conditions. The results are presented in Table 4 and clearly show that *ANB1* expression is repressed aerobically in all three strains. These results were confirmed in an RNA blot, where no *ANB1* mRNA could be detected under repressed conditions in the wild-type and the histone mutant strains (data not shown).

To determine whether this lack of sensitivity to the histone mutations was a peculiarity of the *ANB1* gene or a general property of Rox1-repressed genes, *lacZ* fusions were constructed to the regulatory regions of the hypoxic *COX5B* and *AAC3* genes, and the level of repression of each was measured in the wild-type and H3 and H4 mutant backgrounds. As shown in Table 4, neither gene is expressed strongly even under anaerobic (derepressed) conditions. Nonetheless, it is clear that neither histone deletion has a dramatic effect on repression; the largest effect is on the *AAC3* gene that is derepressed only twofold in cells carrying the H3 mutation. These results strongly suggest that hypoxic gene repression does not require an interaction between the Rox1-Tup1/Ssn6 repression complex and the amino terminal regions of histones H3 and H4.

To test the effect of the histone aminoterminal deletions on the expression of a gene known to have differences in nucleosome phasing between the repressed

TABLE 4

Repression of hypoxic genes is unaffected by H3 and H4 terminal deletions

Gene[a]	Strain[b]	Derepressed[c]	Repressed	Fold repression[d]
ANB1	WT	187	2.3	81
	H3ΔN	134	1.8	74
	H4ΔN	120	2.0	60
COX5B	WT	1.6	0.8	2.0
	H3ΔN	1.5	0.7	2.1
	H4ΔN	2.9	0.9	3.2
AAC3	WT	3.8	0.4	9.5
	H3ΔN	1.7	0.4	4.2
	H4ΔN	1.7	0.3	5.7
SUC2	WT	23	2.5	9.2
	H3ΔN	16	4.7	3.4
	H4ΔN	19	7.2	2.6

[a] β-Galactosidase assays were carried on permeabilized cells transformed with the *ANB1* (YCp(33)AZ), *COX5B*, or *AAC3 lacZ* fusions described in materials and methods. Invertase assays were carried out to determine levels of expression of the genomic *SUC2* gene.

[b] The strain P1/I8 was used carrying a plasmid with a wild-type H3 and H4 gene (WT) or a plasmid with either the H3 N-terminal deletion and wild-type H4 gene (H3ΔN) or the H4 N-terminal deletion and the wild-type H3 gene (H4ΔN) as described in materials and methods.

[c] Cells carrying the *ANB1*, *COX5B*, and AAC3 lacZ fusions were grown on selective medium either anaerobically (derepressed) or aerobically (repressed). For invertase assays, cells were grown in YP medium containing either 2% raffinose (derepressed) or 4% glucose (repressed).

[d] Fold repression was determined by dividing the derepressed value by the repressed value.

and derepressed states, we assayed invertase activity in the wild-type and histone mutant strains, and these results are presented in Table 4 also. In the wild-type strain, *SUC2* expression was repressed about 9-fold in cells grown in glucose (repressed) as compared to raffinose (derepressed). The H3 and H4 deletions resulted in a 2.7-fold and 3.6-fold derepression, respectively. However, it should be noted that significant levels of repression still occurred in both deletion mutants, indicating that, as with the hypoxic genes, there appear to be alternative mechanisms of repression.

DISCUSSION

Rox1-binding site: Rox1 binds to the consensus sequence YYYATTGTTCTC to mediate repression, but many of the demonstrated or putative Rox1-binding sites in hypoxic genes contain mismatches (see Table 1). To determine how these natural variations would effect Rox1-binding and whether there was a correlation between the level of repression of a gene and the strength of binding of Rox1 to its regulatory region, we carried out a mutational analysis of the core sequence ATTGTT and the base pair immediately preceding it. We found that the proposed consensus sequence had the highest affinity for Rox1; any single base pair substitution tested resulted in decreased affinity. Some of the substitutions were better tolerated, reducing the binding affinity by less than fivefold. These included a change in the first position of the core sequence to a T/A, a change in the last position of the core to either C/G or A/T, and a change in the Y/P in the base pair immediately upstream from the core to A/T.

The relevance of these studies to repression *in vivo* was demonstrated by an evaluation of the effect of a subset of the above point mutations on repression of the *ANB1* gene. For the three cases tested, there was an excellent correlation between the reduction of Rox1 affinity *in vitro* and the reduction in repression *in vivo*. This analysis can be extended to an evaluation of naturally occurring repression sites in hypoxic genes. As expected, there is general correlation between the conservation of the core sequence and the strength of repression of the known hypoxic genes. The upstream region of the *CYC7*, *ERG11*, and *ROX1* genes contains Rox1 sites that deviate at the last position of the core sequence. *ERG11* and *CYC7* each contain two sites that differ at this base pair, while *ROX1* contains one site that differs and one site that contains the core sequence. Variations at this base pair reduce the affinity of Rox1 by 4- to 5-fold, and one would predict that lower levels of repression are directed from these sites. Accordingly, these genes are only partially repressed by Rox1; *CYC7* is repressed only 2-fold (Lowry and Zitomer 1988), *ERG11* is repressed 7-fold (Turi and Loper 1992), and *ROX1* is autorepressed 14-fold (Deckert *et al.* 1995a,b). The *HEM13* gene is also repressed only 16- to 20-fold (Keng 1992), although it contains four Rox1-binding sites. Three of these sites match the consensus core sequence, but one of these contains an A/T at position three that should reduce binding 5-fold. The fourth site has an A/T rather than a T/A base pair at the first

position of the core sequence, and this substitution decreases the Rox1-binding affinity by about 4-fold. It is possible that these two substitutions account for the lack of complete repression of *HEM13* under aerobic conditions. In contrast, the regulatory regions of the hypoxic genes *ANB1*, *AAC3*, and *HMG2* contain four, two, and two Rox1 sites, respectively, which all match the core consensus sequence. These genes are very tightly regulated by Rox1 (Thorsness *et al.* 1989; Lowry *et al.* 1990; Sabova *et al.* 1993).

The Rox1 affinity for its binding sites cannot explain the extent of repression of all the hypoxic genes. For example, *OLE1* is expressed under aerobic conditions despite the presence of two sites that conform with the consensus sequence within 40 bp of each other (Stukey *et al.* 1990). Additional factors such as spacing of the sites or their position relative to other regulatory elements may be more important for some cases.

Other HMG proteins bind to sequences similar to the Rox1 core site. In the case of the human activator SRY, a strict DNA consensus sequence has not been established because the target genes for regulation are unknown. However, several different experiments identified the sequence A/TTTGTT as a high-affinity site for SRY, and a comparison of SRY affinity for different DNA targets demonstrated that SRY binds tightest to the Rox1 core site ATTGTT (Haqq *et al.* 1994; Harley *et al.* 1994). It is not surprising that Rox1 and SRY recognize the same DNA site, because the two HMG domains show extensive similarity and all SRY residues proposed to make contact with bases at the recognition site are conserved in Rox1 (Balasubramanian *et al.* 1993; Werner *et al.* 1995). Also, a mutational analysis and modeling studies of Rox1 indicate a functional homology (Deckert 1997).

Arrangement of Rox1 sites: In the prototype hypoxic gene *ANB1*, the four Rox1-binding sites are arranged in two clusters that we have termed operators. An analysis of operator A demonstrated that the two sites act synergistically in repression, but this synergy did not result from cooperative Rox1 binding. These observations suggest that additional factors, such as the corepressors Tup1 and Ssn6, are responsible for the synergistic effect observed in the cell. Repression could depend on the formation of a multiprotein complex at the operators, which interferes with transcriptional activation.

We also explored the spacing requirements for the two Rox1 sites comprising operator A. The spacing between the sites varied in increments of 5 bp from 21 to 41, and the results indicated that spacing did not play a large role in determining the degree of repression. These experiments also altered the helical phasing of the sites and so also demonstrated that Rox1 does not depend strongly on the positioning of binding sites on the same face of the DNA helix. Given that Rox1 bends DNA at an angle of 90°, this conclusion was somewhat surprising. Two bends originating from Rox1 sites on the same face of the helix would cause the DNA molecule to fold back on itself in a U-shape, while bends originating from sites on opposite sides would lead to a Z-shape, a very different topology. It is possible that the overall topology of the DNA is influenced more by the higher-order complex involving the Tup1/Ssn6 complex. It should be noted that our findings that there is not a strong influence of spacing on repression are consistent with the general arrangement of repression sites in the regulatory regions of other hypoxic genes, where the distance between neighboring sites varies from 20 to 150 bp, and even the orientation of the binding sites is different in many cases.

The role of chromatin in repression: We report here that aminoterminal deletions of the histones H3 and H4 do not derepress *ANB1* expression and have little effect on the expression of two other hypoxic genes. Although at odds with the model of nucleosome involvement in Tup1/Ssn6 repression, our findings are perhaps not so surprising. Experimental evidence has pointed to two alternative mechanisms of repression. On one hand, mutations in histone genes suppress Tup1/Ssn6-mediated repression, and Tup1 has been demonstrated to interact with histones H3 and H4 (Edmundson *et al.* 1996). Furthermore, chromatin structure analyses with α2-Tup1/Ssn6 and Mig1-Tup1/Ssn6 repressible genes suggested that chromatin alternations accompany repression (Mattallana *et al.* 1992; Cooper *et al.* 1994). On the other hand, repression by Tup1/Ssn6 is suppressible by mutations in subunits of the RNA polymerase II holoenzyme (Wahi and Johnson 1995; Song *et al.* 1997), and *in vitro* transcription carried out with a naked DNA template was repressible by Tup1 (Redd *et al.* 1997). These data support a model in which repression is achieved through direct interactions of the repression complex and the basal transcriptional machinery assembled at the TATA box. It is quite possible that the Tup1/Ssn6 complex represses both through alterations in chromatin structure and direct interaction with the RNA polymerase II holoenzyme. One or the other of these mechanisms may be more important to repression of a particular gene or regulon, while other genes may be repressed through both mechanisms. Eliminating one mechanism may result in complete derepression of some genes and, perhaps, only partial or no derepression of others.

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