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Mutational Analysis of Rox1, a DNA-Bending Repressor of Hypoxic Genes in *Saccharomyces cerevisiae*

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Rox1 is a repressor of the hypoxic genes of *Saccharomyces cerevisiae*. It binds to a specific hypoxic consensus sequence in the upstream region of these genes and represses transcription in conjunction with the general repression complex Tup1-Ssn6. In this study, we demonstrated that the first 100 amino acids comprising the HMG domain of Rox1 were responsible for DNA binding and that when bound, Rox1 bent DNA at an angle of 90°. A mutational analysis resulted in the isolation of seven missense mutations, all located within the HMG domain, that caused loss of DNA binding. The effect of these mutations on the structure of Rox1 was evaluated on the basis of the homology between Rox1 and the human male sex-determining protein SRY, for which a structural model is available. The failure to isolate missense mutations in the carboxy-terminal three-quarters of the protein prompted a deletion analysis of this region. The results suggested that this region was responsible for the repression function of Rox1 and that the repression information was redundant. This hypothesis was confirmed by using a set of fusions between sequences encoding the *GAL4* DNA-binding domain and portions of *ROX1*. Those fusions containing either the entire carboxy-terminal region or either half of it were capable of repression. Repression by selected fusions was demonstrated to be dependent on Ssn6.

Baker's yeast, *Saccharomyces cerevisiae*, responds to limiting oxygen in the environment by inducing a set of hypoxic genes. Among the proteins encoded by these genes are selected enzymes in heme, sterol, and fatty acid biosyntheses that utilize oxygen as an electron acceptor, alternate cytochrome subunits such as subunit Vb of cytochrome oxidase and iso-2-cytochrome *c*, and an alternate ADP/ATP translocator (see reference 52 for a review). While the transcription of these genes is activated by a variety of different transcriptional activators, all are repressed by the hypoxic repressor Rox1 (52). Rox1 binds to a specific hypoxic consensus sequence that is located in the promoter region of each of the hypoxic genes (2, 22, 52). The expression of the *ROX1* gene is regulated by heme, the synthesis of which is oxygen dependent, at least in part through the heme-dependent transcriptional activator Hap1 (7, 19, 23). Thus, when oxygen tension is high, Rox1 accumulates in the cell, binds to the upstream regions of the hypoxic genes, and represses their transcription. When oxygen levels are low, *ROX1* expression is uninduced, thereby allowing derepression of the hypoxic genes.

Repression of the transcription of the hypoxic genes by Rox1 depends upon at least two other proteins, Tup1 and Ssn6 (2, 43, 50). These two proteins form a complex (42, 47) that serves as a general repressor; it is involved in transcriptional repression of **a**-specific mating type genes (18, 21), glucose-repressed genes (5, 34, 36, 40, 41, 46), and genes whose products control flocculence (11) and plasmid stability (38). In those cases studied, the Tup1-Ssn6 complex functioned in conjunction with the following sequence-specific DNA-binding proteins: Rox1 for the hypoxic genes, the α2-Mcm1 complex for **a**-specific genes (18), and Mig1 for glucose-repressed genes (26, 39). On the basis of the ability of the Tup1-Ssn6 complex to repress a reporter gene when Ssn6 was fused to the DNA-binding bacterial protein LexA, Keleher et al. (18) proposed that the function of the sequence-specific DNA-binding proteins in repression is to anchor the Tup1-Ssn6 complex to specific genes, targeting them for repression. Experimental evidence that supports models in which the repression complex interacts with the basal transcriptional machinery (17) or alters chromatin structure (6) has been obtained.

The *ROX1* gene encodes a protein of 368 amino acids containing an HMG domain in the first 100 residues, followed by a glutamine-rich stretch of 22 residues (2). The remaining region of the protein bears no significant similarity to any other protein in the GenBank database. The HMG domain comprises a motif found in a number of other sequence-specific DNA-binding regulatory proteins as well as a number of nonspecific binding proteins, including the high-mobility-group I proteins from which the motif gets its name (see reference 15 for a review). This domain is responsible for the DNA binding of all HMG proteins, and the sequence-specific proteins in which it has been studied also bend DNA (15). Nuclear magnetic resonance structures have been deduced for one of the HMG domains in the rat HMG1 protein (44) and for the DNA-HMG domain complex of the human SRY protein (16, 45). The purpose of the present study was to characterize the binding of the Rox1 protein to DNA and to carry out a mutational analysis of the *ROX1* gene in order to define important residues involved in DNA interactions as well as those involved in repression.

MATERIALS AND METHODS

Strains, cell growth, and transformations. The *S. cerevisiae* strain RZ53-6Δ*rox1* (*MAT*α *trp1-289 leu2-3,112 ura3-52 ade1-100*, *rox1*::*LEU2*) was described previously (7). The following *S. cerevisiae* strains were constructed for this study by standard methods of yeast genetics (32): MZ14-61 (*MAT*α *his3 leu2 trp1 lys2-801 rox1*::*LEU2 tif51A*::*TRP1 ura3*::*AZ4* Gal$^-$), MZ12-1 (*MAT*α *trp1 leu2 ade1 rox1*::*LEU2 ura3*::*AZ4*), MZ18-23 (*MAT*α *trp1 leu2 his3 ura3 gal4*Δ*532 ade2*

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rox1::*LEU2*), and MZ27-6 (*MAT*α *trp1 leu2 his3 ura3-52 gal4*Δ*532 rox1*::*LEU2 ssn6*::*LEU2* Ade$^-$).

The growth of yeast cells for RNA blots, immunoblots, and β-galactosidase assays has been described previously (2, 7). Yeast cells were transformed as described previously (20).

The *Escherichia coli* strain HB101 was maintained and transformed as described previously (1).

Enzymes and general methods for plasmid constructions. Plasmid constructions were carried out according to standard protocols (1). Enzymatic reactions were carried out under the conditions recommended by the vendors. Most restriction enzymes, T4 DNA ligase, and the Klenow fragment of DNA polymerase were purchased from New England Biolabs. *Taq* polymerase was purchased from Perkin-Elmer. When PCR was used for plasmid constructions, only 12 cycles were carried out with 100 ng of template to minimize mutations. Synthetic oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer.

General plasmids. YCp(33)*ROX1*e contains a 2.8-kb *Hin*dIII fragment carrying the *ROX1* coding sequence with the c-*myc* epitope 9E10 inserted at the carboxy terminus plus 1.2 kb of 5′ flanking sequences and 0.5 kb of 3′ flanking sequences from YEp(112)*ROX1*-2(9E10) (2) inserted into the *URA3*-centromeric vector YCplac33 (14) lacking the *Xba*I site in the polylinker.

The HMG domain (codons 1 through 103) of the wild-type and mutant derivatives of *ROX1* were subcloned into the *E. coli* expression vector pMAL-c2 (New England Biolabs) as follows. The HMG domain was amplified by PCR using the appropriate wild-type or mutant template and the primers 5′-CGCG GTACCAATCCTAAATCCTCTACACCT and 5′-CGGCCTGCAGTTACTGT TGCTCGATTTCCTTC. The amplified fragments were digested with *Kpn*I plus *Pst*I and ligated into the *Kpn*I-*Pst*I sites of p*MALE-ROX1* (2). In the resulting plasmids the entire *ROX1* coding sequence of the p*MAL-ROX1* plasmid was replaced by the amplified HMG domain, generating p*MAL-ROX1(HMG)* or equivalent mutant plasmids.

The plasmid used for the DNA bending experiments, pCY4-R1Op, was constructed by insertion of the synthetic Rox1 binding site, 5′-TCCCATTGTTCT CGA and 5′-GATCTCGAGACCAATGGGAAGCT, into the *Sac*I-*Bgl*II sites of pCY4 (30).

YCp(33)AZ was constructed by subcloning the *Sma*I fragment containing the *ANB1-lacZ* fusion from YCpAZ6 (22) into the *Sma*I site of YCplac33.

YCp(33)AZ-UAS$_{GAL}$ was constructed by insertion of two copies of the synthetic Gal4 binding site, 5′ TCGACGGACGACTGTCCTCCGAGCTCG and 5′-TCGACGACGTCGGAGGACAGTCGTCCCG, into the *Xho*I site of YCp (33)AZ.

***ROX1* deletion plasmids.** Diagrams of the deletion plasmids are contained in Fig. 5.

(i) YCp(22)*R1*Δ*100*/*123*. A 1.5-kb fragment containing 1.2 kb of *ROX1* 5′ flanking sequences plus codons 1 to 99 of the coding sequence was generated by PCR amplification using YCp(33)*ROX1*e as a template and the M13/pUC reverse sequencing primer (New England Biolabs) and 5′-CCGGTACCTACTC GAGTTCCTTCAAAAGTAGTTGC as primers. The latter primer introduced *Xho*I and *Kpn*I sites immediately after codon 99. This fragment was digested with *Kpn*I plus *Hin*dIII and inserted into the *Kpn*I-*Hin*dIII sites of the *TRP1*-centromeric vector YCplac22 (14). A 1.2-kb fragment containing *ROX1* codons 124 to 368 plus 500 bp of 3′ flanking sequences was generated by PCR amplification using YCp(33)*ROX1*e as a template and the M13/pUC sequencing primer (New England Biolabs) and 5′-GGCTCGAGCCCTTTAACAACAATATAGTTCT TATG as primers. The latter primer generated an *Xho*I site immediately 5′ to codon 124. This fragment was digested with *Eco*RI plus *Xho*I and ligated into the plasmid constructed as described above.

(ii) YCp(22)*R1*Δ*100*/*175*. A 1.1-kb *Bgl*II-*Eco*RI fragment containing codons 176 through 368 plus 500 bp of 3′ flanking sequence was excised from Ycp(22) *ROX1*H (7). YCp(22)*R1*Δ*100*/*123* was digested with *Xho*I plus *Eco*RI, and the large fragment containing the vector plus the 5′ flanking sequences and the coding sequence from codon 1 through codon 99 was purified. The two fragments were ligated together with a synthetic adapter consisting of 5′-TCGACCATTG TG and 5′-GATCCACAATGG.

(iii) YCp(22)*R1*Δ*100*/*245*. A 0.9-kb *Age*I-*Eco*RI fragment containing codons 246 through 368 plus 500 bp of 3′ flanking sequences was excised from YCp(22) *ROX1*H. YCp(22)*R1*Δ*100*/*123* was digested with *Xho*I plus *Eco*RI, and the large fragment containing the vector plus the 5′ flanking sequences and the coding sequence from codon 1 through codon 99 was purified. The two fragments were ligated together with an adapter consisting of 5′-TCGAGCTCGCA and 5′-CC GGTGCGAGC.

(iv) YCp(22)*R1*Δ*177*/*368*. YCp(22)*ROX1*H was digested with *Bgl*II, the single-stranded ends were filled in with Klenow fragment, and the plasmid was digested with *Xba*I, generating a 0.9-kb fragment extending from the *Xba*I site in the 5′ flanking region to codon 176. YCp(22)*ROX1*H was digested with *Bst*EII, the single-stranded ends were filled in, the plasmid was digested with *Xba*I, and the large vector fragment was purified. The two fragments were ligated together.

(v) YCp(22)*R1*Δ*194*/*245*. A 1-kb *Xba*I-*Pvu*II fragment containing 5′ flanking and coding sequences through codon 193 was excised from YCp(22)*ROX1*H. YCp(22)*ROX1*H was digested with *Age*I, the single-stranded ends were filled in, the plasmid was digested with *Xba*I, and the large vector fragment was purified. The two fragments were ligated together.

(vi) YCp(22)*R1*Δ*194*/*357*. The 1-kb *Xba*I-*Pvu*II fragment from YCp(22)*ROX1*H described above was ligated with the vector fragment of YCp(22)*ROX1*H generated by digestion with *Bst*EII, filling in of the single-stranded ends, and digestion with *Xba*I.

(vii) YCp(22)*R1*Δ*247*/*368*. YCp(22)*ROX1*H was digested with *Age*I plus *Bst*EII, the single-stranded ends were filled in, and the plasmid was recircularized by ligation.

(viii) YCp(22)*R1-t104*. A 1.5-kb fragment containing 5′ flanking sequences and codons 1 through 103 was generated by PCR amplification of the template YCp(22)*Rox1*H using the reverse primer and 5′-CCGGTGACCTACTGTTGC TCGATTTCCTTC as primers. The latter template placed a termination codon followed by a *Bst*EII site immediately 3′ to codon 103. A 0.75-kb *Xba*I-*Bst*EII fragment was excised from the PCR product and ligated into the *Xba*I-*Bst*EII sites of YCp(22)*ROX1*H.

(ix) YCp(22)*R1-t123*. A 1.5-kb fragment containing 5′ flanking sequences and codons 1 through 123 was generated by PCR amplification of the template YCp(22)*Rox1*H using the reverse primer and 5′-GGCGGTACCTATTGTAAT TGCGGTTGTTACTG as primers. The latter template placed a termination codon followed by a *Bst*EII site immediately 3′ to codon 123. A 0.8-kb *Xba*I-*Bst*EII fragment was excised from the PCR product and ligated into the *Xba*I-*Bst*EII sites of YCp(22)*ROX1*H.

***GAL4-ROX1* fusion plasmids. (i) pMAR1.** A 1.6-kb *Eco*RI-*Pst*I fragment containing the entire *ROX1* coding sequence plus 500 bp of 3′ coding sequences from p*MALE-ROX1* was ligated into the *Eco*RI-*Sal*I sites of pMA424 (24) with an adapter consisting of 5′-TCGACGCTAGCCTGCA and 5′-GGCTAGCG.

(ii) pMAR1(X-C). A 1.3-kb *Xho*I-*Bam*HI fragment containing the *ROX1* coding sequence from codon 124 to codon 368 plus 500 bp of 3′ sequences from YCp(23)*R1*Δ*100-123* was ligated with pMA424 digested with *Eco*RI and *Sal*I with an adapter consisting of 5′-ATTCGAAGGATCCCGCGG and 5′-TCGAC CGCGGATCCTCG.

(iii) pMAR1(X-A). pMAR1(X-C) was digested with *Age*I and *Sal*I, the ends were filled in, and the plasmid was recircularized.

(iv) pMAR1(A-C). A 0.9-kb *Xho*I-*Bam*HI fragment from YCp(22)*R1*Δ*100-245* was ligated with pMA424 with the adapters used for the construction of pMAR1 (X-C).

RNA blots, immunoblots, β-galactosidase assays, and DNA sequence analysis. RNA blotting was carried out with fragments for the gene-specific *ANB1*, *ROX1*, and *ACT1* probes as described previously (50). The 1.4-kb *Hin*dIII *HEM13* probe was obtained from plasmid pUC19::*HEM13*, which contained a 1.8-kb *Sac*I-*Sph*I insert in pUC19 (obtained from R. Labbe-Bois) (49).

Immunoblots were probed for the epitope-tagged Rox1 protein as described previously (2).

β-Galactosidase assays were carried out as described previously (31). Assays were carried out multiple times and with at least two independent transformants.

DNA sequence analysis was carried out as described previously (33).

***ROX1* mutagenesis.** Hydroxylamine mutagenesis of YCp(33)*ROX1*e was carried out as described previously (32). The mutagenized plasmid was transformed into *E. coli* HB101 cells, and the transformation mixture was plated on six plates. The transformants from these plates were maintained in separate pools. The colonies were scraped from each plate, and DNA was prepared from the cells and used to transform the yeast strain MZ14-61 to select for mutants.

PCR mutant pools were generated as follows (51). A 1.6-kb fragment containing the *ROX1* 5′ flanking sequences plus codons 1 through 357 was amplified from plasmid YCp(33)*ROX1*e by using the primers 5′-AATTGGAAATCTGG TAGG and 5′-CTAGTTTTAGCGGTGACC. PCR was carried out under the conditions recommended by the vendor for 30 cycles with 10 ng of template. Three separate reactions were performed to obtain independent pools. The reaction products were digested with *Xba*I and *Bst*EII and fractionated on an agarose gel, and the desired fragment was purified and ligated into the *Xba*I-*Bst*EII sites of YCp(33)*ROX1*e. The ligation products were transformed into HB101 cells, and DNA was prepared from the transformants and used to transform MZ14-61 to select for mutants.

Purification of Rox1 and gel retardation. The HMG domain derivatives of Rox1 were partially purified from *E. coli* extracts as described previously (2). The purity of the fusion protein was determined by sodium dodecyl sulfate gel electrophoresis (1) to be well over 75% in all cases, and the concentrations of protein were determined by the Bradford assay (Bio-Rad). Where indicated, the Rox1 HMG domain was cleaved from the maltose-binding protein with the protease Xa as recommended by the vendor (New England Biolabs).

Full-length Rox1 containing an amino-terminal addition of six histidines was expressed in a baculovirus expression system (Invitrogen) and purified through a nickel column (Invitrogen), as will be described in detail elsewhere.

Gel retardation experiments were carried out with a 32-bp synthetic double-stranded DNA containing one copy of the 12-bp hypoxic consensus sequence as described previously (2) or a set of restriction fragments derived from pYC4-R1Op (see Fig. 1A).

RESULTS

Rox1 bends DNA. Since several HMG domain proteins have been reported to bend DNA (3, 6, 8–10, 12, 27, 28), the ability

of Rox1 to induce bending was assayed. If a protein induces a bend in DNA, the rate of migration through polyacrylamide of the DNA-protein complex will depend upon both the angle and the position of the bend within the DNA fragment (37, 48). Bending activity was measured both for the HMG domain of Rox1 expressed in *E. coli* as a maltose-binding fusion [maltose-binding protein (MBP)-Rox1(HMG)] and for the full-length protein expressed in insect cells and containing six histidine residues at the amino terminus which facilitated purification on a nickel column. This latter system was required because bacterial cells produced extensive degradation of the full-length Rox1 as assayed by immunoblotting (data not shown).

To assay for bending, a plasmid that contained a direct repeat of the 381-bp pBR322 *Eco*RI-*Bam*HI fragment flanking the hypoxic consensus sequence was used to generate a set of fragments of identical size and sequence (circularly permuted) but with the binding site in a different position in each case as illustrated in Fig. 1A. The migration of the protein-DNA complex for the MBP-Rox1(HMG) fusion protein (Fig. 1B, lanes 2 to 6), the Rox1(HMG) protein cleaved free of the MBP with the protease Xa (Fig. 1B, lanes 8 to 12), and the intact Rox1 protein (Fig. 1C) showed the same dependence upon the position of the binding site. The angle of the bend was calculated from this change in mobility as a function of the position of the binding site (9) to be 90° for all three proteins. The locus of flexure (the site of the bend [9]) was calculated to be at the binding site, as expected.

Isolation of Rox1 mutants. Rox1 has at least two functions: DNA binding, mediated through the HMG domain as demonstrated above, and repression, mediated through a presumed interaction with the Tup1-Ssn6 complex. To determine the amino acid residues critical to these functions, a selection for mutations in the *ROX1* gene was devised. The aerobically expressed *TIF51A* gene and the Rox1-repressed hypoxic *ANB1* gene are homologs encoding the essential protein eIF5A (25, 35). Cells carrying a null allele of *TIF51A* cannot grow aerobically unless *ANB1* is expressed constitutively as in a *rox1* deletion strain. A *tif51A rox1* strain which also contained an *ANB1-lacZ* disruption of the *URA3* gene rendering the cells uracil auxotrophs was constructed. When this strain was transformed with a plasmid carrying both *URA3* and *ROX1* genes, no Ura^+ transformants were obtained; the plasmid-borne *ROX1* gene resulted in aerobic repression of *ANB1* and, consequently, cell death due to an eIF5A deficiency. Thus, the selection for *ROX1* mutants was carried out by mutagenizing the *URA3-ROX1* plasmid in vitro, transforming the mutant plasmid pool into the strain described above, and selecting for transformants on plates lacking uracil. Colonies were tested for blue color on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) plates (aerobic expression of the *ANB1-lacZ* fusion) confirming that the putative mutants expressed *ANB1* constitutively.

Most of the *ROX1* mutants isolated in initial selections proved to contain nonsense or frameshift alleles which were not of primary interest. Therefore, the following scheme for the characterization of putative mutants was developed to screen out such alleles. Immunoblotting was carried out on extracts prepared from mutant clones. Since the mutagenized *ROX1* allele encoded the c-*myc* epitope at its carboxy terminus, premature termination by a nonsense mutation would not yield a visible band. Also, since Rox1 represses its own expression (7), *rox1* missense mutations caused increased protein accumulation. An example of this derepression can be seen in Fig. 2 for most of the *rox1* missense mutants ultimately characterized. (One, *rox1-G50D*, was isolated in an original round before the epitope-tagged clone was adopted for use in the mutagenesis

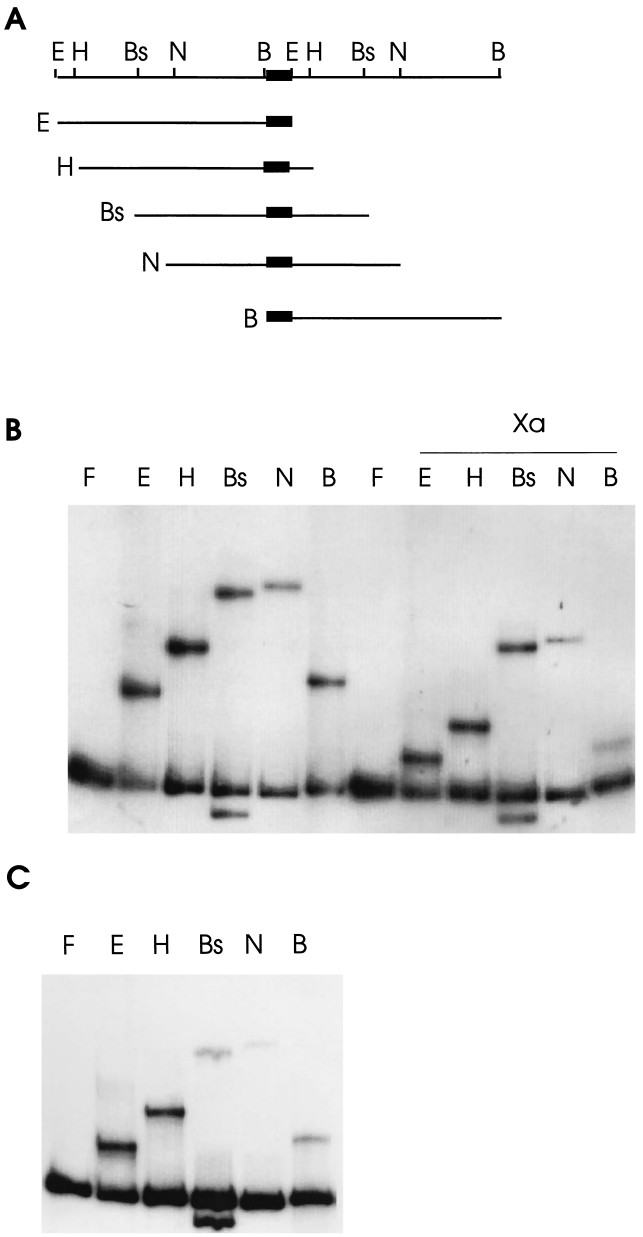

FIG. 1. Rox1 bends DNA. (A) A map of the direct repeat of the 381-bp *Eco*RI-*Bam*HI fragment from the plasmid pCY4 is presented at the top as a solid line. The thickened segment between the repeats represents the 14-bp insert containing the Rox1 binding site. The abbreviations and positions of the restrictions sites within the repeats are as follows: E, *Eco*RI (position 1); H, *Hin*dIII (position 32); Bs, *Bst*NI (position 133); N, *Nhe*I (position 232); and B, *Bam*HI (position 378). The five lines below the map represent the fragments used in the gel retardation experiment, with the restriction enzyme used to generate each fragment represented on the left. (B and C) An autoradiograph of the gel retardation experiment carried out on an 8% polyacrylamide gel with 25 ng of MBP-Rox1(HMG) (B) or 5 ng of full-length Rox1 (C) and 15,000 cpm of the indicated DNA fragment. The protein used for the last five lanes, indicated by Xa, was digested with 0.1 μg of protease Xa, which cleaved between the MBP and the Rox1(HMG). The symbol at the top of each lane represents the fragment used as indicated in panel A; F represents free DNA, indicating the lane in which a sample of the *Eco*RI fragment lacking protein was loaded. Two bands are visible at the bottom of the lanes designated Bs because of a contaminating DNA fragment from the enzyme digest of the plasmid.

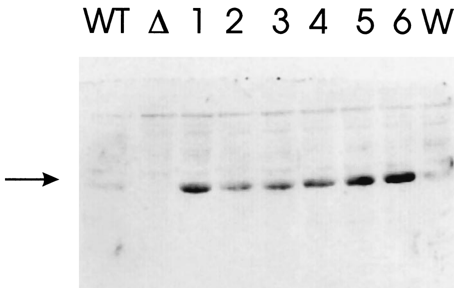

FIG. 2. ROX1 mutants overexpress the full-length protein. Immunoblotting was carried out with 15 μg of crude extracts from cells of the *rox1*Δ strain MZ12-1 grown on SC-uracil medium (32) and carrying the following plasmids: WT, YCp(33)*ROX1*e; Δ, YCplac33 (lacking a *ROX1* gene); lane 1, YCp*rox1-N15D*; lane 2, YCp*rox1-F17S*; lane 3, YCp*rox1-F20S*; lane 4, YCp*rox1-S46P*; lane 5, YCp*rox1-G50N*; and lane 6, YCp*rox1-Y84C*. The arrow indicates the position of the Rox1 protein. The blot was developed by using the ECL system (Amersham).

scheme.) DNA was prepared from the mutants fulfilling this criterion and used to transform *E. coli* cells to ampicillin resistance to recover the plasmid. The plasmid was transformed into the yeast MZ12-1 strain, which was similar to the strain used for the original selection but contained a wild-type *TIF51A* allele, and the mutant phenotype was confirmed by measuring the expression of the *ANB1-lacZ* fusion. The sequence of the *ROX1* coding region in mutant plasmids passing this test was determined.

Seven different mutants were identified, and the locations of the mutations in each are shown in Fig. 3. All contained missense mutations in the HMG domain. The mutant alleles were designated by the single-letter code for the wild-type amino acid, followed by the codon number, followed by the single-letter code for the mutant amino acid. All eight mutant alleles contained only a single base pair change, with the exception of *G50N*, which had a double mutation at codon 50. Mutants *G50D* and *G50N* were isolated from hydroxylamine mutagenesis, and *G50D* was isolated three times from different mutant pools. The other mutants were isolated from the PCR mutagenesis, and *N15D* and *F17S* were isolated twice and three times, respectively, from independent pools.

The effect of each of these mutations on the expression of the *ANB1-lacZ* fusion was determined by β-galactosidase assays performed on mutant extracts. The results, presented in Table 1, show that all caused complete loss of repression activity, with the exception of *Y84C*, which showed only a 3.2-fold repression of *ANB1-lacZ* expression. These results agreed well with determinations of *ANB1* mRNA levels in an RNA blot (data not shown). It should be noted, however, that the level of repression of *ANB1* expression in the *Y84C* mutant may not be an accurate reflection of Rox1 activity. Since Rox1 autorepresses its own synthesis, *rox1* mutants can have as much as a 10-fold increase in protein levels, as is evident in Fig. 2. Thus, the 3.2-fold repression in the *rox1-Y84C* mutant was achieved in a cell with substantially increased levels of the mutant Rox1 protein.

TABLE 1. Repression of *ANB1-lacZ* expression in *ROX1* missense mutant[a]

Allele	β-Galactosidase activity (U)	Repression (fold)
Wild type	5.0 ± 3.3	18
*rox1*Δ	89 ± 22	1
rox1-N15D	77 ± 14	1.2
rox1-F17S	104 ± 10	0.9
rox1-F20S	81 ± 7.5	1.1
rox1-S46P	61 ± 9.3	1.5
rox1-G50N	76 ± 2.8	1.2
rox1-Y84C	28 ± 3.9	3.2

[a] Strain MZ12-1 was transformed with YCp(33) plasmids carrying the indicated *ROX1* alleles. Cells were grown on SC-uracil medium, and the assays were performed at least twice with two independent transformants.

DNA binding activity of mutant Rox1 HMG domains. To determine the DNA binding activity of the mutant Rox1 (HMG) proteins, the coding sequences for the mutant HMG domains were subcloned into the MBP fusion vector and expressed in *E. coli*. The fusion proteins were purified, and DNA binding was assayed by gel retardation. No DNA binding could be detected for most of the mutant proteins, even at concentrations 1,000 times greater than those shown for the wild-type protein in Fig. 4. The two exceptions were the N15D and F17S proteins, which bound DNA weakly. As can be seen in Fig. 4A, 2.5 μg of N15D protein (lane 6) was required to bind an amount of DNA equivalent to that bound by 10 ng of wild-type protein (lane 3), a 250:1 ratio. The F15S protein bound DNA even less strongly. The same results were obtained for the Rox1(HMG) cleaved from the MBP (data not shown). Thus, the lack of repression activity of the mutant Rox1 proteins can be explained by their loss of DNA binding activity. It should be noted that the one protein that showed some repression activity in vivo, Y84C, did not bind DNA in vitro. It is likely that the protein was denatured during purification or that the mutant fusion protein could not fold properly in *E. coli*.

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                    S                                        M                  Y
Consensus       KBP NAa  a    B       P             K  G  W  L    K  a       B  H      a
                                                       N
                    D S  S                         P   D                                  C
                    ↑ ↑  ↑                         ↑   ↑                                  ↑
Rox1          KIPRPKNAFILFRQHYHRILIDEWTAQGVEIPHNSNISKIIGTKWKGLQPEDKAHWENLAEKEKLEHERKYPEYKYKPVRKSKKK
              :: || ||||::    :| :: |         :|| ||| :|  || |   :|  : : | |  : |  ||| |||:| || |
SYR           RVKRPMNAFIVWSRDQRRKMALENPRM-----RNSEISKQLGYQWKMLTEAEKWPFFQEAQKLQAMHREKYPNYKYRPRRKAKML
               ↓   ↓                              ↓    ↓          ↓  ↓
               L   I                              M    R          I  S
                    __________________         ______________  _______________________
                         helix 1                    helix 2              helix 3
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FIG. 3. Rox1 mutations and comparison with the mutations and sequence of SRY. The HMG domains of the Rox1 and SRY proteins are aligned to maximize the similarities; identical residues are indicated with vertical lines, and conservative substitutions are indicated with colons. The mutations isolated in this study are presented above the Rox1 sequence, and the mutations isolated in SRY are indicated below the SRY sequence (4, 29). A consensus sequence (15) for most of the known HMG proteins is presented in bold at the top (B, basic amino acids R and K; a, aromatic amino acids F, W, and Y). The three α-helices in the HMG domain are indicated. The DNA substitutions that gave rise to the amino acid substitutions are as follows for the *ROX1* coding strand, for which the bases are numbered starting from the A in the ATG translational initiation codon: A-43 to G (A43G) for N15D, T50C for F17S, T59C for F20S, T116C for S46P, G129A for G50D, G128A and G129A for G50N, and A251G for Y84C.

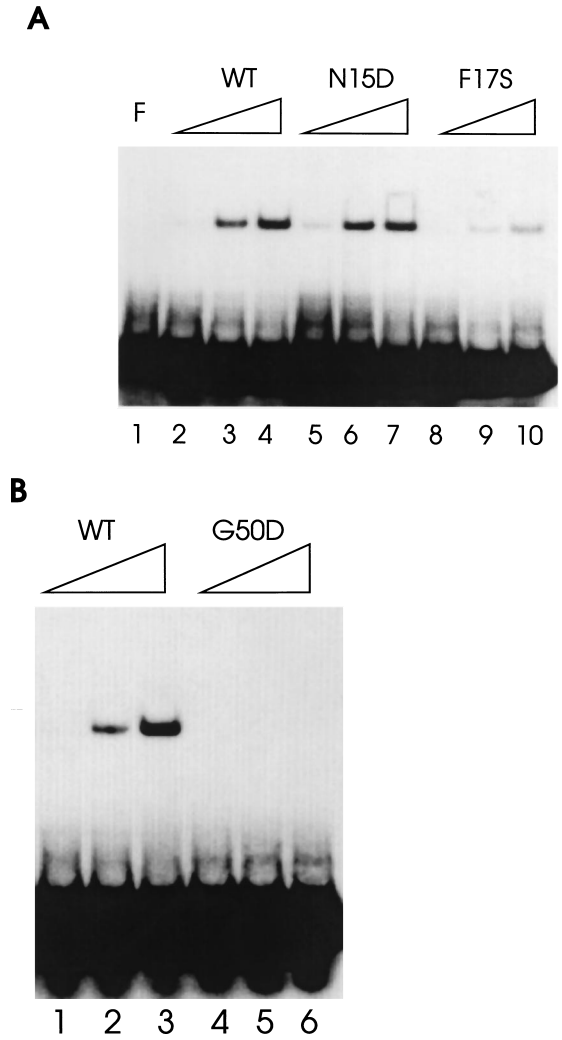

FIG. 4. The mutant Rox1(HMG) proteins have reduced or no DNA binding activity. Gel retardation was carried out with 15,000 cpm of the 32-bp hypoxic DNA fragment and with various amounts of the mutant MBP-Rox1(HMG) proteins. No protein was added to lane F. (A) The lanes contained the following proteins in the amounts indicated: lanes 2 to 4, wild-type protein (2, 10, and 20 ng, respectively); lanes 5 to 7, MBP/Rox1(HMG)N15D protein (0.5, 2.5, and 5 μg, respectively); and lanes 8 to 10, MBP/Rox1(HMG)F17S protein (0.5, 2.5, and 5 μg, respectively). (B) The lanes contained the following proteins in the amounts indicated: lanes 1 to 3, wild-type protein (2, 10, and 20 ng, respectively); and lanes 4 to 6, MBP/Rox1(HMG)G50D protein (0.5, 2.5, and 5 μg, respectively).

Deletion analysis of the Rox1 C terminus. No missense mutations in the *ROX1* coding sequence outside the HMG domain were isolated from the mutageneses of the entire *ROX1* gene described above. In order to focus on the almost three-quarters of the coding sequence outside this DNA binding domain, PCR amplification using a variety of restriction fragments from the non-HMG domain was carried out. These fragments were then ligated into the coding sequence to restore the intact gene, and the selection for *rox1* mutants was applied. Approximately 100 Ura^+ *ANB1-lacZ* constitutive mutants were screened by immunoblotting seeking *rox1* mutants that produced full-length protein; none were found. These results suggested that this region was quite tolerant of amino acid substitutions.

As an alternative to missense mutations, we constructed a series of deletions in the carboxy-terminal region. Plasmids carrying these deletions were transformed into a *rox1* deletion strain carrying an *ANB1-lacZ* fusion, and the transformants were assayed for β-galactosidase activity as a measure of repression activity. Figure 5 shows the deletion constructs and the results of these assays. The data indicate that the HMG domain alone is insufficient for repression; transformants carrying the *rox1-t104* and *rox1-t123* alleles showed no repression activity. One other deletion allele, Δ*177/368* (deletion of codons 177 to 368), also showed no repression activity, and one, Δ*194/357*, showed partial repression. The lack of repression activity of these rather large deletions could be due to a failure to express or properly localize the mutant protein or to the deletion of a repression domain. The results obtained with deletion alleles that retain activity are more revealing and provide some insight into why we failed to isolate missense mutations. The *rox1* deletion alleles Δ*100/123*, Δ*100/175*, Δ*100/245*, Δ*194/245*, and Δ*247/368* had little effect on the ability of Rox1 to repress *ANB1-lacZ* expression. Of particular interest is the fact that Δ*100/245* and Δ*247/368* are nonoverlapping deletion alleles that in combination delete the entire carboxy terminus and yet both retained substantial repression activity, strongly suggesting that the repression domain is redundant.

Because *ROX1* expression is autorepressed, it was possible that some of the deletion alleles that showed full repression of the *ANB1-lacZ* fusion were actually partially defective but that increased protein levels compensated for lowered activity. To measure expression levels of the *ROX1* mRNA in transformants carrying the deletion alleles, RNA blotting was carried out and the blot was probed for *ROX1* RNA as well as the RNAs for *ANB1*, *HEM13*, a gene that is only partially repressed by Rox1, and the actin gene, *ACT1*, as a control (Fig. 6). The results clearly show that transformants carrying the *rox1* deletion alleles Δ*100/123*, Δ*100/175*, and Δ*194/245* (lanes 3, 4, and 5) contained nearly wild-type levels of *ROX1* mRNA (lane 1), indicating that these alleles retained close to full repression activity. On the other hand, transformants carrying the alleles *rox1*Δ*100/245* and *rox1*Δ*247/368* (lanes 6 and 7), which repressed *ANB1* expression 7.5- and 5.9-fold, respectively, contained increased *ROX1* mRNA and, therefore, must have lost some repression activity. The transformants carrying deletion alleles which showed reduced levels of repression, Δ*194/357*, Δ*177/368*, and *t104* (lanes 8, 9, and 10), also showed substantially derepressed *ROX1* mRNA levels, as expected. The levels of *ANB1* and *HEM13* RNA which accumulated in the deletion transformants were as predicted from the *ANB1-lacZ* expression and confirmed that the fusion enzyme reflected mRNA accumulation.

To quantitate the incomplete repression of *ROX1* in some of the deletion mutants and to rule out the possibility that the levels of mutant *ROX1* mRNA were due to alterations in RNA stability resulting from sequences deleted from the RNA, a *ROX1* deletion strain was cotransformed with a centromeric plasmid containing the upstream region and translational initiation codon of *ROX1* fused to *lacZ* plus the various *ROX1* deletion plasmids, and β-galactosidase assays were carried out. The results are presented in Fig. 5. As reported previously, expression of the *ROX1-lacZ* fusion was 9.6-fold greater in a *ROX1* deletion mutant than it was in a deletion mutant transformed with a wild-type *ROX1* gene. Repression was 3- to 5-fold in transformants carrying the Δ*100/123*, Δ*100/175*, and Δ*194/245*; it decreased to 2.4-fold in transformants carrying Δ*100/245* and Δ*247/368*; and it was eliminated in transformants carrying Δ*177/368*. These results were in agreement with the RNA blot results. As expected, in most cases in which the deletion protein only partially repressed *ROX1* (Δ100/245,

Plasmid	ANB1/lacZ Act.	ANB1/lacZ Rep.	ROX1/lacZ Act.	ROX1/lacZ Rep.
Wildtype	5.0±3.3	17	27±3.7	9.6
rox1Δ	83 ±18	1	260±33	1
Δ100/123	7.5±4.1	11	49±10	5.3
Δ100/175	7.4±3.1	11	64±5.3	4.1
Δ100/245	11 ±3.8	7.5	107±14	2.4
t104	90 ±19	0.9	178±15	1.5
t123	85 ±23	1	186±18	1.4
Δ177/368	81 ±24	1	279±26	0.9
Δ194/245	8.2±2.1	10	75±9.7	3.5
Δ194/357	29 ±3.8	2.9	176±32	1.5
Δ247/368	14 +2.4	5.9	109+19	2.4

FIG. 5. Repression of *ANB1-lacZ* and *ROX1-lacZ* expression by Rox1 carboxyl-terminal deletions. The levels of β-galactosidase activity (Act.; expressed in units) in extracts from aerobically grown MZ12-1 cells (*ANB1/lacZ*) and RZ53-6Δ*rox1* cells (*ROX1/lacZ*) transformed with the indicated plasmids were determined. Symbols: filled rectangle, the HMG domain from residue 1 through residue 101; horizontal bars, the glutamine-rich region from residue 102 through residue 123; empty rectangle, the carboxyl-terminal region from residue 124 to residue 368; dashed line, the portion of Rox1 deleted. Rep., fold repression.

Δ194/357, and Δ247/368), *ANB1* was almost completely repressed. Presumably, the mutant Rox1 protein levels were increased sufficiently to compensate for the decrease in activity.

To ensure that the repression observed with these deletions was dependent on the Tup1-Ssn6 repression complex, each deletion plasmid represented in Fig. 5 and the *ROX1-lacZ* fusion plasmid were cotransformed into the *rox1*Δ *ssn6*Δ strain MZ18-23 and β-galactosidase activity was determined. Neither the wild-type *ROX1* plasmid nor any of the deletion derivatives that had shown some repression activity in an *SSN6* wild-type background displayed any repression activity in the *ssn6*Δ background (data not shown).

The carboxy-terminal region of Rox1 can repress when fused to the Gal4 DNA-binding domain. If repression is mediated through the carboxy-terminal region of Rox1, it should be possible to transfer repression activity by fusing this region to a different DNA-binding domain. Such fusions were generated by inserting various regions of the *ROX1* coding sequence into plasmid pMA424, which contains the sequences encoding the *GAL4* DNA-binding domain driven by the *ADH1* promoter. These fusions were transformed into a *rox1*Δ *gal*Δ strain along with an *ANB1-lacZ* fusion derivative that carried two copies of the Gal4 binding site inserted between the Rox1 operators. These constructs and the results of the β-galactosidase assays performed on cells transformed with them are presented in Fig. 7. The Gal4 DNA-binding domain alone showed no repression activity (pMA424) compared with the control without a *GAL4-ROX1*-containing plasmid, while either the intact Rox1 protein (which repressed through the operator sequences) or the fusion protein containing the entire Rox1 (which could repress in principle through the upstream activation sequence for galactose [UAS_{GAL}] and operator sites) repressed expression about ninefold. Fusions containing either the entire carboxy-terminal region (residues 124 to 368) or either half of this region (residues 124 to 247 or 246 to 368) produced significant levels of repression, from 3.2- to 5.1-fold. While repression was less strong with these carboxy-terminal fusions, the results clearly demonstrate that repression activity can be transferred with this region and that the information in this region is redundant, since either half transferred repression activity.

To determine whether this repression activity was dependent on the Tup1-Ssn6 complex, pMA424 or pMAR1(X-C) and the reporter construct were cotransformed into the *rox1*Δ *gal4*Δ *ssn6*Δ strain MZ27-6. The levels of β-galactosidase activity expressed in the transformants were 22.2 ± 8.6 U for pMA424 and 30.7 ± 10 U for pMAR1(X-C). This lack of repression in the *ssn6*Δ background indicated that repression by the Gal4-Rox1 fusions was mediated through the Tup1-Ssn6 complex.

In summary, two firm conclusions concerning repression by Rox1 and the Tup1-Ssn6 complex can be drawn from the results presented here. First, repression is mediated through the carboxy-terminal region of Rox1. Second, the repression information in this region is redundant.

DISCUSSION

The HMG domain of Rox1. The results presented here indicate that Rox1 binds to DNA in a fashion similar to that of other site-specific HMG domain proteins. First, Rox1 bends DNA at an angle of approximately 90°; SRY (90° [9]) and LEF-1 (130° [12]) have previously been shown to bend DNA. Second, most of the missense mutations isolated in the HMG domain of Rox1 map to residues that are highly conserved in the HMG family of proteins, as indicated in Fig. 3. Third, most of these mutations cluster into the same regions of the HMG domain as do missense mutations in the human SRY gene (Fig. 3). Fourth, the site-specific HMG domain proteins bind to similar DNA sites (9). Given these similarities, we assumed that the structure of the Rox1-DNA complex is similar to that of SRY and attempted to deduce the effects of the amino acid substitutions in the Rox1 mutants on the basis of the published structure for the SRY HMG domain. The SRY HMG domain is shaped like the letter C. The DNA binds to the inside of the C, and isoleucine 68 sticks out into the C and is partially intercalated between the two TA base pairs at positions 2 and

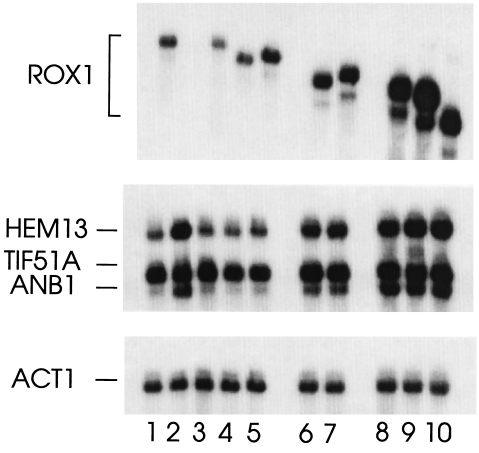

FIG. 6. Repression of hypoxic mRNA levels by Rox1 C-terminal deletions. RNA was prepared from RZ53-6Δ*rox1* cells grown aerobically and carrying the following plasmids: lane 1, YCp(22)*ROX1*H (wild type); lane 2, YCplac22 (this was the control lacking a *ROX1* gene); lane 3, YCp(22)*R1Δ100/123*; lane 4, YCp(22)*R1Δ100/176*; lane 5, YCp(22)*R1Δ194/245*; lane 6, YCp(22)*R1Δ100/245*; lane 7, YCp(22)*R1Δ247/368*; lane 8, YCp(22)*R1Δ194/357*; lane 9, YCp(22)*R1Δ177/368*; lane 10, YCp*R1-t104*. A 30-μg portion of RNA was loaded in each lane, and the blot was probed successively with a *ROX1* probe, a mixture of *ANB1* and *HEM13* probes, and an *ACT1* probe. The variation in migration rates of the *ROX1* mRNAs as seen in this autoradiograph reflects the sizes of the different deletions.

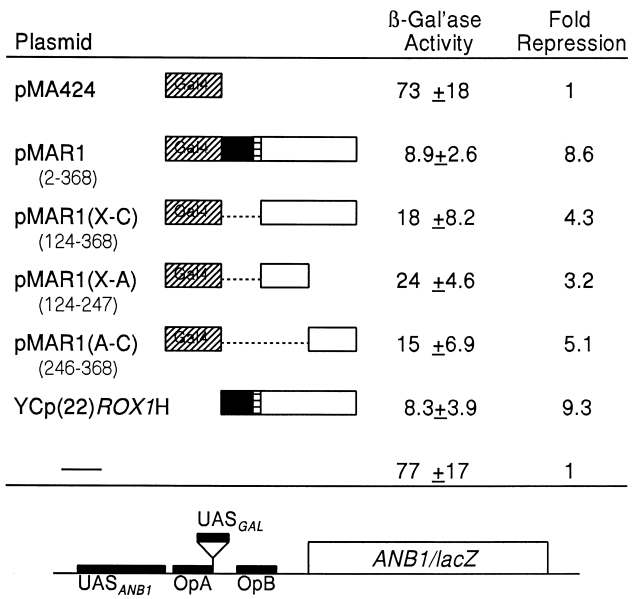

FIG. 7. Repression of *ANB1-lacZ* expression by Rox1-Gal4 fusions. The levels of β-galactosidase (β-Gal'ase) activity in extracts from aerobically grown MZ18-23 cells cotransformed with the indicated fusion plasmids and YCp(33)AZ-UAS$_{GAL}$ which is diagrammed at the bottom, were determined. The symbols are the same as those for Fig. 5, with the addition that the hatched boxes represent the Gal4 DNA-binding domain.

3 in the core SRY site, 5′(A/T)TTGTT (16, 45). This intercalation distorts the helix, aiding in the bending. Rox1 contains an isoleucine in the homologous position (position 18), and the above-mentioned core consensus sequence for SRY is contained within the hypoxic consensus sequence (PyPyPyATTGTTCTC). Computer modeling studies suggest that Rox1 can fold into the same structure as SRY (43a). Thus, using the SRY model, we should be able to explain why the missense mutations in the HMG domain caused the greatly weakened binding or loss of binding reported here.

First, the derepression phenotypes of the *rox1* missense mutations were not due to decreased protein expression; all the mutants tested accumulated higher than wild-type levels of Rox1 protein (Fig. 2). Therefore, barring nuclear localization problems, the loss of repression was almost certainly due to the lost or greatly decreased DNA binding measured in vitro. The most severe mutations were those of *F20S*, *S46P*, and *G50D*; the mutant proteins showed no DNA binding activity in vitro and no repression activity in vivo. The *S46P* allele places a proline in α-helix 2 (Fig. 3), and the presence of the α-helix-breaking residue may well disrupt the folding of the protein. In addition, the equivalent serine in SRY contacts the DNA at bp 1 of the core sequence presented above. The aromatic ring of F-20 sits in a hydrophobic pocket composed of a number of aromatic residues, Y-24, W-53, and W-64, that appears to hold helixes 1 and 2 in position relative to each other. The substitution of the hydrophilic serine for phenylalanine may disrupt this pocket. G-50 lies in a segment of α-helix 2 that encloses part of this hydrophobic pocket, and the substitution of either aspartate or asparagine for the glycine would insert a charged residue into the pocket, again probably disrupting it or preventing the folding of the protein. The two mutant proteins N15D and F17S retained some DNA binding activity, and while the reduction in binding was certainly sufficient to explain the loss of repression activity, the residual binding suggests that the mutant proteins fold properly to some degree. The equivalent asparagine in the SRY protein contacts bp 3 and 4 within the core sequence, and, therefore, the substitution of the negatively charged aspartate for N-15 in Rox1 probably interferes with these contacts. The SRY phenylalanine equivalent to F-17 in Rox1 contacts the two TA base pairs into which the neighboring isoleucine is intercalated (bp 2 and 3 of the core sequence). The substitution of a serine at this position would no doubt affect these contacts. Finally, the Y84C mutant protein retained some repression activity. The equivalent tyrosine in SRY contacts bp 5 of the core sequence, and the cysteine substitution in Rox1 probably affects this contact. No doubt this mutation also affected the in vitro stability of the protein, since it did not bind DNA in our assays. Whether this in vitro stability plays a role in the decreased in vivo repression is not yet clear. In summary, it is possible from this model to gain some insight into why most of the mutations isolated caused loss of protein function. Four of the amino acid substitutions affect residues that probably contact DNA, and we believe that reversion studies, studies of binding to altered DNA sites, and further mutational analyses combined with physical studies will allow a detailed structure-function correlation for Rox1.

The repression domain of Rox1. We have proposed here that the carboxy-terminal region of Rox1 is necessary for repression and that the information required is redundant. The most obvious function for this repression domain is that it binds the Tup1-Ssn6 complex, which is required for repression by Rox1. The Tup1-Ssn6 complex serves as a general repressor in yeast cells, repressing transcription of the hypoxic genes, **a** mating type genes, catabolite-repressed genes, flocculence genes, and a variety of others. In each of the first three cases mentioned above, repression by the general repressor is mediated through a sequence-specific DNA-binding protein, i.e., Rox1, α2, and Mig1, respectively. Keleher et al. (18) demonstrated that when the Tup1-Ssn6 complex was artificially anchored to a site upstream of a reporter gene by fusion of Ssn6 to the DNA-binding protein LexA, transcriptional repression resulted, leading to a model in which the specific DNA-binding proteins serve as anchors for Tup1-Ssn6 and repression is controlled at the level of the DNA-binding protein accumulation (as in the case of Rox1) or activity. Thus, in accordance with this model and the results of the present study, the HMG domain of Rox1 binds to DNA while the carboxy-terminal region mediates the Tup1-Ssn6-dependent repression. It is formally possible that there is not a direct interaction between Rox1 and the Tup1-Ssn6 complex but that rather a novel, as yet unidentified protein interfaces between them. Only binding studies with purified proteins will address this question conclusively.

It should be noted that there are some curious features about the Rox1 repression domain within the context of a proposed simple interaction of Rox1 with the Tup1-Ssn6 complex. First, there is no obvious sequence motif common to the three proteins, Rox1, α2, and Mig1, that are proposed to interact with the Tup1-Ssn6 complex. Either there is a motif that is too subtle to be readily discerned or the three proteins each interact with a different region of the complex, as has been suggested previously (43). Second, on the basis of the deletion analysis, the Rox1 repression domain appears to be large and redundant, although again, it is not obvious from the protein sequence which regions are responsible for the redundancy.

Does DNA bending play a role in repression? The observation that Rox1 bends DNA at an angle of approximately 90° raises the possibility that Rox1-induced DNA bending is important for transcriptional repression. It is possible that the anchoring of the Tup1-Ssn6 complex to DNA is enhanced by a

topological alteration in the DNA. While neither Tup1 nor Ssn6 has any specific DNA binding activity, the possibility that the complex recognizes bent DNA has not been tested. Also, in repeating the experiments of Keleher et al. (18), we artificially anchored Tup1 or Ssn6 to the promoter region of a reporter gene through a Gal4-Tup1 or a Gal4-Ssn6 fusion, respectively. While repression was observed, it was quite weak, suggesting that repression may involve more than simply anchoring these factors to DNA.

DNA bending does appear to play an important role in the function of several sequence-specific HMG proteins. For example, one mutation in the HMG domain of the human SRY protein causes a 20° decrease in the bending angle but wild-type-like binding affinity. This mutation leads to a severe sex-reversal phenotype, strongly suggesting an essential role of DNA bending in protein function (29). Similarly, transcriptional activation by the LEF-1 protein has been proposed to be mediated through LEF-1's bringing proteins bound to physically separated sites on the DNA into contact through bending of the DNA. Replacement of the LEF-1 HMG domain with the DNA-binding domain of either lexA or Gal4 resulted in a protein with weakened transcriptional activation capability (6, 12, 13). Interestingly, in our parallel experiment presented here, the Gal4–carboxy-terminal Rox1 fusions had about half as much repression activity as the intact Rox1. In an extreme case of induced topological changes in DNA, the *Xenopus* upstream binding factor (UBF) protein, a transcriptional activator of the genes coding for rRNA, contains multiple HMG domains which cause the formation of a disk-like DNA-protein structure, designated an enhancesome, within the enhancer region of the genes coding for rRNA (3). Whether DNA bending plays a role in transcriptional repression by Rox1 remains to be determined.

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