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Direct Role for the Rpd3 Complex in Transcriptional Induction of the Anaerobic *DAN*/*TIR* Genes in Yeast▽‡

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Saccharomyces cerevisiae **adapts to hypoxia by expressing a large group of "anaerobic" genes. Among these, the eight *DAN*/*TIR* genes are regulated by the repressors Rox1 and Mot3 and the activator Upc2/Mox4. In attempting to identify factors recruited by the DNA binding repressor Mot3 to enhance repression of the *DAN*/*TIR* genes, we found that the histone deacetylase and global repressor complex, Rpd3-Sin3-Sap30, was not required for repression. Strikingly, the complex was instead required for activation. In addition, the histone H3 and H4 amino termini, which are targets of Rpd3, were also required for *DAN1* expression. Epistasis tests demonstrated that the Rpd3 complex is not required in the absence of the repressor Mot3. Furthermore, the Rpd3 complex was required for normal function and stable binding of the activator Upc2 at the *DAN1* promoter. Moreover, the Swi/Snf chromatin remodeling complex was strongly required for activation of *DAN1*, and chromatin immunoprecipitation analysis showed an Rpd3-dependent reduction in *DAN1* promoter-associated nucleosomes upon induction. Taken together, these data provide evidence that during anaerobiosis, the Rpd3 complex acts at the *DAN1* promoter to antagonize the chromatin-mediated repression caused by Mot3 and Rox1 and that chromatin remodeling by Swi/Snf is necessary for normal expression.**

The *Saccharomyces cerevisiae DAN*/*TIR* genes are among a large group of genes that are upregulated during adaptation to anaerobic growth (37, 59, 65, 66). These genes code for cell wall mannoproteins, which play a significant role in cell wall permeability. The kinetics of expression of these genes ranges from 30 minutes to 3 hours following the onset of anaerobiosis (1, 59). This suggests that as the cells descend towards anaerobiosis, certain requirements that are necessary for survival in that milieu are incrementally satisfied by specific alterations in gene expression. The importance of the *DAN*/*TIR* genes is further underlined by the fact that disruption of some of them, such as *TIR1*, *TIR3*, and *TIR4*, abrogates anaerobic growth (1), indicating that the corresponding proteins play essential functions during anaerobic adaptation. Moreover, it appears that a complex programmed cell wall remodeling occurs during adaptation to anaerobiosis, as shown by the fact that the major aerobic cell wall mannoproteins encoded by *CWP1* and *CWP2* are replaced by their anaerobic counterparts, encoded by the *DAN*/*TIR* genes, under those conditions (1).

The precise mechanisms by which the *DAN*/*TIR* genes are regulated are still being elucidated. We showed earlier that these genes are regulated by heme, which is synthesized only in the presence of oxygen, and by three DNA binding transcription factors (2, 13, 59). The activator Upc2 acts through a consensus site termed *AR1* to induce the expression of these genes in anaerobiosis. Upc2 was also identified as a regulator of the anaerobic sterol transport system (2, 75). It shares extensive homology with Ecm22 and with a *Candida albicans* protein (CaUpc2) (71). The repressors Rox1 and Mot3 function synergistically to efficiently repress these genes in aerobic cultures (60).

In attempting to identify factors which might be recruited by Mot3 to enhance repression of the *DAN*/*TIR* genes and the hypoxic gene *ANB1*, we tested the role of the Tup1/Ssn6 and Rpd3 complexes. These global repressors have been shown to be recruited by DNA binding repressors to remodel chromatin at several promoters (10, 15, 24, 26, 39, 44). We previously found that Tup1/Ssn6 is required for Rox1-mediated repression of the hypoxic gene *ANB1* but not for repression of *ANB1* by Mot3 (60). Tup1/Ssn6 also plays a role in repressing *DAN1*, but this again appears to occur via a separate mechanism from that used by Mot3 (60). We then tested the role of the histone deacetylase (HDAC) and global repressor Rpd3 in mediating repression of the anaerobic genes. We found that the Rpd3 complex was not required for repression. Surprisingly, it was instead required for the expression of all the *DAN*/*TIR* genes and the hypoxic gene *ANB1*, which are negatively regulated by heme and Mot3.

The *S. cerevisiae* Rpd3 histone deacetylase is a class I HDAC that has been demonstrated to regulate the expression of a large number of genes (7, 36, 51, 52, 56). Two distinct complexes of Rpd3 have been identified, namely, a small complex of 0.6 MDa (Rpd3S) and a large one of 1.2 MDa (Rpd3L) (12, 31). These two complexes share three subunits, namely, Rpd3, Sin3, and Ume1. Rco1 and Eaf3 were found to be specific for the small Rpd3 complex, which negatively regulates transcrip-

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† Deceased.

‡ We dedicate this paper to our friend and mentor Charles V. Lowry, who is greatly missed.

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TABLE 1. Strains used in this study

Strain	Genotype	Source or reference
NSY429	*MAT*α *ura3-52 leu2-3,112 trp1-289 his3*Δ*1* Δ(*hht1-hhf1*) (*hht2-hhf2*) pNS329 (*CEN TRP1 HHF1-HHT1*)	56
NSY430	*MAT*α *ura3-52 leu2-3,112 trp1-289 his3*Δ*1* Δ(*hht1-hhf1*) (*hht2-hhf2*) pNS329 (*CEN TRP1 HHF1-HHT1*) *rpd3*::*LEU2*	56
NSY438	*MAT*α *ura3-52 leu2-3,112 trp1-289 his3*Δ*1* Δ(*hht1-hhf1*) (*hht2-hhf2*) pNS338 [*CEN TRP1 hhf1-8* (H4Δ2-26) *HHT1*]	56
NSY458	*MAT*α *ura3-52 leu2-3,112 trp1-289 his3*Δ*1* Δ(*hht1-hhf1*) (*hht2-hhf2*) pNS358 [*CEN TRP1 HHF1 hht1-2* (H3Δ1-28)]	56
K699	*leu2-3,112 ura3-1 his3-11 trp1-1 can100*	16
PAY304	W303 *MAT*α *SIN3-HA-HIS3*	F. Posas
NOY847	*MAT ura3 his3 trp1 leu2-3,112 rrn5*Δ::*LEU2* Δ(*hht1-hhf1*) Δ(*hht2-hhf2*) pNOY434 [*HIS3 RRN5*-$(HA1)_3$-His_6] pNOY436 (*TRP1 HHT2 myc-HHF2*)	29
W303-1A	*MAT***a** *ura3-1 leu2-3,112 trp1-1 can1-100 ade2-1 his3-11,15* [*PSI*$^+$]	5
MAY6	*sin3*::*KanMX* (W303-1A)	5
MAY7	*rpd3*::*KanMX* (W303-1A)	5
MAY8	*sap30*::*KanMX* (W303-1A)	5
FY1339	*MAT***a** *ura3-52 his3*Δ*200 trp1*Δ*63*	23
FY2081	*MAT***a** *ura3-52 his3*Δ*200 trp1*Δ*63 MOT3-Myc18*::*TRP1*	23
Y4	*MAT***a** *his3*Δ*1 leu*Δ*0 met15*Δ*0 ura3*Δ*0 swi3*::*KanMX*	Resgen
Y5	*MAT***a** *his3*Δ*1 leu*Δ*0 met15*Δ*0 ura3*Δ*0 swi10*::*KanMX*	Resgen
Y6	*MAT***a** *his3*Δ*1 leu*Δ*0 met15*Δ*0 ura3*Δ*0 snf2*::*KanMX*	Resgen
Y7	*MAT***a** *his3*Δ*1 leu*Δ*0 met15*Δ*0 ura3*Δ*0 snf6*::*KanMX*	Resgen
Y41	*MAT*α *ura3-52 leu2-3,112 trp1-289 his3*Δ*1* Δ(*hht1-hhf1*) (*hht2-hhf2*) pMS329 (*CEN ARS ura3*Δ*0 HHT2-HHF1*)	This study
Y42	*MAT*α *ura3-52 leu2-3,112 trp1-289 his31* (*hht1-hhf1*) (*hht2-hhf2*) pMS329 (*CEN ARS ura3*Δ*0 HHT2-HHF1*) *rpd3*::*LEU2*	This study
Y56	Y41 *mot3*::*KanMX*	This study
Y57	Y42 *mot3*::*KanMX*	This study
Y72	Y41 *mot3*::*HIS3 rox1*::*KanMX*	This study
Y85	Y41 *mot3*::*HIS3*	This study
Y102	Y41 *MOT3*-Myc18	This study
Y103	Y42 *MOT3*-Myc18	This study

tional elongation by RNA polymerase II (PolII). The large Rpd3 complex, which includes Sap30, Pho23, Rxt1, Rxt2, Dep1, and Sds3, appears to be the promoter-targeting form of the two complexes. Rpd3 does not bind to DNA and is therefore recruited to promoters by DNA-binding transcription factors. For instance, Rpd3 is recruited by Ume6 to directly repress the expression of *INO1*, a gene involved in inositol biosynthesis (24). Furthermore, repression by Ash1 at the *HO* promoter is mediated by Rpd3 recruitment at these sites (11). Recently, Rpd3 was shown to activate transcription of the osmoresponsive gene *HSP12* when recruited to that promoter in response to activation by the mitogen-activated protein kinase Hog1 transcriptional activator (16). Thus, Rpd3 can function as an activator of transcription in addition to its usual role as a repressor.

Here we show that Rpd3, along with its targets, the N termini of histones H3 and H4, is required for the expression of the anaerobic *DAN*/*TIR* genes. We found that the Rpd3 complex is recruited during anaerobic growth. Genetic analysis shows that Rpd3 is dispensable in the absence of the repressor Mot3, implying a functional interaction between these two regulators. Chromatin immunoprecipitation (ChIP) analysis reveals that Rpd3 is required for nucleosome loss at the induced *DAN1* promoter and for stable binding of the activator Upc2. The chromatin remodeling complex Swi/Snf also plays a pivotal role in the regulation of these genes. Taken together, these observations define a novel chromatin-mediated regulatory mechanism responsible for *DAN*/*TIR* induction.

MATERIALS AND METHODS

Yeast strains. The strains used in this work are listed in Table 1. Strains Y41 and Y42 were derived from NYS429 and NSY430, respectively, as follows. NYS429 and NSY430 were transformed with a 4.5-kb EcoRI-BglII-PvuII fragment from the Trp blaster plasmid (4). The resulting Trp$^-$ Ura$^+$ colonies were screened for resistance to 5-fluoroorotic acid to test for the loss of the *URA3* marker.

Strains Y56 and Y57 were generated by transformation of Y41 and Y42, respectively, with a PCR fragment obtained from the proprietary *mot3*::*KanMX* strain derived from BY4741 (76). The knockout fragment was verified by PCR. Strain Y72 was made by transforming strain Y41 with a PCR fragment that was amplified from a *mot3*::*HIS3* strain (42), using primers homologous at positions −964 and +1880 to generate strain Y85. A *rox1*::*KanMX* disruption PCR fragment (76) was then introduced into Y85. Both knockout fragments were verified by PCR. Strains Y102 and Y103 were generated as follows. The *MOT3*-Myc_{18} PCR product was amplified from strain FY2081 (23), using primers homologous at +891 and +2330. The PCR fragment was transformed into strains Y41 and Y42 and then verified by PCR.

Plasmids. The plasmid YCp22*UPC2*(G888A) was described elsewhere (2). YCp33*RPD3* and YEp112*RPD3* were generated as follows. An XbaI-PstI PCR fragment containing the *RPD3* gene was obtained from NSY429 (56), using primers homologous at −994 and +1386. The PCR product was digested with XbaI and PstI and ligated into YCp33 and YEplac112 which had been digested with the same enzymes, generating YCp33*RPD3* and YEp112*RPD3*, respectively. The plasmids YCp33*RPD3*H188A and YEp112*RPD3*H188A were generated by excising a 0.7-kb BglII-MluI fragment from YEplac112LexA*RPD3*H188A (25) and subcloning it into YCp33*RPD3* and YEp112*RPD3*, respectively, which had been digested with the same enzymes.

YCp22 *UPC2*-HA_3 was constructed as follows. YCp22*UPC2* (2) was digested with HindIII and BglII and ligated with a linker oligonucleotide, AGCTTTGA GCGAGA or AACTCGCTCTCTAG. The new plasmid was digested with ClaI and ligated with another linker oligonucleotide, CGATGACTACTACTAA CTTTAGCGATTTCTCGAG or CGCTCGAGAAATCGCTAAAGTTAGT

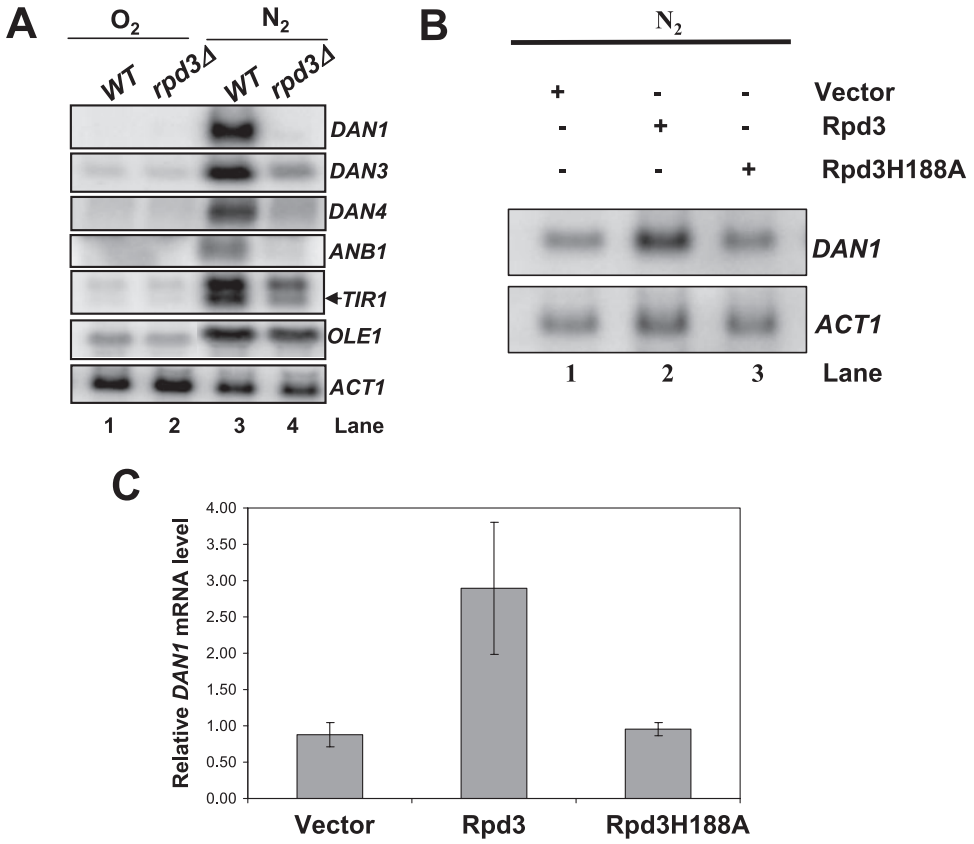

FIG. 1. Rpd3 is required for expression of anaerobic proteins. (A) Wild-type and *rpd3*Δ strains were grown aerobically or anaerobically for 2 h. RNAs were isolated for a Northern blot, which was hybridized with [^{32}P]dCTP-labeled probes for *DAN1*, *DAN3-4*, *TIR1*, *ANB1*, *OLE1*, and *ACT1*. The *TIR1* probe also hybridizes to another band, which corresponds to *TIR2* mRNA (2). (B) *rpd3*Δ yeast cells were transformed with the following plasmids: YCp33 (vector), YCp33Rpd3, and YCp33Rpd3H188A. These cells were grown anaerobically for 2 h in the appropriate medium. Total RNA was isolated and analyzed by Northern blotting, using probes for *DAN1* and *ACT1*. (C) Relative *DAN1* mRNA levels were quantified by phosphorimaging (Molecular Dynamics) and normalized to the *ACT1* internal loading control. The values represent the averages for two independent experiments, and standard deviations are indicated. Note that yeast cells were grown in Ura-deficient medium for the experiment of panel B, resulting in lower expression levels of *DAN1* than those for cells grown in YPD medium, as for panel A.

AGTAGTCAT. The modified plasmid was cut with XhoI and inserted with a three-hemagglutinin (HA_3) PCR fragment amplified from template pMPY-HA_3 (57), resulting in YCp22*UPC2HindIII*/*BglII*-HA_3. Finally, a *UPC2* fragment was excised from YCp22*UPC2*, using AgeI and NdeI, and ligated into YCp22*UPC2HindIII*/*BglII*-HA_3 which was digested with the same enzymes.

Cell growth, yeast transformation, and Northern analysis. Yeast cells were grown at 30°C under aerobic and anaerobic conditions in yeast extract-peptone-dextrose or synthetic complete medium. Anaerobic cultures were bubbled with high-purity nitrogen and harvested in late log phase after 2 h of anaerobic growth. Yeast transformation was performed according to the LiAc TRAFO (lithium acetate/single-stranded carrier DNA/polyethylene glycol) method as described previously (19). For Northern analysis, RNAs were extracted and analyzed as described previously (78). Briefly, 10 μg of RNA was run in agarose-formaldehyde gels and blotted onto nylon membranes in 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate). The membranes were then baked for 2 h and hybridized with probes labeled by random priming. Northern blots were quantitated on a Molecular Dynamics phosphorimager.

Western blotting. Western analysis was performed with whole-cell extracts that were prepared by glass bead lysis. Protein concentrations were determined by the Bradford assay (Bio-Rad). Twenty-microgram samples of denatured proteins in 5% 2-mercaptoethanol were fractionated in 10 to 12% polyacrylamide gels for 1 h at 120 V and transferred to Millipore polyvinylidene difluoride membranes at 30 V for 1 h. The membrane was blocked for 1 h at room temperature in BLOTTO A solution (100 mM Tris, pH 7.6, 0.1% Tween 20, 5% nonfat dry milk) and incubated overnight at 4°C with new BLOTTO A solution containing primary antibody. The blot was washed three times for 10 min each in TBST (100 mM Tris, pH 7.6, 0.1% Tween 20, 0.9% NaCl) and incubated with the secondary antibody, horseradish peroxidase-linked anti-rabbit immunoglobulin, for 1 h. The blot was again washed three times for 10 min each in TBST. Detection of the appropriate protein was revealed with an ECL detection kit and exposure to Kodak X-Omat radiographic film (Eastman Kodak Co., Rochester, NY).

ChIP. Chromatin immunoprecipitation was performed as previously described (18, 34). Briefly, formaldehyde cross-linked samples were sonicated to a size range of 0.2 to 0.6 kb. Immunoprecipitated (IP) samples were eluted according to standard protocols using the following antibodies: 20 μl hemagglutinin antibody (Y-11, SC-805; Santa Cruz Biotechnology), 20 μl c-Myc antibody (A-14, SC-789; Santa Cruz Biotechnology), 3 μl histone H3 antibody (06-755; Upstate Cell Signaling Solutions), and 4.0 μl acetylated histone H4 antibody (06-866; Upstate Cell Signaling Solutions). For analysis, 2.0 μl of IP DNA and 1.0 μl of a 1:100 dilution of input DNA were amplified by quantitative radioactive PCR for 22 cycles, and the products were separated in a 7.5% nondenaturing polyacrylamide gel. Each ChIP experiment was repeated two or three times, and multiple repeats of the same PCR were performed to test for pipetting errors. Quantitation was determined by using a Molecular Dynamics phosphorimager. Primers used for amplification are available upon request.

RESULTS

Rpd3 is required for the expression of anaerobic genes. In our effort to delineate the molecular mechanisms by which the *DAN*/*TIR* genes and the hypoxic gene *ANB1* are regulated, we reported earlier the isolation of Mot3 as a repressor of these

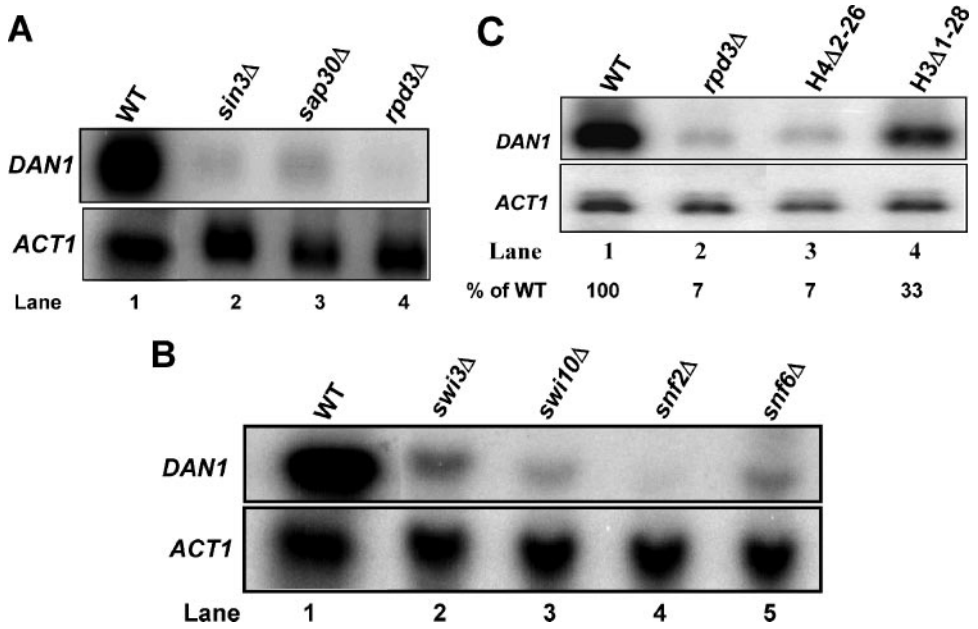

FIG. 2. The large Rpd3 complex and its targets are required for *DAN1* expression. Yeast strains were grown aerobically and shifted to anaerobic growth. (A) Northern blots for wild-type (WT), *sap30*Δ, *sin3*Δ, and *rpd3*Δ strains. (B) Northern blots for wild-type, *swi3*Δ, *swi10*Δ, *snf2*Δ, and *snf6*Δ strains. (C) Northern blots for wild-type and *rpd3*Δ strains and strains with N-terminal deletions of histones H4 (*hhf1*Δ*2-26*) and H3 (*hht1*Δ*1-28*).

heme-repressed genes (60). To elucidate the mechanism of repression by Mot3, we tested in that study the role of the Tup1/Ssn6 complex. This global repressor has been shown to be recruited by DNA binding repressors to remodel chromatin at several promoters (10, 39, 44, 62, 74, 77). We observed constitutive expression of the *DAN1* and *ANB1* genes in a *tup1*Δ/*ssn6*Δ strain, suggesting that the global repressor Tup1/Ssn6 is indeed required for efficient repression of the genes. However, we found that Tup1/Ssn6 is required for Rox1 repression of the *ANB1* gene but not for repression by Mot3 at that promoter (60). Ectopic expression of Rox1 and Mot3 in *tup1*Δ/*ssn6*Δ strains in anaerobic cultures showed that Rox1 can no longer repress, while Mot3 is still capable of repressing the expression of the *ANB1* gene (60).

We then tested whether the histone deacetylase and global repressor Rpd3 has a role in mediating repression of the anaerobic genes. We found that Rpd3 was not required for repression of the anaerobic genes. We did not observe constitutive expression of any of the anaerobic *DAN/TIR* genes or the hypoxic gene *ANB1* in an *rpd3*Δ strain in aerobic cultures (Fig. 1A, lanes 1 and 2). Surprisingly, Rpd3 was required for the expression of the anaerobic genes which are negatively regulated by Mot3 (Fig. 1A, lane 4). Interestingly, two anaerobically expressed genes, namely, *OLE1* (Fig. 1) and *TIP1* (not shown), which are not controlled by either heme or Mot3, were not regulated by Rpd3. These results implicate Rpd3 as part of the *DAN/TIR* and *ANB1* regulatory circuit responding to a heme-deficient milieu by inducing the activation of these anaerobic genes.

Enzymatic activity of Rpd3 is necessary for expression of the anaerobic genes. To determine whether the enzymatic activity of Rpd3 is needed for the expression of the anaerobic genes, we first grew yeast cells in the presence and absence of the histone deacetylase inhibitor trichostatin A (TSA). Northern blot analysis showed a significant reduction in expression of the anaerobic gene *DAN1* (data not shown), suggesting that Rpd3 function is indeed required for full activation of the *DAN/TIR* genes. However, TSA is not a specific inhibitor of Rpd3, as it also affects the activities of all class I and II histone deacetylases (67). Moreover, several studies have demonstrated that TSA affects a wide range of cellular processes, including apoptosis, differentiation, and DNA synthesis (30, 32, 69). Therefore, it is not clear whether the reduction in the expression of *DAN1* is due to the loss of deacetylase activity of Rpd3 or through other cellular abnormalities caused by the drug. To resolve the possible indirect effects of trichostatin A on *DAN1* expression, we utilized an enzymatically defective form of Rpd3 (Rpd3H188A) (25). *rpd3*Δ yeast strains transformed with catalytically inactive Rpd3, in contrast with wild-type Rpd3, could not suppress the noninducible phenotype of the *rpd3*Δ strain (Fig. 1B and C), implying that Rpd3's enzymatic activity is needed for transcriptional activation.

The large Rpd3 complex and its targets are involved in activation of anaerobic genes. To determine which Rpd3 complex (small or large) contributes to *DAN1* activation and to identify other chromatin-related factors that regulate *DAN1* expression, we tested more than 50 strains from a yeast knockout library (76). We observed a nearly absolute requirement for some of the known components of the Rpd3 complex (Rpd3, Sin3, and Sap30) (Fig. 2A) and a partial requirement for other factors, such as Pho23, Ume6, Sds3, and Ume1 (not shown). Since Sap30, Pho23, and Sds3 are components of the Rpd3L but not the Rpd3S complex (12, 31), these results strongly suggest that the large Rpd3 complex is the one required for the induction of *DAN1*. Some of the subunits of the Swi/Snf complex were also needed for *DAN1* expression (Fig. 2B). We also observed a partial requirement for a few factors in other categories (Taf14, Ada2, Ada3, Ccr4, and Caf130)

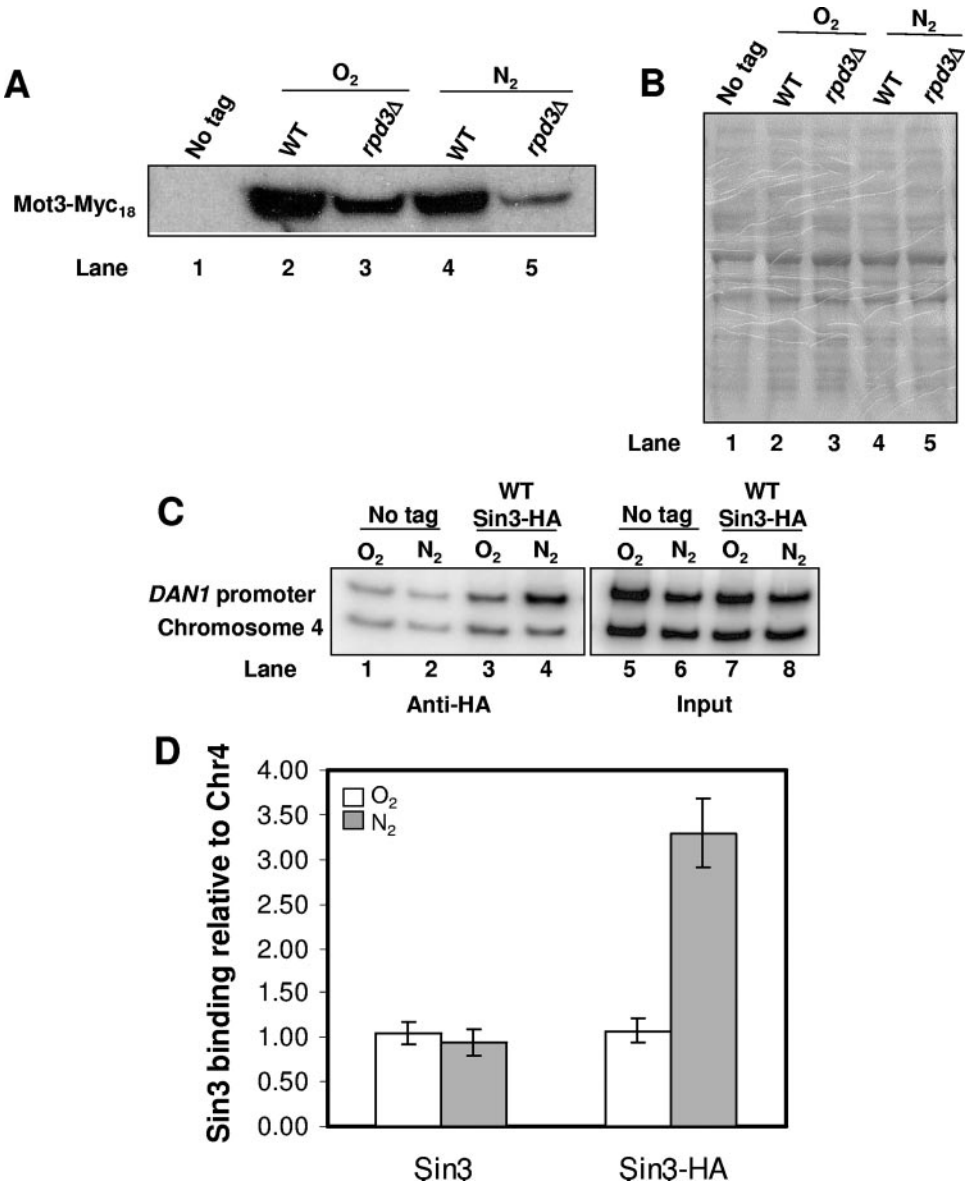

FIG. 3. The Rpd3 complex is directly involved in activation of the anaerobic genes. (A) Mot3-Myc_{18} (Y102 and Y103) and untagged (Y41) cells were grown aerobically or shifted to anaerobic growth for 2 h in the appropriate medium. Extracts prepared from these cells were subjected to immunoblotting with a monoclonal antibody against the Myc epitope. (B) To determine equal loading of the samples, the proteins blotted onto the nylon membrane were stained with Ponceau reagent prior to being blocked with milk. (C) Sin3-HA_3 (PAY304) and untagged (K699) cells were grown aerobically or shifted to anaerobic growth for 2 h in the appropriate medium and subjected to ChIP using anti-HA antibodies. Samples were used to amplify the *DAN1* regulatory region, using primers that span the activator Upc2 site, together with a control PCR that amplified a region of chromosome IV that is outside any open reading frames. The labeled PCR products were quantitated by using a PhosphorImager. (D) The graph represents the ratios of *DAN1* promoter IP samples to input samples relative to the ratio of chromosome IV (Chr4) IP samples to input samples. The values represent the averages for two independent experiments, and standard deviations are indicated.

(data not shown). These results suggest that Rpd3-mediated activation of *DAN1* occurs via the same Rpd3 complex previously identified as a global repressor and underscore the role of chromatin in anaerobic gene expression.

Since the amino termini of histones H3 and H4 are known substrates of Rpd3 (26, 53, 54), we hypothesized that these histone tails might be needed for *DAN1* expression. Northern blot analysis showed that yeast strains carrying N-terminal deletions of histone H4 (*hhf1Δ2-26*) and, to a lesser extent, histone H3 (*hht1Δ1-28*) showed substantial reductions in *DAN1* expression (Fig. 2C). We also found substantially reduced anaerobic expression of *DAN1* in a yeast strain expressing H4 with K5,8,12,16Q mutations (strain NSY491) (56; data not shown); we did not examine the H3(K4,9,14,18,23,27Q) mutant. These results, which are in contrast to the more typical upregulation caused by these mutations (43, 55, 68), indicate that the H3 and H4 amino termini are needed for activation and that the histone H4 tail is particularly important.

Rpd3 is directly involved in activation of *DAN1*. Rpd3 could regulate the expression of the anaerobic genes indirectly by

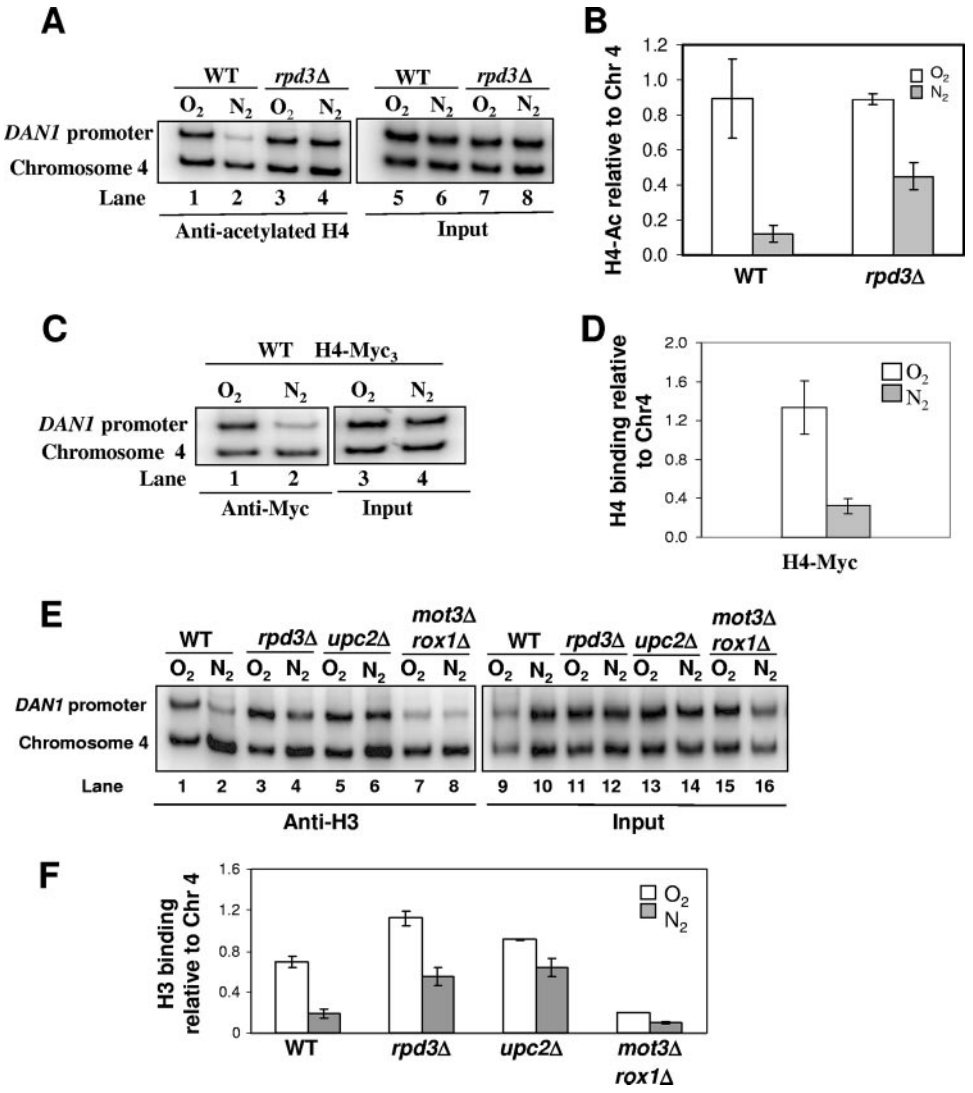

FIG. 4. The Rpd3 complex is needed to remodel chromatin at the DAN1 promoter during anaerobic growth. (A) Wild-type and *rpd3*Δ strains. (C) Myc_3-H4 strain. (E) Wild-type, *rpd3*Δ, *upc2*Δ, and *mot3*Δ *rox1*Δ strains. Yeast strains were grown aerobically or shifted to anaerobic growth for 2 h. Chromatin was cross-linked with formaldehyde and immunoprecipitated with the indicated antibodies. The samples were analyzed as described in the legend to Fig. 3. The graphs (B, D, and F) were generated as described in the legend to Fig. 3. The values represent the averages for two independent experiments, and standard deviations are indicated. Chr4, chromosome IV.

inhibiting the expression of a repressor, particularly Mot3. However, Western blot analysis showed no increase, and in fact a decrease, in Mot3 protein levels during anaerobic growth in an *rpd3*Δ strain (Fig. 3A and B, compare lanes 4 and 5). Since this result does not rule out regulation by Rpd3 of an unidentified repressor, we next tested whether the Rpd3 complex is recruited to the *DAN1* promoter during hypoxia. For this purpose, we performed ChIP experiments, using HA_3-tagged Sin3 as a surrogate for Rpd3, which is somewhat refractory to the standard cross-linking protocol used for ChIP (36). Since Sin3 and Rpd3 are intimately associated in the Rpd3 repressor complex, Sin3 binding is expected to provide an accurate reflection of Rpd3 binding (16, 24). We found 3.5-fold enrichment of Sin3 binding and, by inference, the Rpd3 complex at the UAS region of the *DAN1* promoter in anaerobic compared to aerobic cultures (Fig. 3C, compare lanes 3 and 4, and Fig. 3D). These results strongly suggest that Rpd3 is directly involved in transcriptional activation of the anaerobic genes.

The Rpd3 complex is required for nucleosome loss during *DAN1* expression. Having established that Rpd3 is present at the *DAN1* promoter during induction and that both its enzymatic activities and histone targets are needed for transcriptional activation, we hypothesized that one possible mechanism by which Rpd3 participates in the regulation of the anaerobic genes is by deacetylating its histone targets, particularly histone H4. We monitored levels of acetylated histone H4 at the *DAN1* promoter in the induced and repressed states by ChIP analysis. We observed a drastic reduction in the level of acetylated histone H4 in anaerobic wild-type cells (Fig. 4A, compare lanes 1 and 2). This reduction was greatly diminished in the *rpd3*Δ mutant (Fig. 4A, compare lanes 2 and 4). We next

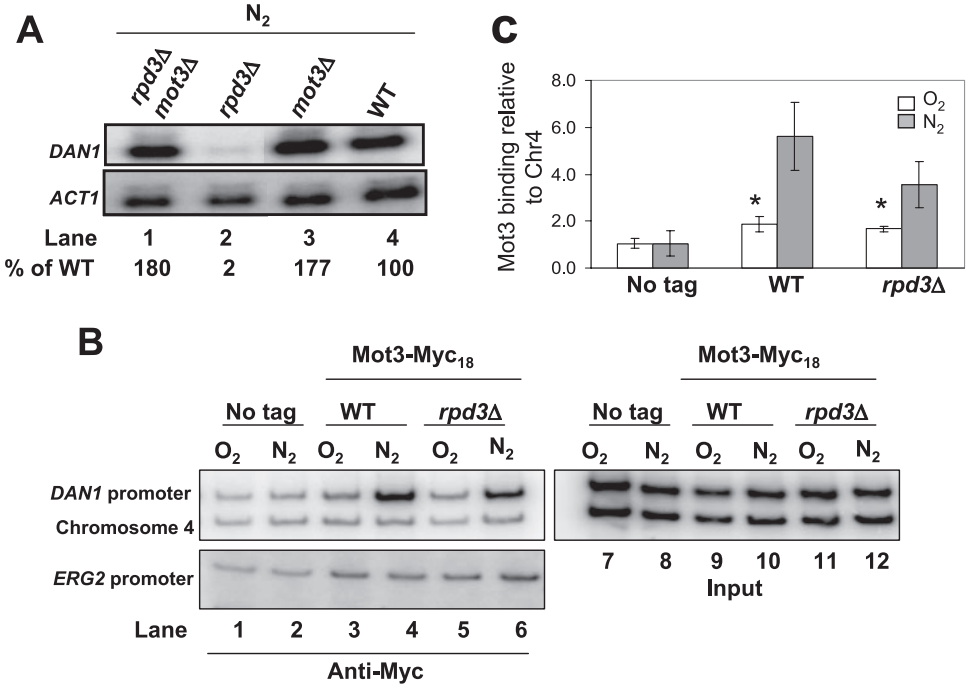

FIG. 5. Rpd3 antagonizes Mot3 function. (A) Northern blots from wild-type, *rpd3*Δ, *mot3*Δ, and *mot3*Δ *rpd3*Δ strains grown aerobically and shifted to anaerobic growth for 2 h. (B and C) Mot3-Myc_{18} (Y102 and Y103) and untagged (Y41) cells were grown aerobically or shifted to anaerobic growth for 2 h. Chromatin was cross-linked with formaldehyde and immunoprecipitated with the indicated antibodies. The samples were analyzed as described in the legend to Fig. 3. *ERG2* was used as a positive control (23). The values represent the averages for four independent experiments, and standard deviations are indicated. Asterisks indicate *P* values of <0.01 by Student's *t* test for the difference between the wild-type or *rpd3*Δ samples and the untagged, uninduced samples. Chr4, chromosome IV.

investigated whether the reduction in acetylated histone H4 at the *DAN1* promoter during anaerobic induction could reflect a loss in overall nucleosome density. For this purpose, we performed ChIP using Myc-tagged histone H4 as well as a pan-histone H3 antibody that recognizes histone H3 regardless of its modification state. These experiments revealed a substantial decrease in histone H4 (Fig. 4C) and histone H3 (Fig. 4E, compare lanes 1 and 2) protein levels at the *DAN1* promoter in wild-type cells under anaerobic conditions. This loss in histone density was dependent on Rpd3 (Fig. 4E, compare lanes 2 and 4). This suggests that the Rpd3 complex acts upstream of a requisite chromatin remodeling step during *DAN1* activation. We also demonstrated a correlation between *DAN1* activation and a loss in histone density, as shown by the constitutive and noninducible mutant *mot3*Δ*rox1*Δ and *upc2*Δ strains, respectively (Fig. 4E, lanes 5 to 8).

Rpd3 is needed to counteract Mot3-mediated repression of *DAN1*. The anaerobic genes that require Rpd3 for activation are negatively regulated by heme and Mot3 (13, 27, 59, 60). Therefore, we decided to probe for genetic interactions between Rpd3, Mot3, and other factors which regulate the expression of the anaerobic genes. We first asked whether the constitutive expression of *DAN1* in *mot3*Δ yeast cells in aerobic cultures required Rpd3. We found that the constitutive phenotype was epistatic, i.e., the *DAN1* mRNA level in the double mutant strain (*rpd3*Δ *mot3*Δ) was the same as that in the single mutant *mot3*Δ strain (data not shown), suggesting that Mot3 and Rpd3 are involved in the same transcriptional regulatory function or pathway. In anaerobic cultures, the inhibitory effect of the *RPD3* deletion was suppressed in *mot3*Δ yeast cells, indicating that the Rpd3 complex is required to counteract repression by Mot3 (Fig. 5A). This was surprising because Mot3 had been assumed to repress only under aerobic conditions, and it implies that Rpd3 antagonizes Mot3 repression following the onset of hypoxia.

We next investigated whether the Rpd3 complex antagonizes Mot3 binding at the *DAN1* promoter following the onset of hypoxia. We found that in wild-type cells, Mot3 is present at the *DAN1* promoter under both repressed and induced conditions (Fig. 5B, compare lanes 3 and 4, and Fig. 5C, where Student's *t* test indicates that ChIP of Myc-tagged Mot3 in aerobic wild-type or *rpd3*Δ yeast cells is greater than that in an untagged sample [$P < 0.01$]). This binding was not affected when *RPD3* was deleted (Fig. 5B, compare lanes 4 and 6). We concluded that Rpd3 antagonizes Mot3 function, but not by inhibiting Mot3 binding at the *DAN1* promoter.

Rpd3 is required for Upc2 to induce *DAN1* and for stable Upc2 binding at the *DAN1* promoter. The fact that both Upc2 (2) and the Rpd3 complex are required for the expression of the anaerobic genes suggests some kind of functional interaction between these two factors. It is possible that one factor facilitates the binding of the other. To determine whether they functionally interact, we performed an epistasis test. When combining the dominant constitutive phenotype of the Upc2G888D allele with the poorly inducible phenotype of the *rpd3*Δ strain, we found that *DAN1* expression in the double mutant was strongly reduced compared to that in the single mutant Upc2G888D in aerobic cultures (Fig. 6A, compare lanes 3 and 4). This indicates

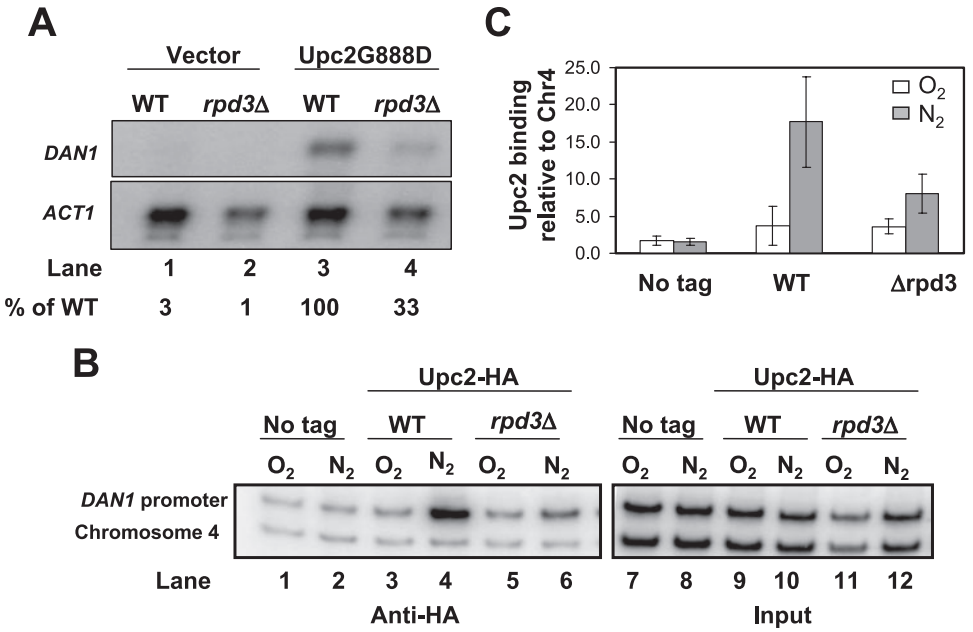

FIG. 6. Rpd3 is needed for stable binding of the activator Upc2. (A) *rpd3*Δ and wild-type strains were transformed with plasmid YCp33UPC2G888D and grown aerobically. Total RNA was isolated and analyzed by Northern blotting, using probes prepared from *DAN1* and *ACT1*. (B) Wild-type and *rpd3*Δ strains were transformed with the plasmid YCp22UPC2-HA and grown aerobically or shifted to anaerobic growth for 2 h. Chromatin was cross-linked with formaldehyde and immunoprecipitated with the indicated antibodies. The samples were analyzed as described in the legend to Fig. 3. (C) The graph was generated as described in the legend to Fig. 3. The values represent the averages for two independent experiments, and standard deviations are indicated. The picture in panel A was assembled from images cut from the same blot. Chr4, chromosome IV.

that Upc2 requires the Rpd3 complex for full activation of *DAN1* expression.

One function of Upc2 is to bind to the *DAN1* promoter and recruit the transcriptional machinery to turn on the expression of the anaerobic genes. We hypothesized that the Rpd3 complex is required for stable binding of Upc2 at the *DAN1* promoter, based on the observation that Rpd3 is needed for Upc2 function. Using ChIP analysis, we found that the activator Upc2, in wild-type cells, binds preferentially to the *DAN1* promoter in anaerobic cultures (Fig. 6B, compare lanes 3 and 4, and Fig. 6C) and that binding is attenuated in cells lacking Rpd3 (Fig. 6B, compare lanes 4 and 6, and Fig. 6C). This experiment further demonstrates a functional relationship between these two regulators and suggests that Rpd3, by counteracting the Mot3-Rox1 repression ensemble, allows stable binding of the activator Upc2 at the *DAN1* promoter during anaerobiosis.

DISCUSSION

In this study, we provide evidence that the Rpd3L histone deacetylase complex is not required for repression of the anaerobic *DAN*/*TIR* genes but, surprisingly, is required for their activation. Our results indicate that Rpd3 enzymatic activity, as well as its principal targets, the histone H3 and H4 tails, is needed for *DAN1* anaerobic expression. Moreover, we found that the Rpd3 complex is present at the *DAN1* promoter under inducing conditions and is needed for chromatin remodeling and stable binding of the activator Upc2 at the *DAN1* promoter. Finally, we show that a principal function of Rpd3 in *DAN1* activation is to overcome repression mediated by Mot3. Together, these results point to a novel mechanism for *DAN*/*TIR* gene activation in which Rpd3 acts to antagonize Mot3 repression and facilitate Upc2 binding and activation.

A positive role for Rpd3 in transcriptional regulation was previously reported. Rpd3 has been shown to counteract transcriptional silencing and is required for the activation of genes involved in cell wall biosynthesis (17, 70). However, the previous works did not rule out indirect effects of Rpd3 and provided no mechanistic insights on how Rpd3 functions to facilitate gene expression. More recently, De Nadal et al. (16) provided evidence for a direct role for the Rpd3/Sin3 complex in the activation of the osmoresponsive genes. Rpd3 was shown to physically interact with the activator Hog1, and Sin3 was found to bind directly to the activated *HSP12* promoter. Furthermore, reduced levels of promoter-associated acetylated histone H4 and increased RNA PolII binding were observed only in the presence of Rpd3. However, the reduced levels of acetylated histone H4 observed in *rpd3*Δ yeast may have been due to nucleosome loss, as we observed for *DAN1* (Fig. 4). Whether Rpd3 activates osmoregulated genes by counteracting repressor function or facilitating activator binding and nucleosome depletion, as we show for *DAN1*, is currently unknown. Other histone deacetylases, particularly HDAC1 and HDAC7, have also been shown to play a positive role in transcriptional regulation (28, 49).

The histone deacetylase requirement for the expression of *DAN1* appears to be specific for Rpd3, since neither overexpression of other histone deacetylases, such as Hos2 or Sir2, in *rpd3*Δ yeast nor deleting those factors and others, such as

Hda1, Hos1, Hos3, Sir1, and Sir3-4, in a wild-type strain affects the expression level of *DAN1* (data not shown). The histone deacetylase Hos2 belongs to the same family as Rpd3 (52, 53) and has been shown to play positive and direct roles in regulation of the *GAL* genes (72) and in promoting the yeast long terminal repeat-retrotransposon Ty1 integration (45), but it does not play a role in *DAN1* regulation.

The histone H3 and H4 tails appear to play essential but distinct roles in the regulation of *DAN1*. This was inferred from Northern blot results, which show a more pronounced effect on *DAN1* expression upon deletion of the histone H4 tail than that with deletion of the histone H3 tail, as well as from epistasis analyses with Upc2 and Mot3 (data not shown). Previous work has shown that, in contrast to the case for *DAN1*, induction of the anaerobic gene *ANB1*, which is not in the *DAN/TIR* family, does not require the histone H3 and H4 amino termini (27), although it does require Rpd3 (Fig. 1). Further work will be needed to clarify the reason for this difference and to determine whether Rpd3 contributes to *DAN1* and *ANB1* activation by the same or distinct mechanisms.

Although both histone H3 and H4 tails are required for full expression of *DAN1* in anaerobic cultures (Fig. 2A), histones are strongly depleted from the *DAN1* promoter under inducing conditions (Fig. 4). Similar observations have been made for the induced *PHO5* and *GAL1-10* promoters (8, 9, 33, 38, 40), and an inverse relationship has been reported between histone density and binding of RNA PolII (58). It is likely that the histone H3 and H4 tails play a role during the early stages of *DAN1* activation. For example, they may facilitate binding of Rpd3 and/or the Swi/Snf complex during activation. In addition, it remains possible that deacetylation of nonhistone proteins, such as Mot3, by Rpd3 at these promoters may be involved in turning on the expression of the anaerobic genes.

We observed Mot3 binding at the *DAN1* promoter under both induced and repressed conditions, and this binding was independent of Rpd3 (Fig. 5). This finding is consistent with our Western blot data showing that Rpd3 does not indirectly regulate *DAN1* by repressing Mot3 expression (Fig. 3A). It appears that more Mot3 protein is bound to the *DAN1* promoter in anaerobic than aerobic cultures. This seems counterintuitive but could be due to masking of the epitope by other bound factors, including nucleosomes, under aerobic conditions. These results are in contrast to another report which showed Mot3 bound to the *ANB1* promoter only under aerobic conditions (44). At present, we do not understand the reason for these contrasting results. Other reports have shown repressors bound to particular promoters in both induced and repressed states (14, 46, 48, 73). In some instances, these repressors behave as activators that can recruit the transcription machinery. It is unclear at this juncture what role Mot3 might play in anaerobic induction. One possibility is that Mot3 binding under activated conditions could facilitate a rapid and efficient return to the repressed state, as speculated for the Sko1-Cyc8-Tup1 repression complex (48).

A loss of Rpd3 results in a reduced level of Mot3 (Fig. 3A). We currently have no explanation for this finding and do not know whether it is a direct or indirect effect. The drastic decreases in Mot3 protein levels observed in *rpd3*Δ strains in anaerobic cultures were probably the result of two distinct inhibitory mechanisms affecting Mot3 expression, namely, a lack of oxygen and a lack of Rpd3. We previously demonstrated that oxygen is required for *MOT3* expression (60), and in this study, we show a reduction in the Mot3 protein level in the absence of oxygen (Fig. 3, compare lanes 2 and 4). In the absence of both oxygen and Rpd3, these two inhibitory mechanisms function synergistically to efficiently decrease Mot3 production (Fig. 3, compare lanes 2 and 4 with lane 5).

The strong requirement for the Swi/Snf components in *DAN1* activation (Fig. 2C), combined with the decrease in histone density, suggests that the Swi/Snf complex may remove nucleosomes in *trans* from this promoter. Nucleosome disruption upon activation has also been observed at other yeast promoters, but those found previously to undergo nucleosome depletion upon activation are not as strongly Swi/Snf dependent as *DAN1* (8, 33, 38, 40, 50, 58). Thus, *DAN1* appears to represent the best candidate identified so far for having nucleosome depletion catalyzed by the Swi/Snf complex in vivo. Other promoters (*SUC2* and *PHO8*) undergo Swi/Snf-dependent chromatin remodeling upon activation, but histone loss has not been shown to occur at these promoters (20, 22). We also found that Swi/Snf is needed to antagonize the repressive effects of Mot3 and Rox1 (data not shown). This may reflect a general requirement for Swi/Snf at genes under the control of global repressors (for example, *SUC2*, *INO1*, and *RNR3*) (22, 47, 61).

One question that arises is how Rpd3, which deacetylates histones, and the Swi/Snf remodeling complex can both be required for *DAN1* activation in light of evidence that the Swi/Snf complex is recruited to acetylated histones under inducing conditions (3, 21, 41, 64). One possibility is that deacetylation of histones or other factors by Rpd3 may act as a platform for efficient binding by the activator Upc2, which then recruits the Swi/Snf complex to trigger nucleosomal eviction. Such roles for acetylation and deacetylation and other posttranslational modifications in the recruitment of activators have been proposed before (6, 35, 63).

Thus, at the *DAN1* promoter, instead of organizing repressive chromatin, Rpd3 is essential for events that lead to dismantling of nucleosomes to facilitate binding of the transcriptional machinery. Based on these results, we propose that Rpd3 is recruited to the *DAN1* promoter under strict anaerobic conditions. The presence of Rpd3 at the promoter counteracts the function of the repressor Mot3, which leads to stable binding of the activator Upc2. Upc2 then recruits the chromatin remodeling complex Swi/Snf to reorganize chromatin, thereby facilitating the binding of the transcriptional machinery, which results in the activation of gene expression.

The participation of the Rpd3 complex in the expression of the anaerobic genes reported here is of special interest for two main reasons. First, the fact that it is recruited during anaerobiosis clearly indicates that the response of the anaerobic genes to oxygen depletion is regulated by chromatin. Second, the paradoxical role of an HDAC complex in activation provides new insight into the relationship between histone modifications and gene regulation.

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