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Recruitment of Tup1-Ssn6 by Yeast Hypoxic Genes and Chromatin-Independent Exclusion of TATA Binding Protein

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The Tup1-Ssn6 general repression complex in *Saccharomyces cerevisiae* represses a wide variety of regulons. Regulon-specific DNA binding proteins recruit the repression complex, and their synthesis, activity, or localization controls the conditions for repression. Rox1 is the hypoxic regulon-specific protein, and a second DNA binding protein, Mot3, augments repression at tightly controlled genes. We addressed the requirements for Tup1-Ssn6 recruitment to two hypoxic genes, *ANB1* and *HEM13*, by using chromatin immunoprecipitation assays. Either Rox1 or Mot3 could recruit Ssn6, but Tup1 recruitment required Ssn6 and Rox1. We also monitored events during derepression. Rox1 and Mot3 dissociated from DNA quickly, accounting for the rapid accumulation of *ANB1* and *HEM13* RNAs, suggesting a simple explanation for induction. However, Tup1 remained associated with these genes, suggesting that the localization of Tup1-Ssn6 is not the sole determinant of repression. We could not reproduce the observation that deletion of the Tup1-Ssn6-interacting protein Cti6 was required for induction. Finally, Tup1 is capable of repression through a chromatin-dependent mechanism, the positioning of a nucleosome over the TATA box, or a chromatin-independent mechanism. We found that the rate of derepression was independent of the positioned nucleosome and that the TATA binding protein was excluded from *ANB1* even in the absence of the positioned nucleosome. The mediator factor Srb7 has been shown to interact with Tup1 and to play a role in repression at several regulons, but we found that significant levels of repression remained in *srb7* mutants even when the chromatin-dependent repression mechanism was eliminated. These findings suggest that the repression of different regulons or genes may invoke different mechanisms.

The general repression complex containing the proteins Tup1 and Ssn6 is a conserved global regulator of transcription in baker's yeast, *Saccharomyces cerevisiae*. This complex regulates a wide variety of gene families or regulons through its recruitment to target genes by association with regulon-specific DNA binding proteins (24, 46). Under repressing conditions, these DNA binding proteins are synthesized, activated, or redistributed and bind to the control region of each gene in the regulon. Examples of these proteins are α2 and the a1-α2 complex (mating type regulon) (45), Mig1 (glucose-repressed genes) (43), Crt1 (DNA damage regulon) (20), and Rox1 (hypoxic regulon) (2).

Tup1 is responsible for the majority of repression by the general repression complex. Previous studies indicated that anchoring Tup1 to DNA resulted in Ssn6-independent repression, while in the reciprocal experiment, anchored Ssn6 still required Tup1 for repression (44). Ssn6 may be an adapter protein, bridging Tup1 to regulon-specific DNA binding proteins (24). A chromatin-dependent repression mechanism has been proposed for many Tup1-repressed genes (15, 23, 31, 41). Tup1 interacts with the amino-terminal domains of the histone H3 and H4 proteins, perhaps allowing direct nucleosome recruitment (13). Also, Tup1 localization often correlates with decreased acetylation of histone tails (4, 7, 10, 13), and some evidence suggests that deacetylation of H3 at Tup1-repressed genes is achieved through interactions between Tup1 and the histone deacetylase Hda1 (47). These data support the model of Tup1-dependent nucleosome recruitment to the TATA box of a gene followed by locking of the nucleosome in place by histone deacetylation. This nucleosome positioning most likely excludes the TATA binding protein (TBP) and thereby blocks subsequent RNA polymerase holoenzyme recruitment and transcriptional activation (28).

The yeast hypoxic genes represent one well-studied Tup1-Ssn6-repressed regulon. *S. cerevisiae* is a facultative aerobe. Under oxygen-limiting conditions, about 70 hypoxic genes that allow more efficient utilization of this limiting electron acceptor are induced (29, 34). These hypoxic genes are regulated by the DNA binding repressor Rox1, the expression of which is controlled by oxygen availability (2, 8, 33, 35). Thus, under aerobic conditions, Rox1 is synthesized and binds in the control region of the hypoxic genes (9). Genetic evidence indicates that when bound, Rox1 recruits the Tup1-Ssn6 complex to effect repression (34, 49).

ANB1, encoding the putative translation initiation factor eIF5a, is the prototype hypoxic gene used in our studies (22). *ANB1* is tightly repressed under aerobic conditions and is induced over 200-fold in cells grown under hypoxic conditions (22, 34). The control region of *ANB1* is divided into two operators, each of which consists of two Rox1 binding sites. The majority of repression observed at *ANB1* is achieved through operator A (OpA), the upstream operator (11). This enhanced repression is due to the binding of transcription factor Mot3 between the Rox1 binding sites (23). Genetic evidence strongly suggests that Mot3 enhances repression by either stabilizing Tup1-Ssn6 recruitment or influencing chromatin structure at

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TABLE 1. Strains used in this study

Strain	Reference or genotype
RZ53-6	2
RZ53-6Δ*rox1*	8
RZ53-6Δ*tup1*	8
RZ53-6Δ*ssn6*	8
RZ53-6Δ*mot3*	23
RZ53-6Δ*rox1* Δ*mot3*	23
RZ53-6Δ*tup1* Δ*mot3*-u	α *ura3 leu2 trp1 ade1 mot3*::*kanMX4 tup1*::*ura3*
MZ90-88Δ*tup1*	α *trp1 ura3*::*AZ4 leu2 ade2 ade3 his3 gal1* Δ *tup1*::*URA3*
JDZ149-32	α *trp1 ura3 leu2 ade1 ade2 spt15*Δ *tup1*::*ura3* + YCp(33)*SPT15*−HA_3
MZ148-148	α *trp1 ura3*::*AZ4 leu2 ade2 ade3 his3 hhf1-8*::*kanMX4 hhf2*/*hht2*::*kanMX4 spt15*Δ + YCp(33)*SPT15*HA_3 + YEp(181)*hhf1-8*
MZ168-24	α *trp1 ura3 leu2 ade1 cti6*::*kanMX4* (RZ53-6 background)
MZ101-18	α *trp1 ura3*::*AZ4 leu2 ade2 ade3 his3 gal1*Δ *hhf1-8*::*kanMX4 hhf2*/*hht2*::*kanMX4* + YEp(195)*HHF1*/*HHT1*
MZ101-18A	α *trp1 ura3*::*AZ4 leu2 ade2 ade3 his3 gal1* Δ*hhf1-8*::*kanMX4 hhf2*/*hht2*::*kanMX4* + YCp(LEU)*hhf1-8*/*HHT1*
MZ101-18*srb7*Δ*7N*	MZ101-18 with *srb7*::*srb7*Δ*7N-hphMX4*
MZ101-18A*srb7*Δ*7N*	MZ101-18A with *srb7*::*srb7*Δ*7N-hphMX4*
MZ101-18*Nub-srb7*Δ*7N*	MZ101-18 with *srb7*::*Nub-srb7*Δ*7N-hphMX4*
MZ101-18A*Nub-srb7*Δ*7N*	MZ101-18A with *srb7*::*Nub-srb7*Δ*7N-hphMX4*

the promoter (23). Under anaerobic conditions, *ROX1* expression ceases, *MOT3* expression is diminished, and repressor localization is eventually lost (8, 23).

There is strong evidence that *ANB1* can be repressed through a chromatin-dependent repression pathway of Tup1, but there is also evidence for a redundant chromatin-independent mechanism. In a previous study, Kastaniotis et al. demonstrated that a nucleosome is positioned over the TATA box of *ANB1* and that a *rox1*, *tup1*, or *mot3* deletion caused the loss of the phased nucleosome (23). The *rox1*Δ or *tup1*Δ allele caused a substantial loss of repression, but the *mot3*Δ allele had only a partial effect. A deletion of the amino-terminus-coding region of *HHF1* (the *hhf1-8* allele), encoding the histone H4 protein, resulted in a loss of the positioned nucleosome. This loss most likely was due to the disruption of Tup1-nucleosome interactions. Surprisingly, this mutation did not cause any derepression; Tup1-mediated repression of *ANB1* remained at wild-type levels in the absence of a nucleosome positioned over the TATA box. Thus, this study provided two lines of evidence that Tup1-dependent repression of *ANB1* expression was not dependent on the positioned nucleosome. First, *mot3*Δ caused a loss of nucleosome positioning but only minor derepression. Second, the amino-terminal deletion in histone H4 also caused a loss of the positioned nucleosome but no derepression. These data demonstrated that *ANB1* could be repressed through a chromatin-independent pathway.

The existence of a second, chromatin-independent Tup1 repression mechanism is supported by other reports of wild-type levels of Tup1-mediated repression in the absence of a positioned nucleosome at other loci (14, 23) and of Tup1-facilitated repression on templates lacking chromatin in whole-cell extracts (19, 40). Proposed models for this mechanism have postulated interactions between Tup1 and members of the mediator complex, which is associated with the carboxy-terminal domain of the large subunit of RNA polymerase II. Recent reports include evidence of Tup1 interactions with Hrs1, Srb10, and Srb7 (18, 27, 37, 48).

In this study, we used chromatin immunoprecipitation (ChIP) to provide biochemical evidence of direct Tup1-Ssn6 recruitment mediated by Rox1 and Mot3 at *ANB1* and *HEM13*, a less tightly regulated hypoxic gene, and to study the requirements for complex formation. We demonstrated that Ssn6 can be recruited by Rox1 or Mot3 in a Tup1-independent manner. ChIP analysis probing for TBP localization at *ANB1* illustrated that Ssn6-Tup1 was capable of excluding TBP binding under repressing conditions, even in the absence of a positioned nucleosome, suggesting that Ssn6-Tup1 blocks holoenzyme recruitment in both chromatin-dependent and chromatin-independent mechanisms. In addition, the profiles of expression of *ANB1* and *HEM13* were determined during the transition from complete repression to maximal induction by Northern analysis, and RNA induction was correlated with Rox1 and Mot3 binding and turnover as well as Tup1 gene localization. During induction, Rox1 and Mot3 dissociation and degradation mimicked expression, while the presence of Tup1 at hypoxic loci appeared to persist well into induction, as reported previously (38).

We also report here that the effects of general transcription factors may be quite different at different Tup1-Ssn6-repressed regulons. Cti6 has been implicated as being an important factor for the induction of Ssn6-Tup1-repressed genes by bridging Tup1 to the SAGA complex, allowing the acetylation and subsequent clearance of the positioned nucleosome (38), but we found no Cti6 effect at hypoxic genes. Similarly, mutations in Srb7 that were previously reported to result in the derepression of some Tup1-regulated genes (18) were shown to have no effect at *ANB1* either in the presence or in the absence of the chromatin-dependent repression pathway.

MATERIALS AND METHODS

Strains and growth conditions. The strains used in this study are listed in Table 1. RZ53-6Δ*tup1*Δ*mot3*-u was constructed by selecting for *tup1*::*ura3* mutants in RZ53-6Δ*tup1* (8) through 5-fluoroorotic acid resistance (3) followed by displacement of *MOT3* with the *mot3*::*kanMX4* construct as previously described (23). JDZ149-32 was constructed through matings between RZ53-6Δ*tup1*Δ*mot3*-u containing YCp(111)*TUP1* and BYΔ2 (6). MZ148-148 was con-

structed through matings between MZ101-18A and BYΔ2 (6). MZ168-24 was constructed through matings between RZ53-6 and BO23AΔ*cti6* (purchased from EUROSCARF) with subsequent matings to RZ53-6Δ*tup1* to achieve *cti6*Δ in the RZ53-6 background. In all cases, haploid strains containing the desired mutations were isolated by using standard yeast genetics (21). *srb7* mutant strains were constructed by displacement of *SRB7* with the mutant allele constructs described below.

Cells were grown at 30°C (with shaking for liquid cultures) in or on yeast extract-peptone-dextrose medium, synthetic complete medium, or synthetic complete medium lacking the appropriate nutrient for selection for plasmid maintenance (21). Cells were grown anaerobically for short terms by bubbling nitrogen through the cultures for various times. Long-term (>4 h) anaerobic growth was achieved by using sealed chambers containing GasPak (Becton Dickinson). Transformations were performed as described previously (21).

Plasmids. All plasmid constructions were carried out by using standard techniques as described previously (1). Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs and Roche Applied Science, respectively, and used in accordance with the manufacturers' recommendations. PCRs were performed with *Taq* polymerase (MBI Fermentas) as recommended by the vendor. Genomic sequences were obtained from the *Saccharomyces* Genome Database maintained at Stanford University. The sequences for all genes discussed are numbered with the adenine of the start codon as +1, bases 5′-ward numbered negatively, and bases 3′-ward numbered positively.

The yeast vectors YEplac112, YCplac22, and YCplac111 have been described elsewhere (16). YCplac23 was made by digesting YCplac22 with *Bgl*II, filling in the resulting overhangs with the Klenow fragment (New England Biolabs), and religating the plasmid, thus removing the *Bgl*II recognition sequence. The *URA3* centromeric *ANB1-lacZ* fusion plasmid YCpAZ33 (11), YEp(195)*ANB1* (23), YCp(33)HA_3-TBP (28), and YCp(LEU)*HHF1*/*HHT1* and YCp(LEU)*hhf1-8*/*HHT1* (36) have been described elsewhere. YEp(112)*HHF1*/*HHT1* was constructed by cloning the *Eco*RI/*Hin*dIII fragment containing *HHF1*/*HHT1* into YEplac112. YCp(22)*SPT15* was constructed by subcloning the 2.4-kb *Eco*RI/*Bam*HI fragment isolated from pRS316 (42) into YCp22. This fragment encodes TBP with the F155S substitution.

YCp(23)*TUP1*-HA_3 consists of *TUP1* cloned as a *Sac*I/*Hin*dIII fragment containing sequences from −2081 to +2379. Three copies of the influenza virus hemagglutinin (HA) epitope tag (HA_3) were added immediately preceding the translational stop codon by PCR. The HA epitope tag was separated from the *TUP1* coding sequences by an *Xho*I site. This site was used to generate the HA epitope-tagged constructs described below by replacing the *TUP1* upstream and coding sequences with those of the indicated gene but retaining the HA epitope tag and *TUP1* 3′ region.

YCp(23)*SSN6*-HA_3 contains the *SSN6* sequences from −791 to +2898 joined to the HA epitope tag and *TUP1* 3′ sequences. YCp(23)*ROX1*-HA_4 contains *ROX1* sequences from −1101 to + 1104 followed by codons encoding four copies of the HA epitope (HA_4) and *TUP1* 3′ sequences. YCp(23)*MOT3*-HA_4 contains *MOT3* sequences from −778 to + 1470 followed by codons encoding HA_4 and *TUP1* 3′ sequences. All epitope-tagged alleles used in this study complemented the respective deletion allele, as determined by the ability to fully repress the *ANB1-lacZ* fusion (data not shown).

YCp(111)*tup1*Δ*98–304* was generated by PCR by joining the *TUP1* sequences from −2081 to + 294 (codon 97) with sequences from +912 (codon 305) to +2379, thus deleting codons 98 to 304 and replacing them with an *Xho*I site.

The *SRB7* gene displacement constructs were constructed as follows. *SRB7* sequences from −683 to +1258 were amplified by PCR and cloned into pBluescript SK(+) (Stratagene). An *Mfe*I site was introduced immediately following the start codon by PCR and joined to a native *Mfe*I site at +19 of *SRB7*, resulting in the deletion of five codons (encoding residues 2 to 6). This construct mimicked the *srb7*Δ*7* mutant allele used by Gromoller and Lehming (18). Finally, an *Eco*RV/*Bam*HI fragment containing *hghMX4* (a hygromycin B resistance allele) was isolated from pAG32 (17) and cloned into the *Eco*RV/*Bam*HI sites in the downstream region of *srb7*Δ*7*. This construct was then integrated into the *SRB7* locus as an *Xho*I/*Xba*I fragment and confirmed by PCR.

The *srb7*::*Nub-srb7*Δ*7N-hphMX4* construct was generated as follows. A *Sal*I/*Mfe*I fragment containing sequences from +1 to +111 of *UBI4* was generated by PCR. This fragment was cloned into the *Sal*I/*Mfe*I sites of p*srb7*::*srb7*Δ*7-hghMX4*, placing the sequences encoding the first 37 residues of *UBI4* upstream of codon 7 of *srb7*Δ*7*. The *srb7*::*Nub-srb7*Δ*7N-hphMX4* construct was then integrated into the *SRB7* locus as an *Xho*I/*Xba*I fragment and confirmed by PCR. This construct mimicked the amino-terminal ubiquitin fusion mutant allele made in the study mentioned above (18).

Enzyme, RNA, protein, and MNase sensitivity assays. β-Galactosidase assays were performed as described previously (21). All assays were carried out multiple times with multiple transformants for each mutant strain examined.

RNA was prepared by using hot acidic phenol, and Northern blotting was carried out as described previously (1). The blots were hybridized to ^{32}P-labeled DNA probes prepared from their respective genes as described previously (1).

Crude protein extracts were prepared by harvesting cells in mid-exponential phase and boiling in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) loading buffer containing β-mercaptoethanol as described previously (1). Proteins were electroblotted onto a nitrocellulose membrane, and the blot was probed with HA antibody (F-7; Santa Cruz Biotechnology, Inc.) and subsequently with horseradish peroxidase-conjugated anti-mouse antibody (sc-2005; Santa Cruz Biotechnology) as described previously (1) and then visualized with Western blotting Luminol reagent (Santa Cruz Biotechnology) in accordance with the manufacturer's recommendations.

MZ90-88Δ*tup1* cells used for micrococcal nuclease sensitivity assays were transformed with YEp(112)*ANB1* as well as either YCp(111)*TUP1* or YCp(111)*tup1*Δ*98–304*. Samples were prepared and analyzed as described previously (23). Naked DNA samples were prepared from the same cells as described previously (23).

ChIP. The recruitment of HA epitope-tagged proteins to specific regions of DNA was measured by the immunoprecipitation of formaldehyde-cross-linked chromatin with antibody against the HA epitope (F-7) and protein A-Sepharose resin (Amersham Biosciences) as described previously (12, 28). Immunoprecipitated complexes were eluted from the protein A-Sepharose resin at room temperature for 30 min by incubation with 0.1 mg of HA peptide (Roche Applied Science) in 100 μl of lysis buffer (28) or, in the case of some Tup1-HA experiments, stripped from the protein A-Sepharose resin by incubation with 100 μl of 1% SDS in Tris-EDTA at 65°C for 15 min. There was no consistent difference between these two methods.

Quantitative analysis by PCR was performed essentially as described previously (28). The oligomer pairs amplified the following regions: *ANB1* regulatory region from −505 to −231, *HEM13* regulatory region from −617 to −388, *FLO1* from −165 to +10, and *RPC1* internal coding region from +2518 to +2765 for the Tup1, Ssn6, Rox1, and Mot3 ChIP assays described below and *ANB1* TATA box from −196 to +10, *HEM13* TATA box from −165 to +10, and *ACT1* from −225 to +10 for the TBP ChIP assays described below. PCR products were separated on 8% polyacrylamide gels, and quantitation was performed as described previously (28). For all oligomer pairs, amplification within the linear range was determined with an input DNA template dilution series.

RESULTS

Ssn6 and Tup1 are recruited to the hypoxic genes by Rox1 and Mot3. Genetic studies indicated that Rox1 recruits Ssn6 and Tup1 to the hypoxic genes under aerobic conditions (35, 49, 50). To visualize the requirements for complex formation, we carried out ChIP experiments. By cross-linking protein-DNA complexes in vivo, followed by immunoprecipitation of a specific member of the complex and detection of specific regions of DNA by PCR amplification, ChIP analysis can reveal under what conditions specific complexes form and what proteins are required for their formation (28). To carry out these experiments, we constructed epitope-tagged versions of Tup1, Ssn6, Mot3, and Rox1 by placing copies of the nine-amino-acid influenza virus HA epitope at the carboxy terminus of each protein. In all versions, the epitope tag did not measurably affect repression, as determined by complementation of the respective deletions.

ChIP analysis demonstrated that Ssn6 was localized to the upstream region of the hypoxic genes *ANB1* and *HEM13* under conditions of repression. The amount of PCR product from *ANB1* OpA was 10 times greater in samples prepared from wild-type cells carrying the *SSN6*-HA allele (wild type) (Fig. 1, lane 1) than in those prepared from cells carrying an untagged allele (lane 2). The OpA region contains two Rox1 binding sites and one Mot3 binding site and is responsible for most of

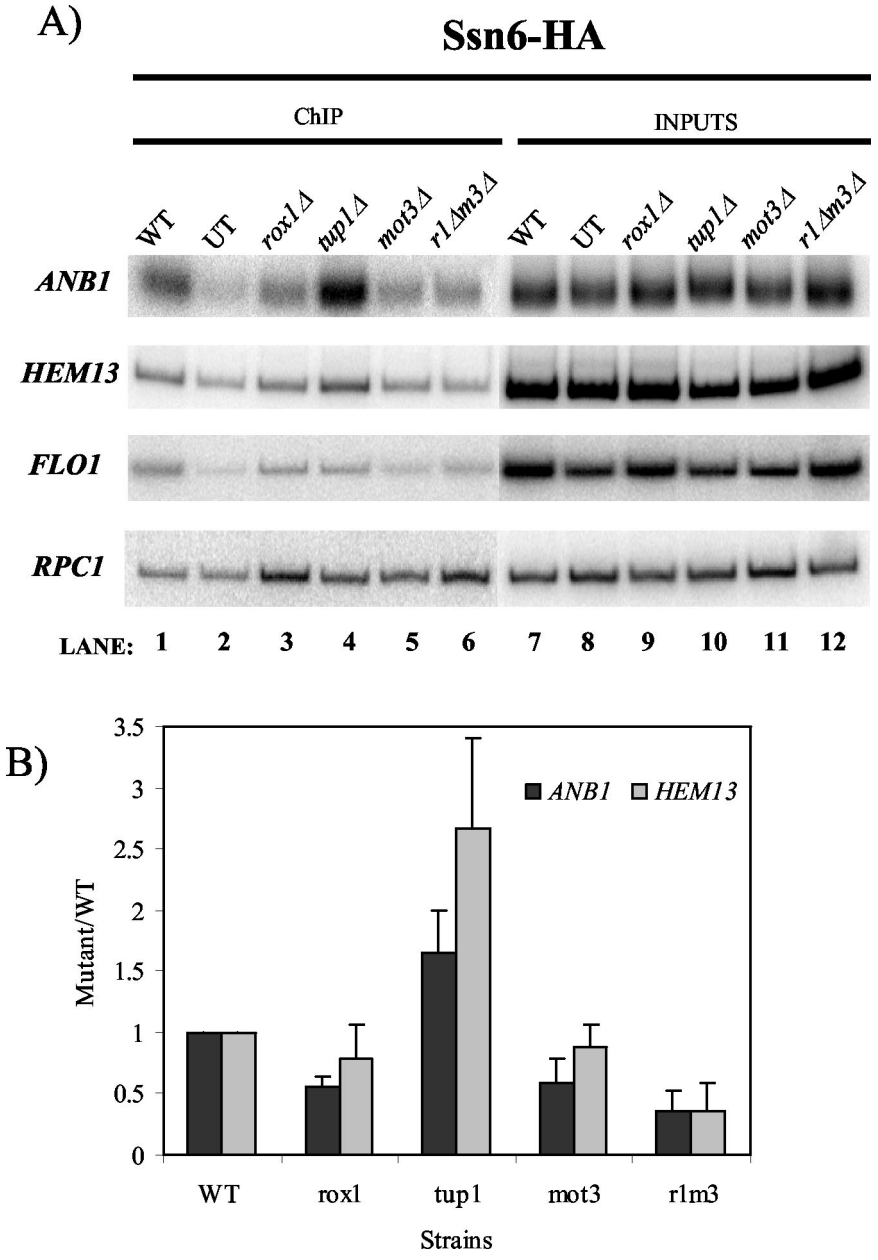

FIG. 1. Ssn6 recruitment to hypoxic genes requires either Rox1 or Mot3 but not Tup1. (A) ChIP assays with a monoclonal antibody against the HA epitope were carried out with YCp(23)*SSN6*-HA_3-transformed RZ53-6 (WT) and its *rox1*Δ, *tup1*Δ, *mot3*Δ, and *rox1*Δ *mot3*Δ (*r1*Δ *m3*Δ) derivatives or with YCplac23-transformed RZ53-6 (UT). Cultures were grown aerobically (repressed) to mid-exponential phase. DNA samples extracted from the ChIP reactions (lanes 1 to 6) and before immunoprecipitation (inputs; lanes 7 to 12) were amplified by PCRs with oligonucleotide primers for the *ANB1*, *HEM13*, and *FLO1* regulatory regions. ^{32}P-dATP was added to the PCRs, and after PAGE, the labeled products were visualized and quantitated by using a PhosphorImager. (B) The radioactivity in the ChIP samples was normalized as follows. For each gene amplified, the input sample was normalized to the wild-type input sample. Then, the value for each ChIP sample was divided by the normalized ratio for its corresponding input sample. The ChIP value for the untagged (UT) sample was then subtracted from those for all other samples. Finally, each normalized ChIP value was divided by the normalized wild-type ChIP value (Mutant/WT). In later experiments, *RPC1* was used as a negative control. In those experiments, each experimental sample was normalized to each corresponding *RPC1* sample. This correction contributed very little to the comparative values. The error bars represent the standard deviations calculated from at least five ChIP experiments. Thus, the wild-type ChIP value is presented as 1.0. The normalized ChIP values were plotted as histograms for *ANB1* and *HEM13*. The values represent the averages of five trials.

the repression of *ANB1*. Under aerobic growth conditions, *ANB1* expression is repressed 250-fold. *HEM13* expression, on the other hand, is repressed only 20-fold, and amplification of the OpA region of *HEM13*, which also contains two Rox1 binding sites and at least one Mot3 binding site, produced only a twofold difference between samples from the cells carrying the tagged allele and those from cells carrying the untagged allele. Similar results were observed for the recruitment of

Tup1 (see below), and these results suggest that there is a correlation between the strength of the signal at a given gene and the strength of repression. Repression of the hypoxic genes is absolutely dependent on Rox1, while Mot3 can augment Rox1-dependent repression.

To determine whether these DNA binding proteins are required for the recruitment of Ssn6, ChIP analysis was carried out with *rox1*Δ, *mot3*Δ, and *rox1*Δ *mot3*Δ deletion strains. As shown in Fig. 1, either Rox1 or Mot3 was sufficient to recruit Ssn6 to *ANB1*; only in the presence of the double deletion did the PCR signal fall to nearly background levels. This result was surprising, since Mot3 cannot repress *ANB1* in the absence of Rox1 (23), suggesting that recruitment is not the sole determinant of repression. Mot3 can recruit Ssn6 as efficiently as Rox1 but cannot form a fully active repression complex. As a control, the recruitment of Ssn6 to the flocculence gene *FLO1* was investigated. *FLO1* is repressed by the Tup1-Ssn6 complex, but repression is independent of Rox1. As shown in Fig. 1A, the *rox1*Δ allele did not affect the formation of the repression complex at *FLO1*. However, the recruitment of Ssn6 in the *mot3*Δ deletion strain was about twofold weaker that that in the *rox1*Δ *mot3*Δ double-deletion strain. There are a number of sequence matches to the Mot3 binding site in the *FLO1* regulatory region, and whether these play a role in augmenting *FLO1* repression has yet to be determined. As a negative control, i.e., a gene that should not bind Rox1, Mot3, Tup1, or Ssn6, we amplified a region from *RPC1*, encoding the large subunit of RNA polymerase III. There was no indication of any Tup1-HA or Ssn6-HA recruitment at this gene, although the *rox1*Δ *mot3*Δ strain showed a consistent increase in signal for Tup1-HA. The significance of this finding, if any, is unclear.

Finally, Ssn6 was recruited to DNA in the absence of Tup1 for both hypoxic genes. There was actually an increase in the ChIP signal in the absence of Tup1 for these genes (Fig. 1A, compare lanes 1 and 4), which probably resulted from the increased expression of Rox1. *ROX1* expression is autorepressed approximately 10-fold under aerobic conditions, and this repression is Tup1 dependent (8, 23). The increase in Rox1 protein levels in a *tup1*Δ mutant would lead to increased occupancy of Rox1 and a consequent increase in Ssn6 recruitment. We believe that this increase was greater at *HEM13* than at *ANB1* because the Rox1 binding sites of *ANB1* OpA were occupied a greater percentage of the time in wild-type cells than the corresponding sites of *HEM13* and, therefore, that Ssn6 occupancy at *ANB1* would not increase as dramatically with an increase in cellular Rox1 levels. It was also possible that some of the increased Ssn6 recruitment was due to the loss of the Tup1-positioned nucleosome and the subsequent opening of operator B (OpB). Both the *ANB1* and the *HEM13* genes have a second set of Rox1 binding sites closer to the coding sequence, designated OpB, but these respective operators contribute only weakly to the repression of *ANB1* (11) and not at all to the repression of *HEM13* (L. G. Klinkenberg, unpublished results). However, the signals for PCR amplification of the ChIP wild-type and *tup1*Δ samples obtained with primer sets located 150 bp upstream or downstream of the OpA-OpB regulatory region of *ANB1* were equally diminished compared to those obtained with the OpA primer set (data not shown), suggesting that the increased Ssn6 recruitment observed in the absence of Tup1 was not due to occupancy at an accessible OpB in the *tup1*Δ strain.

Similar experiments were carried out with epitope-tagged Tup1, with somewhat different results. As described above, Tup1 was recruited more strongly to *ANB1* than to *HEM13*; there was a fivefold greater signal from the *ANB1* gene in wild-type cells containing the Tup1-HA protein than in those containing the untagged protein, while the ratio was only twofold for *HEM13* (Fig. 2A, compare lanes 1 and 2). However, Tup1 recruitment appeared to have a stronger requirement for Rox1 than did Ssn6 localization; the *rox1* deletion reduced the PCR signal as severely as, if not more severely than, did the *rox1*Δ *mot3*Δ double mutation (Fig. 2A, compare lanes 3 and 5), while the *mot3* deletion resulted in nearly wild-type levels of Tup1 recruitment (lane 5). Also, while Ssn6 recruitment did not require Tup1, Tup1 recruitment did require Ssn6 (Fig. 2A, lane 4). As expected, Tup1 localization to *FLO1* was Rox1 independent and was reduced in an *ssn6*Δ strain. Thus, both Ssn6 and Tup1 were recruited to these two hypoxic genes but differed in that Tup1 recruitment was Rox1 dependent while Ssn6 could be recruited by either Rox1 or Mot3. In the case of Mot3 recruitment of Ssn6, the complex formed could not cause repression, possibly due to the absence of Tup1. This is the first suggestion that Ssn6 and Tup1 may not be always complexed together in the cell, albeit in a *mot3* mutant strain. Finally, Tup1 was recruited to the hypoxic genes in an Ssn6-dependent manner, in agreement with a direct Rox1-Ssn6 interaction facilitating general repressor recruitment (23).

The rates of derepression of *ANB1* and *HEM13* are more rapid in a *mot3*Δ strain but are unaffected by an *hhf1-8* or a *cti6*Δ allele. Papamichos-Chronakis et al. recently reported (38) that a newly identified transcription factor, Cti6 (YPL181w), was critical to the induction of Tup1-regulated genes. This study presented evidence for both Cti6-Tup1 and Cti6-SAGA complex interactions that were important for the acetylation and subsequent clearance of the positioned nucleosome responsible for chromatin-dependent repression in the presence of Tup1. The effect of *cti6*Δ on *ANB1* expression under anaerobic conditions was examined in their study, and it was concluded that *ANB1* was uninducible in such a mutant (38). This result and the model that it suggests imply that the limiting factor for *ANB1* induction is the clearance of the nucleosome placed over the TATA box. In the absence of Cti6, the SAGA complex cannot be recruited to *ANB1* for activation, and the positioned nucleosome remains over the TATA box, blocking TBP recruitment and hindering initiation. We attempted to verify the uninducibility of *ANB1* in a *cti6*Δ strain by Northern analysis of hypoxic gene induction.

Total RNA was prepared from cells grown aerobically at various times after the initiation of anaerobiosis. The RNA was then size fractionated by agarose gel electrophoresis and blotted onto a nylon membrane. The induction profiles for the hypoxic genes were visualized by hybridization with gene-specific probes. The kinetics of *ANB1* and *HEM13* RNA induction upon anaerobiosis in wild-type cells are shown in Fig. 3A. After a 30-min delay, during which no induction of *ANB1* RNA was visible, RNA levels increased at 1 h and were nearly fully induced at 2 h of anaerobic growth (Fig. 3A, lanes 1 to 6). Due to extensive sequence homology, the RNA of the aerobic paralog of *ANB1*, *TIF51a*, was also detected and showed an expres-

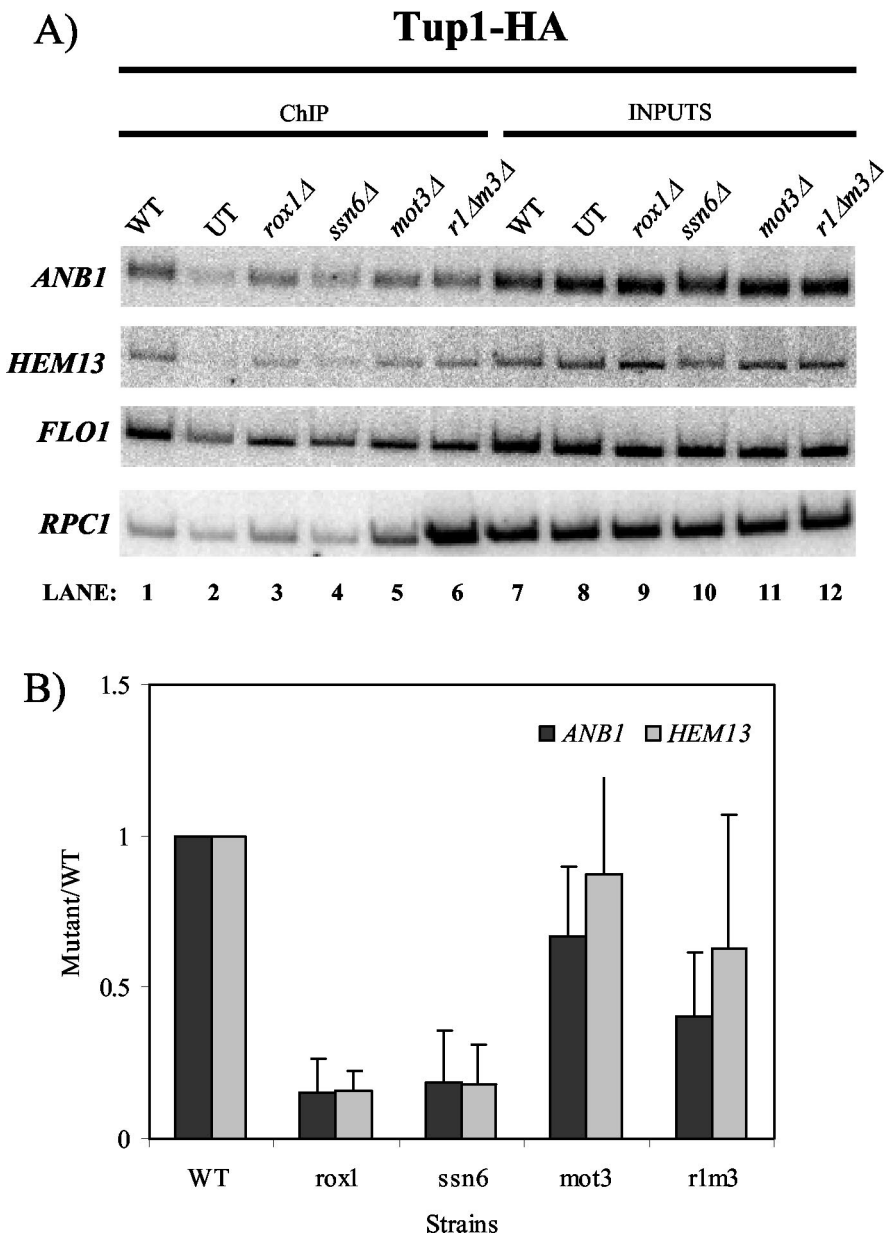

FIG. 2. Tup1 recruitment to hypoxic genes requires Rox1 and Ssn6 but not Mot3. (A) ChIP assays with a monoclonal antibody against the HA epitope were carried out with YCp(23)*TUP1*-HA_3-transformed RZ53-6 (WT) and its *rox1*Δ, *ssn6*Δ, *mot3*Δ, and *rox1*Δ *mot3*Δ (*r1*Δ *m3*Δ) derivatives or with YCplac23-transformed RZ53-6 (UT). Cultures were grown aerobically (repressed) to mid-exponential phase. DNA samples were prepared, amplified, and visualized as described in the legend to Fig. 1. (B) The radioactivity in the ChIP samples was normalized as described in the legend to Fig. 1. The error bars represent the standard deviations calculated from at least five ChIP experiments. The normalized ChIP values were plotted as histograms for *ANB1* and *HEM13*. The values represent the averages of five trials.

sion profile opposite that of *ANB1*, disappearing after 4 h of hypoxia. This aerobic RNA served as an internal control for complete anaerobiosis. *HEM13* was expressed at low levels aerobically, but the kinetics of induction were similar to those of *ANB1* (Fig. 3A, lanes 7 to 12). After an initial 30-min lag, *HEM13* RNA levels increased approximately twofold at 1 h and then continued to increase gradually over the entire time course, reaching fivefold induction after 4 h of anaerobiosis.

Interestingly, the induction profiles for the *cti6*Δ mutant were nearly identical to those for the wild type (Fig. 3B). *ANB1* RNA was first observed after 1 h of hypoxia (Fig. 3B, lane 3), just as in the wild type. Also, *HEM13* was induced at similar rates in wild-type and *cti6*Δ cells, with increased RNA levels observable at 30 min (Fig. 3B, lane 2), followed by a continuous slow increase. These genes were obviously inducible at wild-type rates in the *cti6*Δ strain. The same results were observed in the EUROSCARF strain background (data not shown). The discrepancy between the results of Papamichos-Chronakis et al. (38) and our own is probably due to incomplete anaerobiosis in their study. Their detection of equal lev-

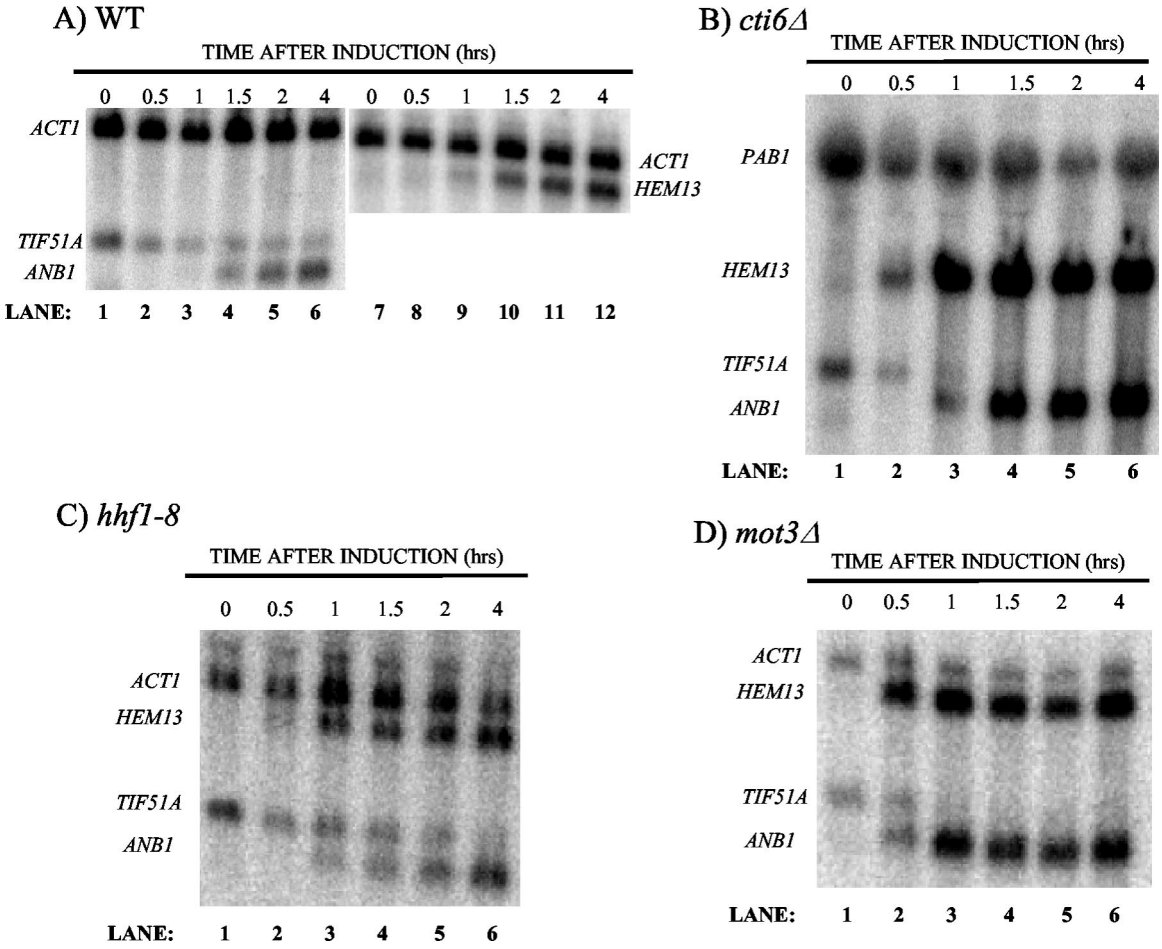

FIG. 3. *ANB1* and *HEM13* induction is unaffected by a deletion of *CTI6* or an amino-terminal deletion in histone H4 but occurs more rapidly in a *mot3*Δ strain. RNA blots were made with total cellular RNA prepared from cells grown aerobically to mid-exponential phase (time zero), and then hypoxia was initiated and maintained by bubbling N_2 through the cultures. Samples were taken at the times indicated. The RNA was hybridized with ^{32}P-labeled probes for *ANB1* (+123 to +465) and *HEM13* (−402 to +803) and either *ACT1* (600-bp internal fragment) or *PAB1* (−81 to +1734) as a control. The positions of the specific RNAs are indicated to the left or right of the blots. While a probe for *TIF51a* RNA was not used, its high degree of similarity to the *ANB1* probe resulted in various degrees of cross-hybridization in each blot. RNA was prepared from RZ53-6 (wild type [WT]) (A), MZ168-24 (*cti6*Δ) (B), MZ148-148 (*hhf1-8*) (C), and RZ53-6Δ*mot3* (*mot3*Δ) (D) cells.

els of *ANB1* and *TIF51a* transcripts in wild-type cells, as shown by Northern analysis, would be equivalent to only partial induction and would be indicative of the incomplete purging of oxygen.

In spite of the lack of a Cti6 effect on hypoxic gene induction, it remained possible that the Tup1-positioned nucleosome was the limiting factor for the induction of *ANB1*, due to the necessity for chromatin remodeling and TATA box clearance. In the absence of the positioned nucleosome, the transcriptional machinery could preassemble at the promoter or assemble much more rapidly upon hypoxia, allowing more rapid induction. It was shown previously that the positioned nucleosome was absent in cells carrying a deletion of the amino terminus of histone H4, although repression remained strong. Therefore, we investigated the induction profiles for *ANB1* and *HEM13* in such a strain (Fig. 3C). Again, no significant difference was observed for the induction of these hypoxic genes in the *hhf1-8* mutant versus the wild type. *HEM13* RNA levels increased 30 min into induction (Fig. 3C, lane 2) and reached maximal induction by 1.5 h (lane 4), while *ANB1* RNA was evident by 1 h of anaerobiosis (lane 3) and the levels reached maximal induction after 2 h (lane 5), as in the wild type. Thus, the presumed requirement for clearance of the positioned nucleosome did not affect the rate of induction.

Mot3 contributes to the repression of *ANB1* by binding to OpA and appears to aid in Ssn6-Tup1 recruitment to *ANB1* and *HEM13*. A deletion of *mot3* results in partial derepression and the loss of the positioned nucleosome at *ANB1* (23). We investigated the effect of Mot3 on induction. Figure 3D shows the induction profiles for *ANB1* and *HEM13* in a *mot3*Δ strain. Interestingly, both hypoxic genes were induced more rapidly in the absence of Mot3. *HEM13* RNA appeared to be nearly fully induced after only 30 min of hypoxia (Fig. 3D, lane 2), while *ANB1* RNA was detectable after only 30 min and appeared to be fully induced by 1 h (lanes 2 and 3). The role of Mot3 during induction and the cause for this rapid gene expression in its absence are currently under investigation.

Rox1 and Mot3 but not Tup1 disassociation from *ANB1* and *HEM13* correlates with RNA induction. Recently, it was demonstrated that Tup1 remains associated with Hog1 kinase-

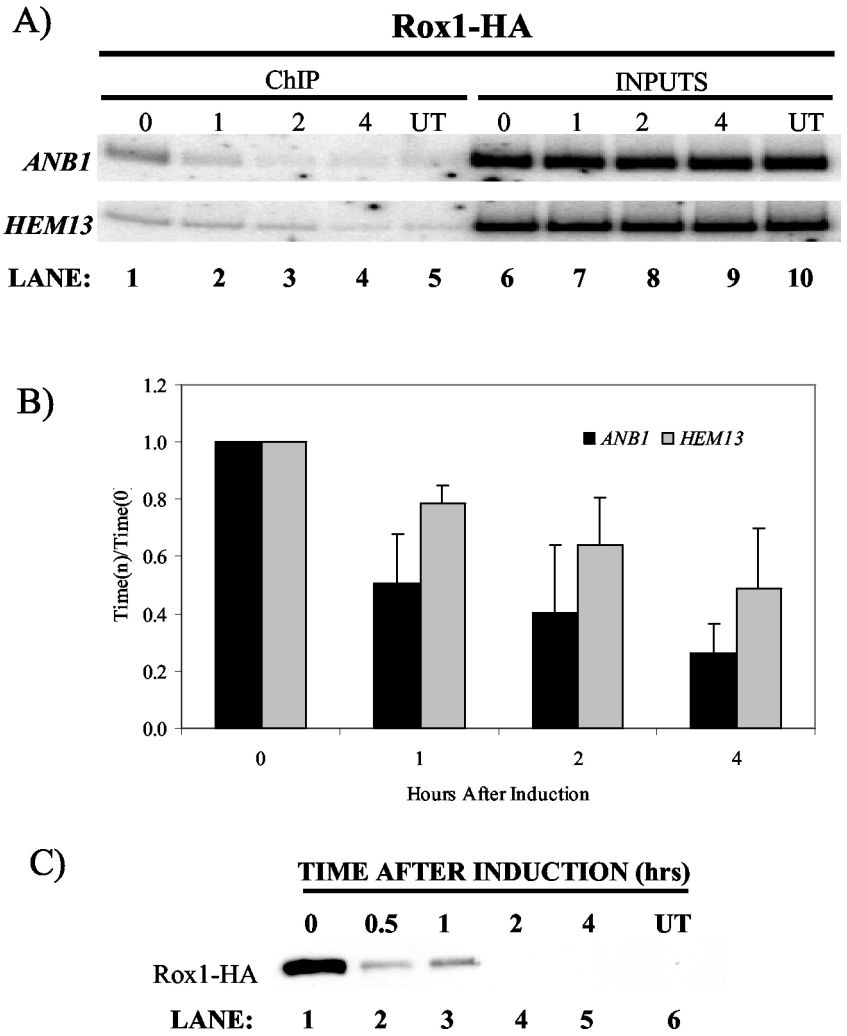

FIG. 4. Rox1 dissociates from hypoxic genes and disappears from cells rapidly after the onset of hypoxia. (A) ChIP analysis with a monoclonal antibody against the HA epitope was carried out with RZ53-6Δ*rox1* cells transformed with YCp(23)*ROX1*-HA_4 (lanes 1 to 4 and 6 to 9) or with YCp(22)*ROX1* (UT) (lanes 5 and 10). Cultures were grown aerobically to mid-exponential phase (time zero; lanes 1, 5, 6, and 10), and then hypoxia was initiated and maintained by bubbling N_2 through the cultures. Samples were taken at the times (in hours) indicated above the lanes. PCR amplification was carried out for both the *ANB1* and the *HEM13* regulatory regions. The ChIP samples were analyzed as described in the legend to Fig. 1. (B) Histograms were generated by using normalized values for the ChIP samples in panel A as follows. For each gene amplified, the input sample was normalized to the aerobic (time zero) input sample. Then, each ChIP sample value was divided by the normalized ratio for its corresponding input sample. Finally, each normalized ChIP value was divided by the normalized aerobic ChIP value [Time(n)/Time(0)]. Thus, the aerobic ChIP value is presented as 1.0. The normalized ChIP values were plotted for *ANB1* and *HEM13*. The values represent the averages of three trials; error bars represent the deviation from the mean observed for the three trials. (C) From the same cultures as those described in panel A, samples were taken at the indicated times and subjected to immunoblotting with a monoclonal antibody against the HA epitope. Equal loading of the samples was determined by staining of the proteins blotted onto a nylon membrane with Ponceau S prior to blocking with milk (data not shown).

regulated genes even after induction by osmotic stress and that in some cases, this Tup1 recruitment was independent of the DNA binding repressor Sko1 (39). The study by Papamichos-Chronakis et al. (38) discussed above also proposed that Tup1 remains associated with the *ANB1* control region after the gene is fully induced by anaerobiosis. This finding disagreed with our model for hypoxic gene induction, which predicts that full induction results from the loss of *ROX1* transcription, degradation of the Rox1 protein and consequently, disassociation of the Tup1-Ssn6 complex from the hypoxic genes (33, 50). Therefore, we examined the association of Rox1, Mot3, and Tup1 with the hypoxic genes during the transition from maximal repression to full induction.

Initially, we confirmed the rapid disappearance of Rox1 from cells and monitored its dissociation from the *ANB1* and *HEM13* loci during induction. ChIP analysis of cells containing a plasmid encoding Rox1-HA demonstrated that after 1 h of anaerobic growth, Rox1 binding at *ANB1* was reduced by one-half and continued to decline over the 4-h induction period (Fig. 4A and B). The levels of Rox1 bound at *HEM13* fell at a similar rate; the apparently slower decline is deceptive, since the background/signal ratio was higher for *HEM13* (discussed

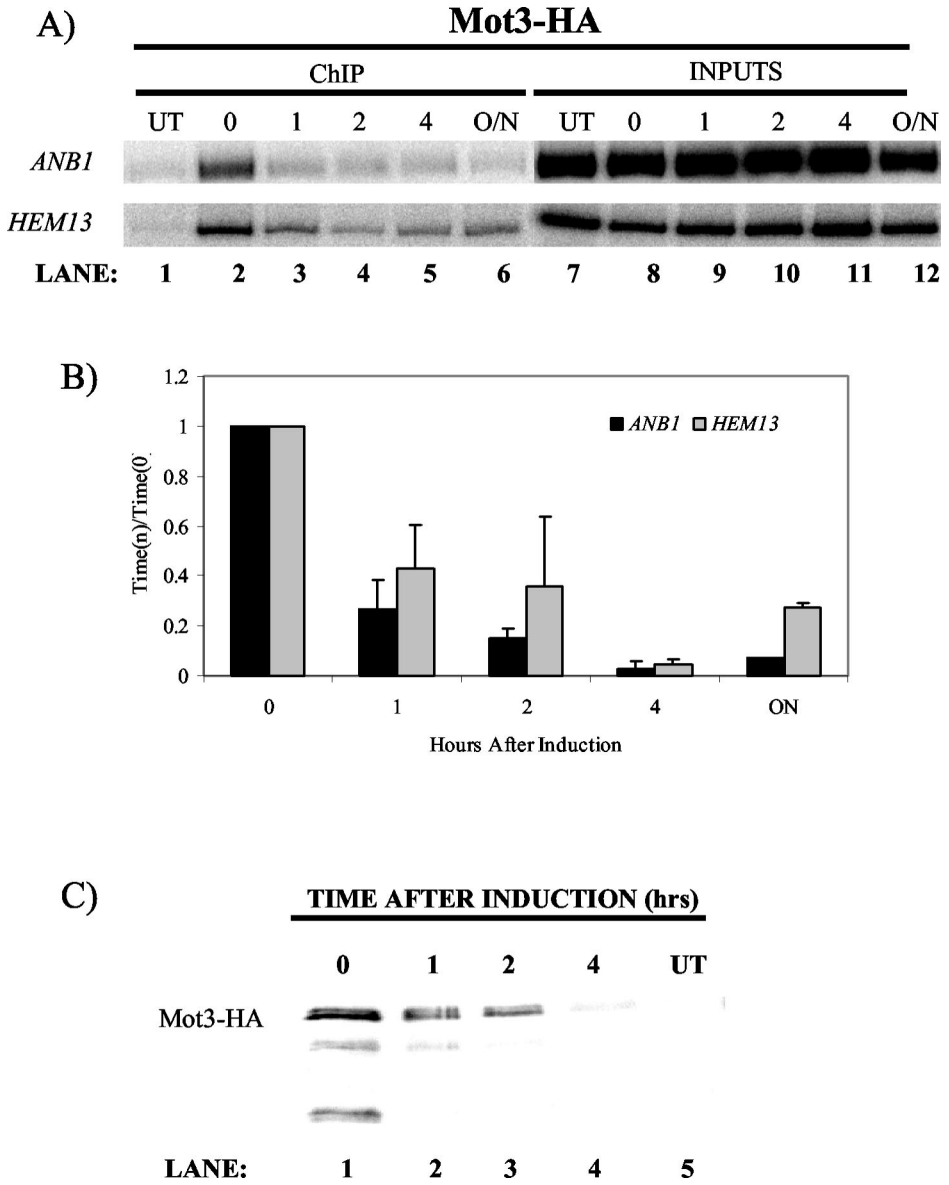

FIG. 5. Mot3 dissociates from hypoxic genes and disappears from cells after the onset of hypoxia. (A) ChIP assays identical to those described in the legend to Fig. 4A for Rox1 were performed with RZ53-6Δ*mot3* cells transformed with YCp(23)*MOT3*-HA_4 and with the addition of a sample grown anaerobically overnight (O/N) (lanes 2 to 6 and 8 to 12). Cells transformed with YCp(22)*MOT3* were used as untagged controls (UT) (lanes 1 and 7). (B) Histograms were generated with ChIP samples normalized as described in the legend to Fig. 4B with the addition of the overnight (ON) sample. The values represent the averages of two trials; error bars represent the deviation from the mean observed for the two trials. (C) Protein samples were prepared from the cells described above during induction and subjected to immunoblotting to detect Mot3-HA as described in the legend to Fig. 4C.

above). This dissociation of Rox1 correlated well with the kinetics of gene induction (Fig. 3A).

The rate of Rox1 disappearance from cells was determined by using Western analysis of samples taken from the same culture as that used for the ChIP analysis described above. Cells were harvested and lysed in SDS gel sample buffer, and the proteins were size fractioned by SDS-PAGE and electroblotted onto a nitrocellulose membrane. Blots were probed with antibody against the HA epitope. As shown in Fig. 4C, Rox1-HA was detected in cells grown aerobically but disappeared from cells rapidly upon the induction of anaerobiosis. After 30 min of hypoxia, Rox1 levels were appreciably lower than those at time zero and continued to drop after 1 h (Fig. 4C, lanes 1 to 3). By 2 h of hypoxia, when *ANB1* and *HEM13* were almost fully induced, Rox1 levels were nearly undetectable (Fig. 4C, lane 4). The disappearance of Rox1 from cells clearly can account for its dissociation from the hypoxic genes (25).

Mot3 binding at the hypoxic genes and the disappearance of Mot3 from cells during induction were somewhat different from the patterns observed for Rox1. Mot3 was bound to the regulatory regions of both *ANB1* and *HEM13* under conditions of repression but disappeared within the first hour of derepression (Fig. 5A and B). Interestingly, Mot3-HA disappeared

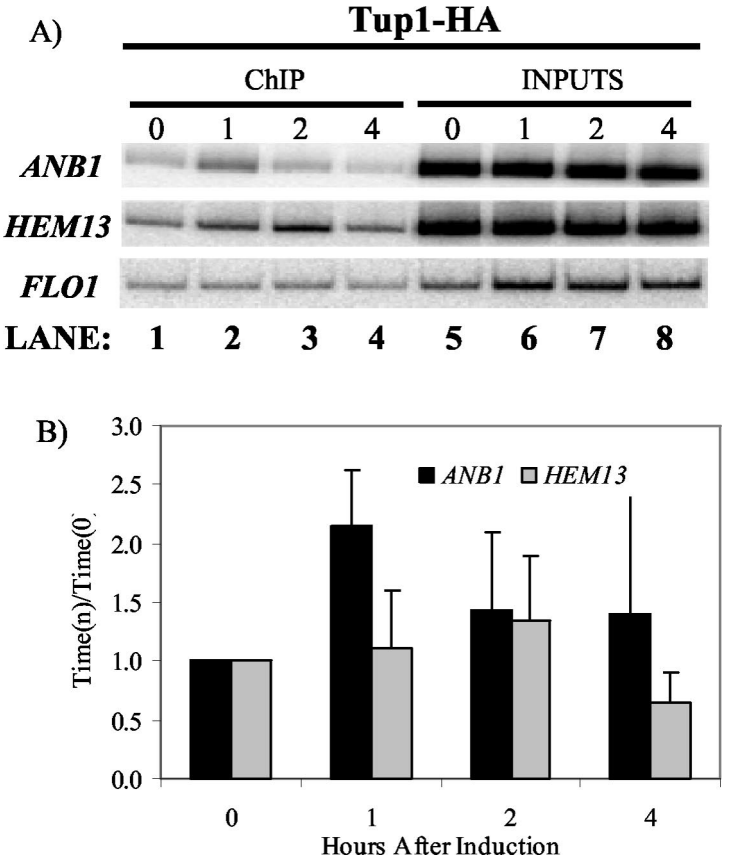

FIG. 6. Tup1-HA persists at hypoxic genes during induction and while transcription occurs. (A) ChIP assays identical to those described in the legend to Fig. 4A for Rox1 were performed with RZ53-6Δ*tup1* cells transformed with YCp(23)*TUP1*-HA_3 (lanes 1 to 8). Cultures were grown aerobically to mid-exponential phase (time zero; lanes 1 and 5), and then hypoxia was initiated and maintained by bubbling N_2 through the cultures. Samples were taken at the times (in hours) indicated above the lanes. PCR amplification was carried out for the *ANB1*, *HEM13*, and *FLO1* regulatory regions. (B) Histograms were generated with ChIP samples normalized to time zero as described in the legend to Fig. 4B. The values represent the averages of three trials; error bars represent the deviation from the mean observed for the three trials.

from cells less rapidly than did Rox1-HA after the initiation of anaerobiosis, as determined by immunoblotting as described above for Rox1 (Fig. 4C). After 1 h of growth under inducing conditions, Mot3 levels were decreased somewhat, and by 4 h, protein levels were significantly diminished. Mot3 may generally be a labile protein, as the smaller Mot3-specific bands in Fig. 5C were observed in cells grown aerobically as well as anaerobically (compare lanes 1 through 4 with lane 5). Interestingly, Mot3 dissociated from the hypoxic genes more rapidly than did Rox1, yet it disappeared from cells more slowly, suggesting that an active mechanism may cause the loss of Mot3 binding under inducing conditions.

We also examined Tup1 occupancy at the hypoxic genes during induction. Our model predicts that the loss of Tup1 occupancy should parallel Rox1 and Mot3 dissociation, as these DNA binding repressors are believed to be necessary for general repressor recruitment. However, this was not the case. During induction, the Tup1 signal at *ANB1* and *HEM13* actually increased (Fig. 6). As expected, Tup1 recruitment to the repressed flocculence gene, *FLO1*, was unaffected by anaerobiosis (Fig. 6A). This apparent increase in Tup1 recruitment to the hypoxic genes during early induction may be genuine or may be due to a conformational change in the general repression complex, allowing transcriptional activation, which results in greater accessibility of the epitope for immunoprecipitation. In any event, Tup1 recruitment at *ANB1* and *HEM13* clearly did not parallel the Rox1 and Mot3 binding discussed above; Tup1 remained at the hypoxic genes during induction and did so in a Rox1- and Mot3-independent manner.

Tup1 excludes TBP from the TATA box at *ANB1* in the absence of the positioned nucleosome. Tup1 is capable of repressing transcription through both chromatin-dependent and chromatin-independent mechanisms. The chromatin-dependent mechanism appears to involve the recruitment of a nucleosome that excludes TBP binding (28), and the chromatin-independent mechanism is defined here as a loss of the positioned nucleosome. One model for chromatin-independent repression consists of putative interactions between Tup1 and the transcriptional machinery (46). In the absence of a positioned nucleosome over the TATA box, Tup1 would trap the transcriptional machinery at the initiation site. This model predicts that TBP would be bound to the TATA box of genes that are repressed by the chromatin-independent mechanism.

To test this model, TBP localization was assayed by ChIP analysis in the absence of the positioned nucleosome. The transcription of *ANB1* increases over 15-fold after 2 h of anaerobiosis (reference 32 and this study). TBP occupancy of the TATA box should increase with transcription. Wild-type cells

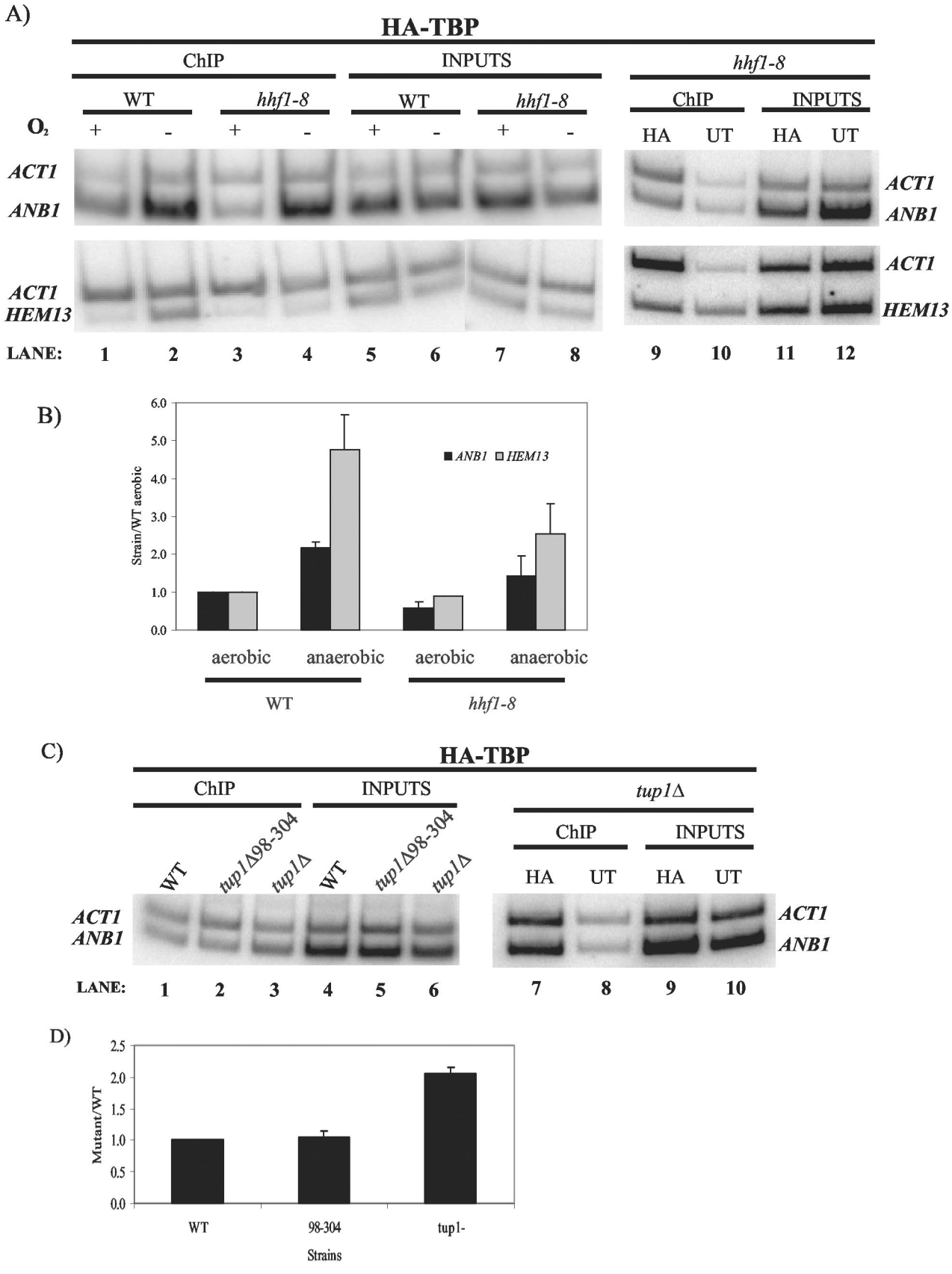

FIG. 7. The repression complex excludes HA-TBP from binding to *ANB1* and *HEM13* even in the absence of a positioned nucleosome. (A) ChIP assays with a monoclonal antibody against the HA epitope were carried out with HA-TBP-containing MZ148-148 cells transformed with

were grown aerobically or anaerobically for 2 h, and a ChIP assay was performed to detect the binding of HA-tagged TBP. Samples from cells carrying a copy of TBP which lacked the HA epitope tag served as a negative control. PCR analyses were carried out with *ACT1*, a constitutively expressed gene, as a positive control. TBP localization to the *ANB1* promoter region increased under inducing conditions (without O_2) compared to repressing conditions (with O_2) (Fig. 7A, lanes 1 and 2). In a strain containing the *hhf1-8* mutation, the TATA box is free of a positioned nucleosome; however, we observed that TBP was still excluded from the *ANB1* promoter under repressing (with O_2) compared to derepressing (without O_2) conditions (Fig. 7A, lanes 3 and 4), and this exclusion occurred at wild-type levels (lanes 1 and 3). This chromatin-independent TBP exclusion was also observed at *HEM13* (Fig. 7A and B), suggesting that, at the hypoxic genes, Tup1 repression both in the presence and in the absence of the positioned nucleosome involves decreased occupancy by TBP.

The deletion of the amino-terminal region of histone H4 has pleiotropic effects in cells, complicating the interpretation of the above results. To provide confirmation, we created a mutant of Tup1 that could not position nucleosomes but that still repressed the hypoxic genes. The domain of Tup1 responsible for histone interactions has been investigated. Using truncated forms of Tup1 and in vitro protein interaction studies, Edmondson et al. (13) demonstrated that residues 120 through 316 are critical for Tup1-histone associations. We constructed an allele in which the codons for residues 98 through 304, including the proposed histone interaction domain, of Tup1 have been deleted (*tup1Δ98–304*). Using a micrococcal nuclease sensitivity assay as previously described (23), we mapped nucleosome positioning in cells carrying the *tup1Δ98–304* mutant allele. Figure 8 illustrates that there was no nucleosome positioned over the TATA box of *ANB1* in cells containing this mutant allele. The micrococcal nuclease cleavage pattern derived from *tup1Δ98–304* cells was identical to that derived from *tup1Δ* cells and naked DNA (23), with cleavage observed flanking the TATA box (Fig. 8, arrows at right). This region was protected by a nucleosome in wild-type cells, as demonstrated previously (23) and verified here. Therefore, the deletion of amino acid residues 98 through 304 of Tup1 generated a Tup1 protein that could not position a nucleosome at *ANB1*.

The Tup1Δ98–304 protein was capable of repression despite the lack of a positioned nucleosome and the large internal deletion. When grown in liquid media, a *tup1Δ98–304* strain exhibited a moderate flocculent phenotype, most likely due to partial derepression of the flocculence genes. Expression studies with the *ANB1-lacZ* reporter demonstrated moderate repression of *ANB1* by Tup1Δ98–304 (Table 2). Repression was 11-fold greater than that in a *tup1Δ* deletion strain and only 2-fold derepressed with respect to the wild type. This repression observed at *ANB1* in the absence of a positioned nucleosome must have been due to the chromatin-independent repression mechanism.

We were interested in determining whether the chromatin-independent TBP exclusion observed in the *hhf1-8* mutant background could be independently verified with the *tup1Δ98–304* strain. Presumably, repression by the Tup1Δ98–304 protein would be due to the same mechanism. As shown in Fig. 7C and D, Tup1Δ98–304 was capable of excluding TBP. Localization of TBP to the TATA box of *ANB1* was observed in the *tup1Δ* strain (Fig. 7C, compare lanes 1 and 3), but occupancy in the *tup1Δ98–304* strain was nearly identical to that in the wild-type strain. These data provided verification of the TBP exclusion by Tup1 observed in the histone H4 amino-terminal deletion strain, independent of the positioned nucleosome.

Srb7 is not essential to chromatin-independent repression of the hypoxic genes. A number of studies have suggested both genetic and physical interactions between Tup1 and components of the mediator complex (18, 27, 37, 48). For example, there is compelling evidence for a physiologically relevant interaction between Tup1 and the essential mediator protein Srb7 (18). Srb7 and Tup1 were demonstrated to interact in vivo and in vitro. A mutant form of Srb7 containing a deletion of the first seven amino-terminal residues (Srb7Δ7) resulted in decreased interaction with Tup1 and derepression of five Tup1-regulated genes representing three regulons (mating type, flocculence, and glucose-repressed genes). This interaction and the amount of Tup1-mediated repression were decreased even further when the amino-terminal half of ubiquitin was fused to the amino terminus of the Srb7Δ7 mutant (Nub-Srb7Δ7).

To determine the possible role of Srb7 in the chromatin-dependent and chromatin-independent mechanisms of repression of *ANB1* expression, we explored the effects of the Srb7 mutants on an *ANB1-lacZ* reporter with or without the positioned nucleosome. The mutant allele encoding Srb7Δ7 was constructed and integrated into the *SRB7* locus. There was negligible derepression of *ANB1* in this mutant strain (Table 3). Cells grown under repressing conditions were only 3-fold

either YEp(112)*HHF1*/*HHT1* (WT) or YEp(181)*hhf1-8*/*HHT1* (*hhf1-8*) and grown to mid-exponential phase aerobically ($+O_2$) and then under hypoxic conditions for 2 h ($-O_2$) (lanes 1 to 8). Also, MZ148-148 cells transformed with YEp(181)*hhf1-8*/*HHT1* and expressing either HA-TBP (HA) or TBP lacking the HA epitope tag (UT) were used for ChIP assays, with untagged TBP serving as a negative control (lanes 9 to 12). PCR amplification was carried out for the TATA box regions of *ANB1* and *ACT1* (upper panel) and *HEM13* and *ACT1* (lower panel). (B) Normalization of the input samples was carried out as described in the legend to Fig. 1. However, the ChIP samples for *ANB1* and *HEM13* were normalized to the *ACT1* ChIP samples, assuming that *ACT1* TBP occupancy was constant. The value for each experimental sample was divided by the value for the wild-type aerobic (repressed) sample for each strain (Strain/WT aerobic) for *ANB1* and *HEM13*. The ratios represent the averages of three trials; error bars represent the deviation from the mean observed for the three trials. The ratios are presented as a histogram with strains labeled as wild type (WT) and *hhf1-8*, aerobic and anaerobic. (C) ChIP assays with a monoclonal antibody against the HA epitope were carried out with HA-TBP-containing JDZ149-32 cells transformed with YCp(111)*TUP1* (WT), YCp(111)*tup1Δ98–304*, or YCplac111 (*tup1Δ*) and with JDZ149-32 cells containing untagged TBP. Cells were grown to mid-exponential phase aerobically. PCR amplification was carried out for the TATA box regions of *ANB1* and *ACT1*. (D) Normalization was carried out as described above to generate a histogram in which the ratios represent the value for each experimental sample divided by the value for the wild-type sample (Mutant/WT). Thus, the wild-type ChIP value is presented as 1.0. The strains tested were wild type (WT), *tup1Δ98–304* (98-304), and *tup1Δ* (tup1−). The ratios represent the averages of two trials; error bars represent the deviation from the mean observed for the two trials.

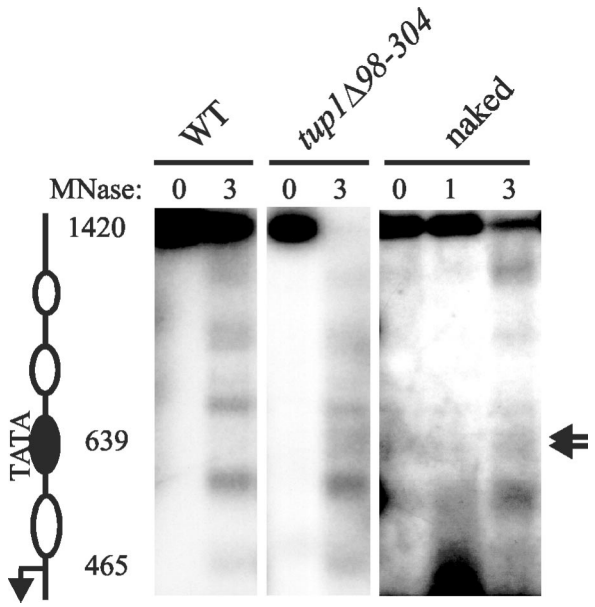

FIG. 8. Deletion of the histone interaction domain of Tup1 results in a loss of the positioned nucleosome. Micrococcal nuclease sensitivity assays were carried out with strain MZ90-88Δ*tup1* transformed with YEp(195)*ANB1* and either YCp(111)*TUP1* (wild type [WT]) or YCp(111)*tup1Δ98–304*. Cells were grown aerobically to mid-exponential phase. After lysis, micrococcal nuclease was added to a final concentration of 3 U/400 μl, and digestion was carried out at 37°C for 10 min. The samples in the lanes marked "Naked" were prepared from MZ90-88Δ*tup1* transformed with YEp(195)*ANB1* and YCp(111)*TUP1* and deproteinated prior to digestion with 0, 1, or 3 U of micrococcal nuclease per 400 μl for 10 min at 37°C. All samples were then deproteinated and digested with *Eco*RI plus *Bgl*II, and Southern analysis was carried out as described previously (23). The diagram on the left represents the *ANB1* gene from the *Bgl*II site at 1 (not shown) to the *Eco*RI site at 1420. Fragment lengths were determined by use of a molecular weight standard (not shown). The ovals represent protected regions, with the filled-in oval representing the positioned nucleosome in wild-type, repressed cells. The hooked arrow represents the translational initiation site. The two arrows on the right indicate the two bands that are visible in the *tup1Δ98–304* and naked digests but not in the wild-type digest.

derepressed compared to wild-type cells and 26-fold repressed compared to cells grown under inducing conditions. To determine the effect of the Srb7Δ7 mutant in the absence of chromatin-dependent repression, a strain that included both mutant *srb7Δ7* and *hhf1-8* alleles was constructed. We reasoned that if the amino-terminal domains of H4 and Srb7 were required for the chromatin-dependent and chromatin-independent mechanisms, respectively, then a strain with deletions of both of these domains would leave Tup1 with no means for repression. However, *ANB1* was still repressed 10-fold in this strain (Table 3), suggesting that Tup1 is capable of repression in the absence of nucleosome and Srb7 interactions.

The more severe allele encoding Nub-Srb7Δ7 was also constructed and integrated, replacing the wild-type allele. This protein was reported to cause greater derepression of Tup1-regulated genes than was the Srb7Δ7 protein. Again, we found no such effect at *ANB1*. Cells containing the Nub-Srb7Δ7 protein were capable of 17-fold repression, with less than 2-fold derepression (Table 3). The *Nub-srb7Δ7/hhf1-8* mutant strain

TABLE 2. Repression of *ANB1-lacZ* by Tup1Δ98–304

Strain[a]	Miller units	Fold repression[b]
Wild type	2.8 ± 1.4	21.3
tup1Δ98–304	5.4 ± 2.2	10.9
*tup1*Δ	59.1 ± 25.3	1.0

[a] Enzyme assays were carried out by using MZ90-88Δ*tup1* transformed with YCp(111)*TUP1* (wild type), YCp(111)*tup1Δ98–304*, or YCplac111 (*tup1*Δ).
[b] β-Galactosidase activity with respect to that in the *tup1*Δ strain.

was 17-fold repressed at *ANB1*, with less than 8-fold derepression (Table 3).

This lack of a dramatic Srb7 effect on the repression of *ANB1* was confirmed by Northern blot analysis. There was no detectable increase in *ANB1* RNA levels in the *Nub-srb7Δ7* and *Nub-srb7Δ7/hhf1-8* strains (Fig. 9). A similar analysis showed no derepression of *HEM13* in the same strains (data not shown). Thus, while Srb7 may play a weak role in chromatin-independent repression, it is not the sole determinant of repression by this mechanism at the hypoxic genes.

DISCUSSION

We have tried to address three questions concerning Tup1-Ssn6 repression of yeast hypoxic genes in this study. First, what are the requirements for recruitment of the general repression complex? Second, how does the complex disassemble to allow induction during derepression? Third, what are the requirements for the chromatin-independent mechanism of repression? In each case, the answers obtained were surprising and challenged our simplistic view of repression and induction.

Requirements for complex association at the hypoxic genes. Four proteins are known to be involved in hypoxic gene repression: the DNA binding proteins Rox1 and Mot3 and the non-DNA binding general repressors Tup1 and Ssn6. Rox1, Ssn6, and Tup1 are required for repression, while Mot3 enhances repression at strongly repressed hypoxic genes, such as *ANB1* and *HEM13* (23). Rox1 and Mot3 bind DNA independently at the OpA sites of both of these genes in vitro, and evidence indicates that Rox1 interacts with Ssn6 (23, 49) and that Mot3 aids in Tup1-Ssn6 recruitment (23). Using ChIP experiments, we demonstrated that the Tup1-Ssn6 complex is indeed physically localized to the OpA sites of the *ANB1* and *HEM13* hypoxic genes in vivo, verifying previously reported genetic evidence for Tup1-Ssn6 repression at the hypoxic regulon (2, 5, 49). This recruitment is abolished in a *rox1*Δ *mot3*Δ double-deletion strain, confirming the well-documented model that Tup1-Ssn6 recruitment requires a specific DNA binding protein. Furthermore, the localization of the complex to the hypoxic genes occurred through Rox1 and Mot3 interactions with Ssn6; Ssn6 was localized to the hypoxic genes in the absence of Tup1, but Tup1 did not localize to the genes in the absence of Ssn6.

All of these findings were anticipated, but we were surprised to find that recruitment of Ssn6 was not sufficient for recruitment of Tup1. While repression is completely abolished in a *rox1*Δ strain (32), this deletion did not eliminate Ssn6 localization; only the deletion of both the *ROX1* and the *MOT3* genes resulted in complete loss of this member of the general repression complex. Thus, Mot3 alone could recruit Ssn6, but Rox1 must be present to recruit Tup1 and form a competent repres-

TABLE 3. Repression of *ANB1-lacZ* in *SRB7* and/or *HHF1* mutant strains

Strain[a]	Mean ± SD Miller units under the following conditions:		Fold repression[b]
	Aerobic	Anaerobic	
Wild type	1.7 ± 1.4	90.1 ± 27.6	52.9
hhf1-8	6.9 ± 3.7	82.7 ± 22.7	12.0
srb7Δ7	3.4 ± 0.1	33.1 ± 3.2	9.7
Nub-srb7Δ7	3.5 ± 1.5	59.2 ± 22.9	16.8
srb7Δ7/hhf1-8	7.2 ± 0.8	80.5 ± 45.8	11.2
Nub-srb7Δ7/hhf1-8	13.3 ± 8.3	95.3 ± 54.5	7.2

[a] Enzyme assays were carried out by using MZ101-18 (wild type), MZ101-18A (*hhf1-8*), MZ101-18*srb7Δ7N* (*srb7Δ7*), MZ101-18*Nub-srb7Δ7N* (*Nub-srb7Δ7*), MZ101-18A*srb7Δ7N* (*srb7Δ7/hhf1-8*), and MZ101-18A*Nub-srb7Δ7N* (*Nub-srb7Δ7/hhf1-8*).

[b] β-Galactosidase activity in cells grown anaerobically for 2 h relative to that in cells grown aerobically.

sion complex. This requirement for Rox1 to recruit Tup1 was not the result of a strong interaction between Rox1 and Tup1; Rox1 could not recruit Tup1 in the absence of Ssn6. We can envision several alternatives for how this Rox1 dependence arises. Rox1 may place Ssn6 in a conformation that allows Tup1 recruitment, or Mot3 alone may alter Ssn6 so that it cannot bind Tup1. It is also possible that Rox1 contacts Tup1 weakly in the presence of Ssn6 to strengthen the Ssn6-Tup1 complex at the hypoxic genes. Further experiments are required to distinguish these possibilities.

Finally, these findings lend support to the hypothesis that Mot3 aids not in Rox1 binding but rather in repression complex recruitment. In vitro DNA binding experiments indicated that Rox1 and Mot3 did not bind to *ANB1* OpA cooperatively (23), and the ability of Mot3 to recruit Ssn6 independently of Rox1 in a *rox1Δ* strain clearly reinforces this scenario.

Induction of the hypoxic genes. Upon oxygen deprivation, *ROX1* transcription ceases and the protein is quickly degraded. Since Rox1 is required for repression, these events would explain derepression. We confirmed here the rapid disappearance of the Rox1 protein from the cell at the onset of hypoxia and demonstrated that the concomitant dissociation of Rox1 from the hypoxic genes correlated well with the appearance of hypoxic gene RNA. Nonetheless, despite this rather tidy picture, Papamichos-Chronakis et al. (38) recently identified transcription factor Cti6 as being important for the induction of Tup1-regulated genes, including *ANB1* (38). They presented evidence for physical interactions between Cti6 and Tup1 and between Cti6 and the SAGA complex. It was hypothesized that during induction, Cti6 bridges Tup1 and the SAGA complex, allowing SAGA to acetylate and remodel the repressive chromatin structure established by Tup1. Cells lacking Cti6 showed either a delayed or a complete loss of induction. They found that *ANB1* was uninducible in a *cti6Δ* strain, a result that we could not reproduce. We observed no significant difference between wild-type and *cti6Δ* strains in the induction of *ANB1* or *HEM13*. It is likely that complete anaerobiosis was not achieved in their experiment, resulting in the lack of *ANB1* induction.

The same study demonstrated that Tup1 was bound to the promoter region of *ANB1* under both repressing and inducing conditions (38). We found this result to be reproducible; Tup1 persisted at the hypoxic genes during early induction, but then its levels declined as the cells approached full induction. This persistence was not due to the presence of Rox1 or Mot3 at these genes, raising the question of what keeps Tup1 there. In addition, at this time we have no evidence for a physiological role for this persistence.

We further investigated the role of chromatin remodeling in the induction of the *ANB1* gene. Tup1 positions a nucleosome over the *ANB1* TATA box, and the need to remove it may delay induction (23). To test this possibility, we measured the appearance of *ANB1* RNA in an *hhf1-8* mutant carrying a deletion of the histone H4 amino terminus. This allele causes

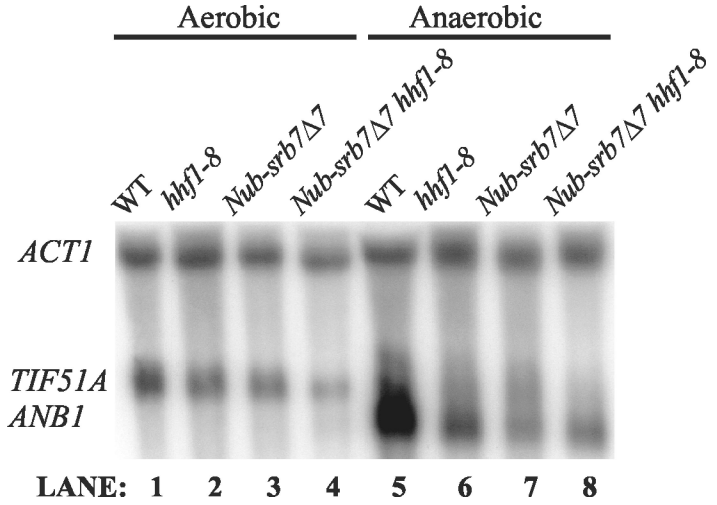

FIG. 9. Srb7 does not play a major role in the chromatin-dependent or chromatin-independent repression of *ANB1*. RNA blotting was carried out with total cellular RNA from MZ101-18 (wild type [WT]), MZ101-18A (*hhf1-8*), MZ101-18*Nub-srb7Δ7N* (*Nub-srb7Δ7N*), and MZ101-18A*Nub-srb7Δ7N* (*Nub-srb7Δ7N/hhf1-8*). Cells were grown aerobically to mid-exponential phase (lanes 1 to 4), and then N_2 was bubbled through the cultures for 2 h to induce hypoxia (lanes 5 to 8). The RNA was hybridized to ^{32}P-labeled probes for *ANB1* and *ACT1* as described in the legend to Fig. 3.

a loss of the positioned nucleosome but does not result in depression. Hence, induction could be measured, and we found that it was identical to that of the wild type. Consequently, the positioned nucleosome does not affect derepression.

Interestingly, in a *mot3*Δ strain, the rates of *ANB1* and *HEM13* induction are more rapid. Since we found that Mot3 dissociated from DNA as fast as did Rox1, it appears that it is not the presence of Mot3 per se that slows induction but rather some memory of its presence. The same type of experiment is not possible with Rox1, since *rox1*Δ cells are completely derepressed, but this memory may require both proteins. Both Rox1 (9) and Mot3 (Klinkenberg, unpublished) bind and bend DNA specifically, making it possible that the memory of Mot3 binding and perhaps Rox1 binding as well is achieved by the persistence of DNA bending. For example, the bending may bring into contact two disparate elements that then remain associated after the Rox1 and Mot3 bending proteins are gone. Also, the dissociation of Mot3 from the hypoxic genes was much faster than the slow loss of Mot3 from hypoxic cells, suggesting that, unlike Rox1 binding, Mot3 binding to DNA may be regulated.

Chromatin-independent repression pathway. According to the model of Tup1 repression, there are two distinct mechanisms, one chromatin dependent and the other chromatin independent. Either can be utilized at *ANB1* (23). Chromatin-independent repression might involve interactions between Tup1 and members of the RNA polymerase II holoenzyme that inhibit transcriptional initiation. According to this model, the transcriptional machinery would be assembled at the promoter but inhibited from initiating transcription. However, we found that this is not true at *ANB1* and *HEM13*. The exclusion of TBP was observed under repressing conditions in the histone H4 mutant in the absence of the positioned nucleosome. The existence of chromatin-independent TBP exclusion was independently supported by studies with the *tup1Δ98–304* allele. The resulting mutant Tup1 (Tup1Δ98–304) is capable of significant repression at *ANB1* and presumably other genes as well, but it is missing all, or an essential part, of the domain required for nucleosome positioning. Assays in which the chromatin structure at the *ANB1* promoter was analyzed demonstrated that Tup1Δ98–304 could not position a nucleosome and must cause repression through some mechanism independent of a positioned nucleosome. Nonetheless, TBP was excluded from *ANB1* at wild-type levels by Tup1Δ98–304, verifying the exclusion observed in the histone mutant.

The mediator complex subunit Srb7 was previously described to be critical for Tup1 repression (18). An interaction between Tup1 and Srb7 likely would occur only if chromatin-independent repression were utilized. To address this possibility, we constructed strains with mutations in the chromatin-dependent (*hhf1-8*) and chromatin-independent (*srb7*) pathways, either alone or in conjunction. Two mutant *SRB7* alleles that had previously been reported to result in derepression of some Tup1-repressed genes were made (18). We hypothesized that if Tup1 required interactions with H4 and Srb7 for the chromatin-dependent and chromatin-independent mechanisms, respectively, then the elimination of both interactions should leave Tup1 with no means of repression and, therefore, complete derepression would result. Surprisingly, Tup1 still repressed *ANB1* significantly in cells carrying these mutations either alone or in combination. Therefore, Tup1 does not solely require Srb7 at *ANB1* for chromatin-independent repression. This result also supports the findings of Lee et al. (30) that there was little or no effect on Tup1-dependent repression of *ANB1* or *RNR2* in cells containing mutations of the mediator complex subunit genes *srb8*, *srb9*, *srb10*, and *srb11*, either alone or in various combinations. There was a very modest loss of repression at *SUC2* in the *srb* mutants. Also, mutations of *srb10* and *srb11*, in conjunction with amino-terminal deletions of H3 or H4, had no effect on the repression of *ANB1* (23, 30). In light of TBP exclusion from the hypoxic genes by Tup1-mediated chromatin-dependent repression and chromatin-independent repression, the lack of a mediator complex effect is not surprising. The transcriptional machinery does not preassemble at the hypoxic genes prior to activation, leaving no opportunity for a Tup1-mediator interaction. It is also possible, however, that there are more than two distinct mechanisms for repression.

The manner by which Tup1 excludes TBP independently of chromatin is unknown; however, the simplest model might include interference with activator protein binding and/or function. This model would explain the lack of transcriptional machinery recruitment but implies that Tup1 interacts with yet another family of proteins. Given the extensive interactions that have been reported for Tup1, this versatile protein may use multiple, redundant contacts and mechanisms to achieve repression.

The results reported here have shaken our confidence in devising a simple, general model for repression by the Tup1-Ssn6 general repressor complex. The simple recruitment model in which repression is solely a function of whether Tup1-Ssn6 is present at a gene must be discarded. We must now consider the importance of the conformation and perhaps the stoichiometry of the repression complex. Also, we must discard the notion that repression occurs through one or two common mechanisms at all regulons. Previous studies with *TUP1* point mutations indicated that various regulons were differentially affected by the same mutations (5, 26). Similar differences are apparent for *trans*-acting factors. Mutations in the gene for the mediator protein Srb10 were reported to affect the repression of *SUC2* (27, 30, 48) and the mating type genes (45) but not of *ANB1* (23, 30), and here we found that mutations in *CTI6* and *SRB7* did not affect *ANB1* induction and repression in the same manner as was reported for other Tup1-Ssn6-regulated genes. Obviously, more knowledge of the mechanisms of repression must be obtained in order to provide a clear understanding of how the general repression complex functions.

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 2000. Current protocols in molecular biology. John Wiley & Sons, Inc., New York, N.Y.
2. **Balasubramanian, B., C. V. Lowry, and R. S. Zitomer.** 1993. The Rox1 repressor of the *Saccharomyces cerevisiae* hypoxic genes is a specific DNA-binding protein with a high-mobility-group motif. Mol. Cell. Biol. **13:**6071–6078.

3. **Boeke, J. D., J. Trueheart, G. Natsoulis, and G. R. Fink.** 1987. 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. Methods Enzymol. **154:**164–175.
4. **Bone, J. R., and S. Y. Roth.** 2001. Recruitment of the yeast Tup1p-Ssn6p repressor is associated with localized decreases in histone acetylation. J. Biol. Chem. **276:**1808–1813.
5. **Carrico, P. M., and R. S. Zitomer.** 1998. Mutational analysis of the Tup1 general repressor of yeast. Genetics **148:**637–644.
6. **Cormack, B. P., and K. Struhl.** 1992. The TATA-binding protein is required for transcription by all three nuclear RNA polymerases in yeast cells. Cell **69:**685–696.
7. **Davie, J. K., R. J. Trumbly, and S. Y. Dent.** 2002. Histone-dependent association of Tup1-Ssn6 with repressed genes in vivo. Mol. Cell. Biol. **22:**693–703.
8. **Deckert, J., R. Perini, B. Balasubramanian, and R. S. Zitomer.** 1995. Multiple elements and auto-repression regulate Rox1, a repressor of hypoxic genes in *Saccharomyces cerevisiae*. Genetics **139:**1149–1158.
9. **Deckert, J., A. M. Rodriguez Torres, J. T. Simon, and R. S. Zitomer.** 1995. Mutational analysis of Rox1, a DNA-bending repressor of hypoxic genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **15:**6109–6117.
10. **Deckert, J., and K. Struhl.** 2001. Histone acetylation at promoters is differentially affected by specific activators and repressors. Mol. Cell. Biol. **21:**2726–2735.
11. **Deckert, J., A. M. Torres, S. M. Hwang, A. J. Kastaniotis, and R. S. Zitomer.** 1998. The anatomy of a hypoxic operator in *Saccharomyces cerevisiae*. Genetics **150:**1429–1441.
12. **Dudley, A. M., C. Rougeulle, and F. Winston.** 1999. The Spt components of SAGA facilitate TBP binding to a promoter at a post-activator-binding step in vivo. Genes Dev. **13:**2940–2945.
13. **Edmondson, D. G., M. M. Smith, and S. Y. Roth.** 1996. Repression domain of the yeast global repressor Tup1 interacts directly with histones H3 and H4. Genes Dev. **10:**1247–1259.
14. **Edmondson, D. G., W. Zhang, A. Watson, W. Xu, J. R. Bone, Y. Yu, D. Stillman, and S. Y. Roth.** 1998. In vivo functions of histone acetylation/deacetylation in Tup1p repression and Gcn5p activation. Cold Spring Harbor Symp. Quant. Biol. **63:**459–468.
15. **Gavin, I. M., and R. T. Simpson.** 1997. Interplay of yeast global transcriptional regulators Ssn6p-Tup1p and Swi-Snf and their effect on chromatin structure. EMBO J. **16:**6263–6271.
16. **Gietz, R. D., and A. Sugino.** 1988. New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene **74:**527–534.
17. **Goldstein, A. L., and J. H. McCusker.** 1999. Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. Yeast **15:**1541–1553.
18. **Gromoller, A., and N. Lehming.** 2000. Srb7p is a physical and physiological target of Tup1p. EMBO J. **19:**6845–6852.
19. **Herschbach, B. M., M. B. Arnaud, and A. D. Johnson.** 1994. Transcriptional repression directed by the yeast alpha 2 protein in vitro. Nature **370:**309–311.
20. **Huang, M., Z. Zhou, and S. J. Elledge.** 1998. The DNA replication and damage checkpoint pathways induce transcription by inhibition of the Crt1 repressor. Cell **94:**595–605.
21. **Kaiser, C., S. Michaelis, and A. Mitchell.** 1994. Methods in yeast genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
22. **Kang, H. A., H. G. Schwelberger, and J. W. Hershey.** 1992. The two genes encoding protein synthesis initiation factor eIF-5A in *Saccharomyces cerevisiae* are members of a duplicated gene cluster. Mol. Gen. Genet. **233:**487–490.
23. **Kastaniotis, A. J., T. A. Mennella, C. Konrad, A. M. Torres, and R. S. Zitomer.** 2000. Roles of transcription factor Mot3 and chromatin in repression of the hypoxic gene *ANB1* in yeast. Mol. Cell. Biol. **20:**7088–7098.
24. **Keleher, C. A., M. J. Redd, J. Schultz, M. Carlson, and A. D. Johnson.** 1992. Ssn6-Tup1 is a general repressor of transcription in yeast. Cell **68:**709–719.
25. **Keng, T.** 1992. *HAP1* and *ROX1* form a regulatory pathway in the repression of *HEM13* transcription in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **12:**2616–2623.
26. **Komachi, K., and A. D. Johnson.** 1997. Residues in the WD repeats of Tup1 required for interaction with α2. Mol. Cell. Biol. **17:**6023–6028.
27. **Kuchin, S., and M. Carlson.** 1998. Functional relationships of Srb10-Srb11 kinase, carboxy-terminal domain kinase CTDK-I, and transcriptional corepressor Ssn6-Tup1. Mol. Cell. Biol. **18:**1163–1171.
28. **Kuras, L., and K. Struhl.** 1999. Binding of TBP to promoters in vivo is stimulated by activators and requires Pol II holoenzyme. Nature **399:**609–613.
29. **Kwast, K. E., L. C. Lai, N. Menda, D. T. James III, S. Aref, and P. V. Burke.** 2002. Genomic analyses of anaerobically induced genes in *Saccharomyces cerevisiae*: functional roles of Rox1 and other factors in mediating the anoxic response. J. Bacteriol. **184:**250–265.
30. **Lee, M., S. Chatterjee, and K. Struhl.** 2000. Genetic analysis of the role of Pol II holoenzyme components in repression by the Cyc8-Tup1 corepressor in yeast. Genetics **155:**1535–1542.
31. **Li, B., and J. C. Reese.** 2001. Ssn6-Tup1 regulates *RNR3* by positioning nucleosomes and affecting the chromatin structure at the upstream repression sequence. J. Biol. Chem. **276:**33788–33797.
32. **Lowry, C. V., M. E. Cerdan, and R. S. Zitomer.** 1990. A hypoxic consensus operator and a constitutive activation region regulate the *ANB1* gene of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:**5921–5926.
33. **Lowry, C. V., and R. H. Lieber.** 1986. Negative regulation of the *Saccharomyces cerevisiae ANB1* gene by heme, as mediated by the *ROX1* gene product. Mol. Cell. Biol. **6:**4145–4148.
34. **Lowry, C. V., and R. S. Zitomer.** 1984. Oxygen regulation of anaerobic and aerobic genes mediated by a common factor in yeast. Proc. Natl. Acad. Sci. USA **81:**6129–6133.
35. **Lowry, C. V., and R. S. Zitomer.** 1988. *ROX1* encodes a heme-induced repression factor regulating *ANB1* and *CYC7* of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **8:**4651–4658.
36. **Megee, P. C., B. A. Morgan, B. A. Mittman, and M. M. Smith.** 1990. Genetic analysis of histone H4: essential role of lysines subject to reversible acetylation. Science **247:**841–845.
37. **Papamichos-Chronakis, M., R. S. Conlan, N. Gounalaki, T. Copf, and D. Tzamarias.** 2000. Hrs1/Med3 is a Cyc8-Tup1 corepressor target in the RNA polymerase II holoenzyme. J. Biol. Chem. **275:**8397–8403.
38. **Papamichos-Chronakis, M., T. Petrakis, E. Ktistaki, I. Topalidou, and D. Tzamarias.** 2002. Cti6, a PHD domain protein, bridges the Cyc8-Tup1 corepressor and the SAGA coactivator to overcome repression at GAL1. Mol. Cell **9:**1297–1305.
39. **Proft, M., and K. Struhl.** 2002. Hog1 kinase converts the Sko1-Cyc8-Tup1 repressor complex into an activator that recruits SAGA and SWI/SNF in response to osmotic stress. Mol. Cell **9:**1307–1317.
40. **Redd, M. J., M. B. Arnaud, and A. D. Johnson.** 1997. A complex composed of Tup1 and Ssn6 represses transcription in vitro. J. Biol. Chem. **272:**11193–11197.
41. **Shimizu, M., S. Y. Roth, C. Szent-Gyorgyi, and R. T. Simpson.** 1991. Nucleosomes are positioned with base pair precision adjacent to the alpha 2 operator in *Saccharomyces cerevisiae*. EMBO J. **10:**3033–3041.
42. **Stargell, L. A., and K. Struhl.** 1996. A new class of activation-defective TATA-binding protein mutants: evidence for two steps of transcriptional activation in vivo. Mol. Cell. Biol. **16:**4456–4464.
43. **Trumbly, R. J.** 1992. Glucose repression in the yeast *Saccharomyces cerevisiae*. Mol. Microbiol. **6:**15–21.
44. **Tzamarias, D., and K. Struhl.** 1994. Functional dissection of the yeast Cyc8-Tup1 transcriptional co-repressor complex. Nature **369:**758–761.
45. **Wahi, M., and A. D. Johnson.** 1995. Identification of genes required for alpha 2 repression in *Saccharomyces cerevisiae*. Genetics **140:**79–90.
46. **Wahi, M., K. Komachi, and A. D. Johnson.** 1998. Gene regulation by the yeast Ssn6-Tup1 corepressor. Cold Spring Harbor Symp. Quant. Biol. **63:**447–457.
47. **Wu, J., N. Suka, M. Carlson, and M. Grunstein.** 2001. *TUP1* utilizes histone H3/H2B-specific HDA1 deacetylase to repress gene activity in yeast. Mol. Cell **7:**117–126.
48. **Zaman, Z., A. Z. Ansari, S. S. Koh, R. Young, and M. Ptashne.** 2001. Interaction of a transcriptional repressor with the RNA polymerase II holoenzyme plays a crucial role in repression. Proc. Natl. Acad. Sci. USA **98:**2550–2554.
49. **Zhang, M., L. S. Rosenblum-Vos, C. V. Lowry, K. A. Boakye, and R. S. Zitomer.** 1991. A yeast protein with homology to the beta-subunit of G proteins is involved in control of heme-regulated and catabolite-repressed genes. Gene **97:**153–161.
50. **Zitomer, R. S., and C. V. Lowry.** 1992. Regulation of gene expression by oxygen in *Saccharomyces cerevisiae*. Microbiol. Rev. **56:**1–11.