MOLECULAR AND CELLULAR BIOLOGY, Apr. 2007, p. 3199–3210 Vol. 27, No. 8
0270-7306/07/$08.00+0 doi:10.1128/MCB.02311-06

Histone Deacetylases RPD3 and HOS2 Regulate the Transcriptional Activation of DNA Damage-Inducible Genes▽

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Received 11 December 2006/Returned for modification 22 January 2007/Accepted 29 January 2007

DNA microarray and genetic studies of *Saccharomyces cerevisiae* have demonstrated that histone deacetylases (HDACs) are required for transcriptional activation and repression, but the mechanism by which they activate transcription remains poorly understood. We show that two HDACs, *RPD3* and *HOS2*, are required for the activation of DNA damage-inducible genes *RNR3* and *HUG1*. Using mutants specific for the Rpd3L complex, we show that the complex is responsible for regulating *RNR3*. Furthermore, unlike what was described for the GAL genes, Rpd3L regulates the activation of *RNR3* by deacetylating nucleosomes at the promoter, not at the open reading frame. Rpd3 is recruited to the upstream repression sequence of *RNR3*, which surprisingly does not require Tup1 or Crt1. Chromatin remodeling and TFIID recruitment are largely unaffected in the Δ*rpd3*/Δ*hos2* mutant, but the recruitment of RNA polymerase II is strongly reduced, arguing that Rpd3 and Hos2 regulate later stages in the assembly of the preinitiation complex or facilitate multiple rounds of polymerase recruitment. Furthermore, the histone H4 acetyltransferase Esa1 is required for the activation of *RNR3* and *HUG1*. Thus, reduced or unregulated constitutive histone H4 acetylation is detrimental to promoter activity, suggesting that HDAC-dependent mechanisms are in place to reset promoters to allow high levels of transcription.

Although the correlation between histone modifications and gene expression was established many years ago (2), the underlying mechanism by which they affect transcription is still largely unknown. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) function in an antagonistic manner to regulate the balance of histone acetylation and gene activity (25, 37, 42). It is widely accepted that histone acetylation by HAT correlates with gene expression, while histone deacetylation by HDACs is associated with gene repression (25, 37, 42).

Histone deacetylases catalyze the removal of acetyl groups from the amino-terminal tails of the core histones, making chromatin inaccessible to the transcriptional machinery (11, 25, 42). Two families of HDACs are found in yeast (*Saccharomyces cerevisiae*), and each family is classified based upon sequence homology among its members. Five HDACs, namely Hda1, Rpd3, Hos1, Hos2, and Hos3, belong to one family, and the other includes Sir2 and Hst1 to Hst4 (25). Two mechanisms have been proposed for how HDACs regulate transcription: targeted and nontargeted. The targeting mechanism involves the direct recruitment of HDACs to promoters by DNA binding proteins or corepressors, such as Ume6 or Tup1, respectively (21, 22, 23, 36, 38, 46, 47). This mechanism results in targeted deacetylation, spanning approximately two nucleosomes over the DNA binding site (22, 38, 47). The second mechanism is poorly understood and results in untargeted, genome-wide histone deacetylation (26, 44, 47), including deacetylation within coding regions. The mechanism of targeted versus global deacetylation was illuminated partly by the discovery of two Rpd3-containing complexes, Rpd3L and Rpd3S (7, 23). Rpd3L was found to be responsible for the targeted deacetylation of the promoters of genes and contains the DNA binding proteins Ume6 and Ash1 (7). Rpd3S, on the other hand, is responsible for deacetylating chromatin within the coding regions of genes and suppresses intragenic transcription. The recruitment of Rpd3S is mediated by the methylation of K36 of histone H3 by the Set2 methyltransferase through the chromodomain of Eaf3 (7, 20, 23).

Historically, HDACs have been considered repressors of gene expression, but recent reports indicate that Rpd3 and Hos2 are required for transcriptional activation. Rpd3 associates with actively expressed genes and is recruited to stress-regulated genes (12, 24, 36, 48). Furthermore, Hos2 is required for the activation of *GAL* genes and *INO1* and deacetylates chromatin within (and is recruited to) the open reading frames (ORFs) of activated genes (45). It was proposed that Hos2 is required to reverse the effects of transcriptional activation within the ORFs of genes to allow for multiple rounds of transcription (25, 45).

In an effort to understand how histone tail modifications regulate gene expression, we examined the requirement for HDACs in regulating DNA damage-inducible genes, specifically *RNR3* and *HUG1*. Our results suggest that *RPD3* and *HOS2* play redundant roles in the transcriptional activation of *RNR3* and *HUG1*. We further show that Rpd3L is recruited to the upstream repression sequences (URS) when the gene is activated. We provide evidence that inhibiting histone acetylation by using HAT mutants or causing constitutive acetylation by mutating HDACs results in activation defects. We

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▽ Published ahead of print on 12 February 2007.

TABLE 1. List of strains used in this study

Strain	Description
BY4705	*MAT*α *ade2*Δ::*hisG HIS3-*Δ*200 LEU2-*Δ*0 ura3-*Δ*0 trp1-*Δ*63 LYS2-*Δ*0 met15-*Δ*0*
JR415	BY4705 *MAT*α Δ*crt1*::*LEU2*
JR460	BY4705 *MAT***a** Δ*hda1*::*HIS3*
JR461	BY4705 *MAT***a** Δ*rpd3*::*LEU2*
JR462	BY4705 *MAT***a** Δ*hos1*::*URA3*
JR463	BY4705 *MAT***a** Δ*hos2*::*LYS2*
JR464	BY4705 *MAT***a** Δ*hda1*::*HIS3*
JR465	BY4705 *MAT***a** Δ*hda1*::*HIS3* Δ*hos1*::*URA3*
JR466	BY4705 *MAT***a** Δ*hda1*::*HIS3* Δ*hos2*::*LYS2*
JR467	BY4705 *MAT***a** Δ*rpd3*::*LEU2* Δ*hos1*::*URA3*
JR468	BY4705 *MAT***a** Δ*rpd3*::*LEU2* Δ*hos2*::*LYS2*
JR469	BY4705 *MAT***a** Δ*hos1*::*URA3* Δ*hos2*::*LYS2*
JR470	BY4705 *MAT***a** Δ*hda1*::*HIS3* Δ*rpd3*::*LEU2* Δ*hos1*::*URA3*
JR471	BY4705 *MAT***a** Δ*hda1*::*HIS3* Δ*rpd3*::*LEU2* Δ*hos2*::*LYS2*
JR472	BY4705 *MAT***a** Δ*hda1*::*HIS3* Δ*hos1*::*URA3* Δ*hos2*::*LYS2*
JR473	BY4705 *MAT***a** Δ*rpd3*::*LEU2* Δ*hos1*::*URA3* Δ*hos2*::*LYS2*
JR474	BY4705 *MAT***a** Δ*hda1*::*HIS3* Δ*rpd3*::*LEU2* Δ*hos1*::*URA3* Δ*hos2*::*LYS2*
JR511	BY4705 *MAT*α Δ*tup1*::*HIS3*
JR507	BY4705 *MAT*α Δ*ssn6*::*LEU2*
LPY3498	*MAT***a** ESA1 *HIS3-*Δ*200 LEU2-3,112 trp1-*Δ*1 URA3-52*
LPY3500	*MAT***a** *esa1*-L254P Δ*esa1*::*URA3*
LPY3430	*MAT***a** *esa1*-L327S Δ*esa1*::*URA3*
LPY3291	*MAT***a** *esa1*Δ::*HIS3* [pLP863, *esa1-*Δ*414 TRP1/CEN*]
JR826	LPY3498, Δ*gcn5*::KanMx
JR827	LPY3500 Δ*gcn5*::KanMx
JR828	LPY3430 Δ*gcn5*::KanMx
JR829	LPY3291 Δ*gcn5*::KanMx
JR943	BY4705 *MAT***a** Δ*rpd3*::*LEU2* Δ*crt1*::*TRP1*
JR944	BY4705 *MAT***a** Δ*hos2*::*LYS2* Δ*crt1*::*TRP1*
JR945	BY4705 *MAT***a** Δ*rpd3*::*LEU2* Δ*hos2*::*LYS2* Δ*crt1*::*TRP1*
JR946	BY4705 *MAT***a** Δ*rpd3*::*LEU2* Δ*hos2*::*LYS2* Δ*tup1*::*HIS3*
JR947	BY4705 *MAT*α Δ*set1*::*URA3*
JR948	BY4705 *MAT*α *RPD3-9*Myc::*HIS3*
JR949	BY4705 *MAT*α *HOS2-9*Myc::*HIS3*
JR950	BY4705 *MAT***a** Δ*rpd3*::*LEU2* Δ*hos2*::*LYS2* Δ*ssn6*::*HIS3*
JR951	BY4705 *MAT***a** Δ*tup1*::*URA3* RPD3-9Myc::*HIS3*
JR952	BY4705 *MAT***a** Δ*crt1*::*TRP1* RPD3-9Myc::*HIS3*
JR953	BY4705 *MAT***a** Δ*tup1*::*URA3* HOS2-9Myc::*HIS3*
JR954	BY4705 *MAT***a** Δ*crt1*::*TRP1* HOS2-9Myc::*HIS3*
JR955	BY4705 *MAT***a** *rpd3* H150A/H151A *hos2*H196A/H197A
JR958	BY4705 *MAT***a** Δ*hos2*::*LYS2 rpd3* H150A/H151A
JR1017	BY4705 *MAT*α Δ*sds3*::KanMx
JR1018	BY4705 *MAT*α Δ*rco1*::KanMx
JR1019	BY4705 *MAT*α Δ*set2*::KanMx
JR1021	BY4705 *MAT*α Δ*sds3*::KanMx Δ*hos2*::*LYS2*
JR1023	BY4705 *MAT*α Δ*rco1*::KanMx Δ*hos2*::*LYS2*
JR1024	BY4705 *MAT*α Δ*set2*::KanMx Δ*hos2*::*LYS2*
JR1027	LPY3498 Δ*sas3*::KanMx
JR1028	LPY3500 Δ*sas3*::KanMx
JR1029	LPY3291 Δ*sas3*::KanMx

propose that HDACs maintain a balance of histone acetylation and deacetylation at active promoters, which is required for multiple rounds of transcription.

MATERIALS AND METHODS

Yeast strains and genetic manipulations. The strains used in this study are listed in Table 1. Deletion mutants were constructed using PCR-based gene deletion cassettes to carry out one-step gene replacement as described earlier (6). Deletions were first identified by PCR analysis and then verified by Southern blotting. Strains were grown in rich medium (YPAD) containing 1% yeast extract, 2% peptone, 20 μg/ml adenine sulfate, 2% dextrose at 30°C. *RPD3* was PCR amplified from the yeast genomic DNA, digested with restriction enzymes KpnI and EagI, and cloned into pRS406. HOS2 was cloned into the SpeI site of pRS404. These plasmids were then used to construct catalytically dead mutants by oligonucleotide site-directed mutagenesis using the QuikChange mutagenesis procedure (Strategene, La Jolla, CA). H150A/H151A and H196A/H197A double amino acid substitutions were introduced into *RPD3* and *HOS2*, respectively. Mutations were confirmed by DNA sequencing. The plasmids were digested with restriction endonuclease and transformed into the corresponding null strains JR461 and JR463. The resulting strains contained the mutants inserted into the chromosomal loci of *RPD3* and *HOS2*. The Δ*gcn5*/*esa1* double mutants were constructed by a method of one-step gene replacement in the *ESA1* mutants, which was described previously (8). The histone H4 mutants and wild-type strains were described previously (14).

RNA analysis and chromatin mapping. Cells were grown to an optical density at 600 nm of 0.6 to 0.8 at 30°C, and an aliquot was removed for the untreated sample. Methyl methanesulfonate (MMS) was added to the remaining culture to a concentration of 0.03% for 2.5 h, unless indicated otherwise. RNA isolation was carried out as described earlier (34), and *RNR3*, *HUG1*, and *scR1* were detected by Northern blotting. Micrococcal nuclease mapping of nucleosome positions was carried out as described previously (52).

Cross-linking and ChIP assay. The chromatin immunoprecipitation (ChIP) assay was performed essentially as described previously (28, 40). Briefly, 200 ml of yeast cultures was grown in YPAD to an optical density at 600 nm of 0.8. Cultures were induced with MMS (0.03% vol/vol) for 2.5 h before cross-linking with formaldehyde (1% vol/vol) for 15 min at room temperature. Formaldehyde cross-linking was quenched by the addition of glycine (125 mM). Cells were harvested and washed twice with Tris-buffered saline, cell extracts were prepared, and the chromatin was sonicated to a length of 200 to 400 base pairs of DNA. Rpd3 cross-linking was performed using two cross-linking agents as described previously (45), except that the formaldehyde cross-linking step was reduced to 15 min. Cells were washed twice with ice-cold phosphate-buffered saline (PBS), resuspended in ice-cold PBS containing 10 mM dimethyl adipimidate-HCl (Pierce, Rockford, IL) and 0.25% (vol/vol) dimethyl sulfoxide, and incubated at room temperature for 45 min with shaking. Cells were then washed twice with ice-cold PBS and resuspended in 1% (vol/vol) formaldehyde in ice-cold PBS for 15 min at room temperature, followed by the addition of 2.5 M glycine to a final concentration of 125 mM for 10 min. Cells were then washed with ice-cold PBS twice and stored at −80°C. Polyclonal antibodies used in this study were described previously (49, 50, 51). Immunoprecipitated and input DNA were analyzed by semiquantitative PCR analysis with primers spanning the core promoter, upstream region, and downstream coding regions of *RNR3*. Coordinates are as indicated (see Fig. 4A). Primer sequences are available upon request. The PCR products were analyzed on 2% agarose gels, stained with ethidium bromide, scanned using the Typhoon system (GE Life Sciences, Piscataway, NJ), and quantified using ImageQuant software. ChIP assays were repeated a minimum of three times using different chromatin preparations.

RESULTS

Histone deacetylases are required for the activation of DNA damage-inducible genes. We have shown that deleting *HDA1* causes constitutive acetylation of histone H3 at DNA damage-inducible genes, but only a weak level of derepression (49). Since HDACs display redundancy at other loci and have different histone tail specificities in vivo (43, 46, 47), we examined the responses of *RNR3* and *HUG1* to deletions of multiple HDAC genes in combination. *RNR3* and *HUG1*/*NORF5* are part of the same regulon and are controlled by the same transcription factors (3, 49, 50). The deletion of HDACs, even in multiple combinations, caused very modest levels of *RNR3* derepression, significantly below those of Δ*ssn6*, Δ*tup1*, or Δ*crt1* mutants (Fig. 1A). The strongest derepression of *RNR3* was observed in the Δ*hda1* strain (~2.5-fold), and progressive deletion of additional HDACs failed to significantly increase expression. Next, we examined modifications of histones and the formation of the preinitiation complex (PIC) in representative mutants (Fig. 1B). Even though progressive deletion of HDAC genes led to increased H3 and H4 acetylation, the level

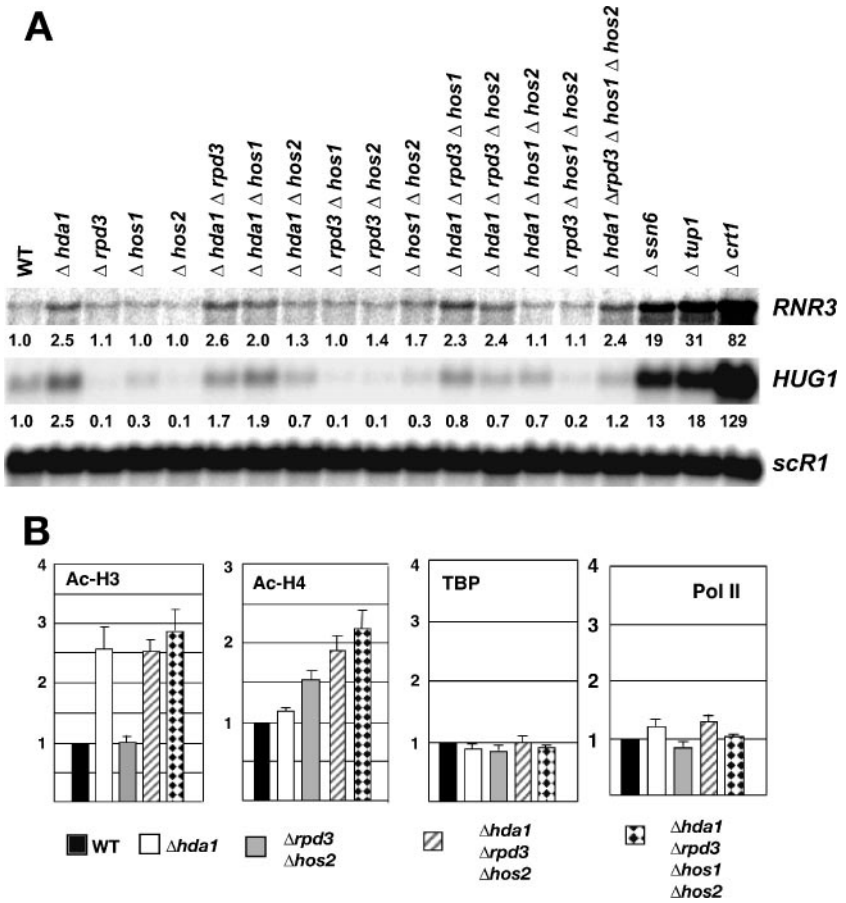

FIG. 1. (A) Deletion of HDACs causes subtle derepression of DNA damage-inducible genes. Northern blots of *RNR3* and *HUG1* mRNA from the mutant strains indicated are shown. Quantification is shown below each blot and has been expressed relative to the signal from the untreated wild-type (WT) cells, which was arbitrarily set to 1.0. RNA levels were normalized to the signal of *scR1*, a loading control. (B) Examination of histone modifications and transcription factor association in multiple HDAC mutants. ChIP was used to monitor histone H3 (K9 and K14) and H4 acetylation (penta-Ac) levels and the recruitment of TBP and RNA polymerase II (8WG16). Data are expressed relative to corresponding levels in wild-type cells, which were arbitrarily set to 1. Data are presented as the averages and standard deviations (error bars) of three independent experiments.

of the recruitment of TATA binding protein (TBP) and RNA polymerase II (Pol II) was not significantly increased. Thus, the failure to observe large increases in *RNR3* mRNA is unlikely to result from defects in elongation or mRNA processing or stability, which theoretically could reduce steady-state mRNA levels. Comparing the patterns of histone acetylation in the mutants indicated that *HDA1* is largely responsible for deacetylating H3, while Rpd3 functions redundantly with Hos1 and Hos2 to deacetylate H4 (Fig. 1B). Thus, the lack of derepression in the mutants cannot be explained by redundancy between the different HDACs.

We next examined whether HDACs are required for the activation of *RNR3* and *HUG1*. Both Rpd3 and Hos2 act in a redundant manner to activate galactose-induced and osmotic stress-responsive genes (12, 45). To address this, we investigated DNA damage-induced transcription in HDAC mutants using MMS. As shown in Fig. 2A, the deletion of *HOS2* caused a weak but reproducible reduction (~65% of the wild type) in *RNR3* transcription. The activation of *RNR3* was not strongly affected in the other single mutants. However, *HUG1* transcription was strongly reduced in both Δ*rpd3* and Δ*hos2* single mutants (~20% of the wild type) (Fig. 2A, compare lane 2 with lanes 6 and 10). We noted previously that despite being regulated by Crt1 and Tup1-Ssn6, *HUG1* and *RNR3* respond somewhat differently to transcription factor mutations (see below) (49). Analysis of the double mutants clearly showed that the combination of Δ*rpd3* and Δ*hos2* mutations caused strong defects in the activation of *RNR3* and *HUG1* (Fig. 2B, lane 12). This effect seems quite specific because other double and triple mutants lacking the Δ*rpd3*/Δ*hos2* combination, such as Δ*hda1*/Δ*rpd3*/Δ*hos1* and Δ*hda1*/Δ*hos1*/Δ*hos2*, show much milder defects in the activation of *RNR3* and *HUG1* (Fig. 2C). Thus, these two HDACs play a redundant, positive role in the transcriptional activation of DNA damage-regulated genes. We have also verified that the activation of *SUC2*, another Ssn6-Tup1-regulated gene, requires *RPD3* and *HOS2* for activation (data not shown). Given that multiple genes regulated by distinct pathways and cellular signals require *RPD3* and *HOS2* for activation, HDACs likely affect a central aspect of transcriptional activation. To investigate the mechanism by which HDACs regulate transcriptional activation, we focused on the Δ*rpd3*/Δ*hos2* double mutant.

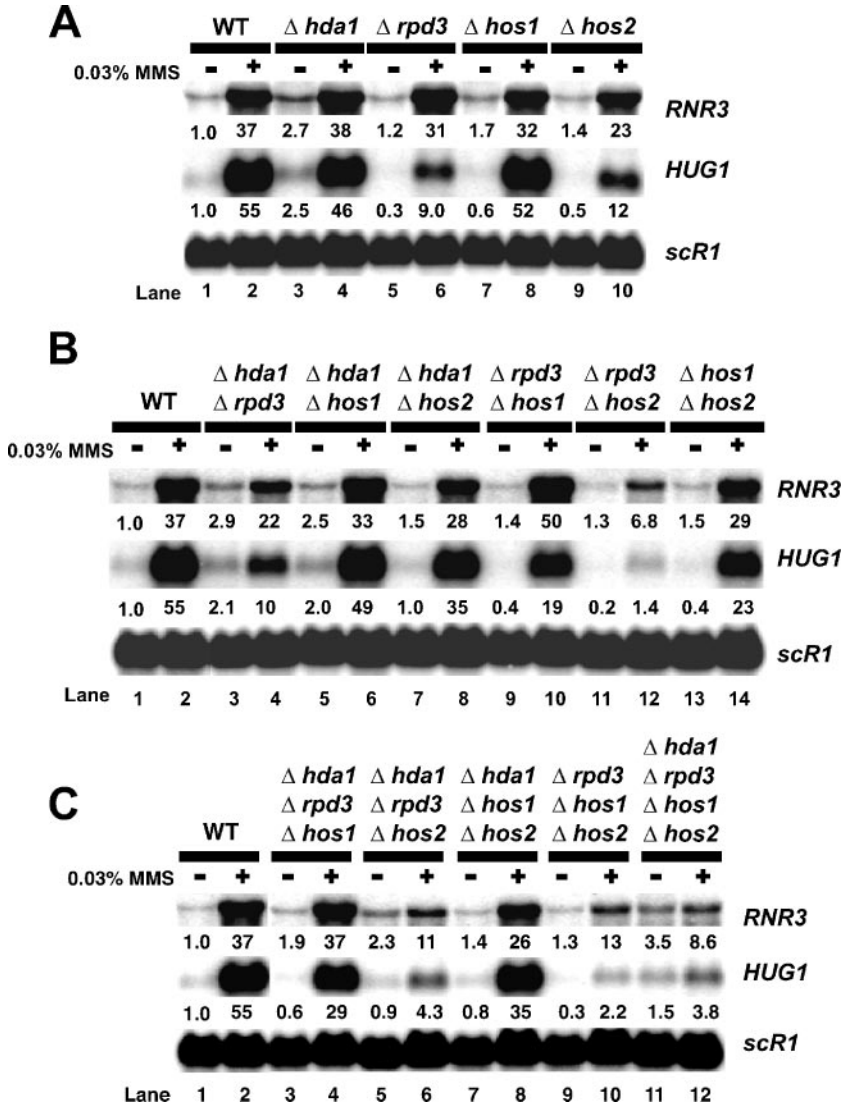

FIG. 2. Activation of *RNR3* and *HUG1* is defective in certain HDAC mutants. Northern blot analysis of *RNR3* and *HUG1* in untreated (−) and MMS-treated (+) (A) single, (B) double, and (C) multiple HDAC mutants. Strains were treated with 0.03% MMS for 2.5 h at 30°C. Data are expressed relative to corresponding levels in untreated wild-type (WT) cells and were corrected for the signal of the loading control (*scR1*).

***RPD3* and *HOS2* directly regulate *RNR3*.** The results thus far suggest that *RPD3* and *HOS2* play an essential role in gene activation. However, the derepression of *RNR3* requires the checkpoint-dependent release of Crt1 and Tup1 from the promoter (18, 50, 51), and the transcription defect could be caused by the failure of the cells to respond to damage signals and release the repressors from the promoter. To rule out this possibility, we examined the recruitment of Crt1 and Tup1 to *RNR3*. The wild type and the Δ*rpd3*/Δ*hos2* double mutant were untreated or treated with 0.03% MMS and subjected to the ChIP assay. Polyclonal antibodies to Crt1 and Tup1 were used to immunoprecipitate chromatin. The results in Fig. 3 show that Crt1 is recruited to an equal level in these two strains in the absence of DNA damage and is released from the promoter in treated cells. The level of Crt1 cross-linking is slightly higher in the double mutant after MMS treatment, however. Next, we examined the cross-linking of Tup1 to *RNR3*. Delet-

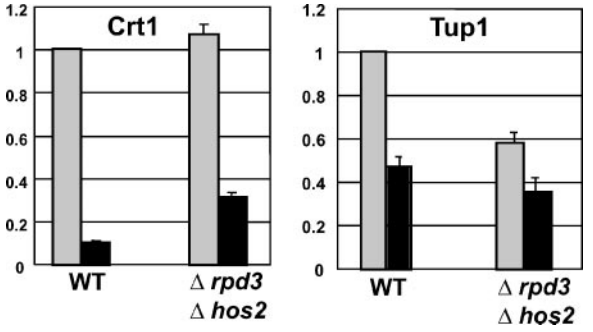

FIG. 3. Repressor and corepressor release in Δ*rpd3*/Δ*hos2* mutant cells. ChIP monitoring the association of Crt1 and Tup1 with the URS of *RNR3*. Results from untreated cells (gray bars) and cells treated with 0.03% MMS (black bars) are displayed. Data are presented as the averages and standard deviations (error bars) of at least three independent experiments. WT, wild type.

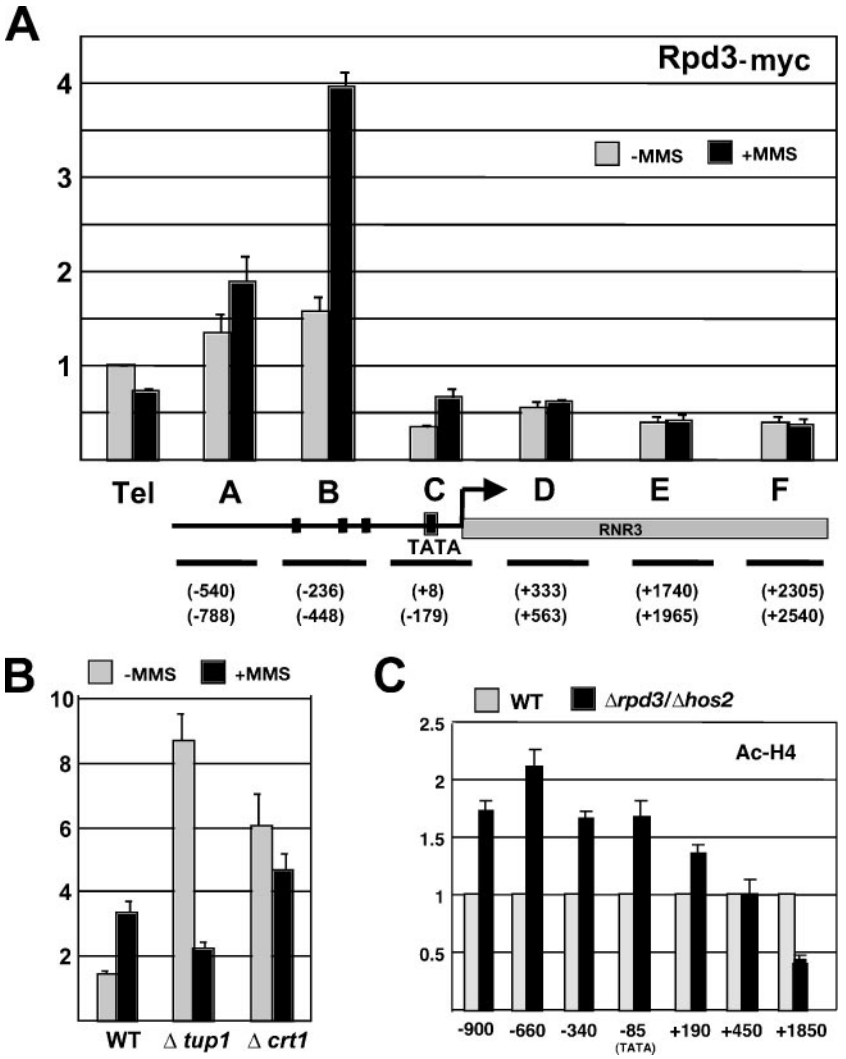

FIG. 4. Rpd3 recruitment to the URS of *RNR3* coincides with transcription. (A) Cross-linking of Rpd3-myc over *RNR3* in a wild-type (WT) strain. Results from untreated cells (gray bars) and cells treated with 0.03% MMS (black bars) are displayed. A schematic representation of the PCR fragments from *RNR3* is given. The severalfold increase in Rpd3 cross-linking is represented with respect to the IP signal from a subtelomeric region, which was arbitrarily set as 1.0. (B) Rpd3-myc association in Δ*tup1* and Δ*crt1* cells over the URS region (−236/−448). Gray and black bars represent data from untreated and MMS-treated cells, respectively. (C) The levels of histone H4 acetylation were examined across *RNR3* in Δ*rpd3*/Δ*hos2* mutant cells. Data are presented relative to corresponding levels in wild-type cells. Error bars indicate standard deviations.

ing both *RPD3* and *HOS2* caused a small reduction in Tup1 cross-linking in untreated cells, consistent with data of others showing that deleting HDACs weakens Tup1 association with promoters (9). Importantly, treating the mutant with MMS resulted in a reduction in Tup1 cross-linking to a level very similar to that observed in wild-type cells. This result further supports the notion that Ssn6-Tup1-mediated repression is alleviated in the Δ*rpd3*/Δ*hos2* double mutant by MMS treatment, indicating that the DNA damage signal transduction pathway is intact in the Δ*rpd3*/Δ*hos2* mutant. Collectively, these results suggest that the impaired activation in the double mutant is not caused by defects in repressor and corepressor release from the promoter and is likely to be direct.

If Rpd3 and Hos2 directly participate in the activation of *RNR3*, we expect that they will be recruited when the gene is transcribed. The recruitment of an epitope-tagged version of *RPD3* (Rpd3-myc) to *RNR3* was examined by using a modified ChIP procedure (45). As shown in Fig. 4A, Rpd3 cross-linking in uninduced cells over the URS of *RNR3* was approximately 1.5-fold above the levels detected within a subtelomeric region (Tel) and was significantly increased when cells were treated with the DNA-damaging agent MMS. Interestingly, cross-linking of Rpd3 was the strongest over the URS, with significantly lower levels detected over the promoter and ORF. Thus, Rpd3 is predominantly recruited to the regulatory region of *RNR3* and its recruitment coincides with transcription. Unfortunately, we have not been successful in detecting the cross-linking of Hos2 to *RNR3*. However, given that Rpd3 and Hos2 cooperate in the regulation of DNA damage-inducible genes, we expect that Hos2 is present at *RNR3* and the failure to cross-link it is due to technical limitations.

Tup1 and Crt1 cross-linking is strongest over the URS re-

gion of *RNR3* (50), and Rpd3 and Hos2 have been shown to interact with the Ssn6-Tup1 corepressor complex (10, 46), suggesting that Ssn6-Tup1 or Crt1 may regulate Rpd3 recruitment. Next, we examined whether Crt1 or Tup1 is required for the recruitment of Rpd3 to *RNR3*. Rpd3 cross-linking was examined in strains deleted of either *CRT1* or *TUP1* before and after MMS treatment. Deleting *CRT1* or *TUP1* resulted in constitutive Rpd3 recruitment in untreated cells (Fig. 4B), suggesting that Tup1 and Crt1 inhibit Rpd3 binding under the repressed condition. Surprisingly, we found that treating the Δ*tup1* mutant with MMS resulted in a significant reduction in Rpd3 cross-linking, but the level of cross-linking was still greater than that observed in untreated wild-type cells. In contrast, treating the Δ*crt1* mutant with MMS resulted in only a small decrease in Rpd3 cross-linking, which is most likely insignificant. The cause of this difference is unclear, but we know that deleting *TUP1* only partially derepresses *RNR3* and the partial derepression in the Δ*tup1* mutant cannot be increased further by MMS treatment (51). The combined effects of reduced release of Crt1 (51) and reduced recruitment of Rpd3 (Fig. 4B) may account for the lack of inducibility of *RNR3* in the Δ*tup1* mutant.

Rpd3 cross-linking to *RNR3* was restricted to the URS, while Rpd3 and Hos2 cross-link to the ORF of *GAL1* to deacetylate chromatin within the ORF (45). This suggests that Rpd3 and Hos2 regulate histone acetylation within the promoter of *RNR3*. This result was confirmed by performing the ChIP assay in the double mutant using an antibody recognizing acetylated histone H4. Figure 4C shows that deleting *RPD3* and *HOS2* increased histone H4 acetylation over the promoter-URS region of *RNR3*, but no increase in acetylation was detected within the ORF. Only a small increase was observed at the very beginning of the ORF (+190), which may be attributed to the size of the sheared chromatin (200 to 400 bp). We did observe elevated histone H4 acetylation significantly upstream of the peak of Rpd3 cross-linking, for example, over the −900 and −600 region. Possible explanations for this observation are that Hos2 has a broader localization pattern than Rpd3 does or that transcription factors controlling the upstream gene recruit HDACs.

Rpd3L targets deacetylation to the promoter region. Recent reports indicate that two Rpd3-containing complexes, Rpd3S and Rpd3L, are required for widespread and targeted histone deacetylation, respectively (7, 23). Rpd3S contains fewer subunits and cooperates with *SET2* to maintain histone deacetylation within the ORFs of genes. Rpd3L, on the other hand, contains additional subunits and targets deacetylation to promoters. We next determined which Rpd3 complex regulates *RNR3*. Sds3 is a subunit unique to Rpd3L, and it is required for the structural integrity of the complex (7). Rco1 is unique to Rpd3S and is required for its assembly (7). We constructed strains containing a deletion of these genes individually or in combination with a Δ*hos2* mutation. Deleting *RCO1* had no significant effect on the activation of *RNR3*, and the Δ*rco1*/Δ*hos2* double mutant activated the gene to a level equal to that of the Δ*hos2* mutant (Fig. 5A), suggesting that Rpd3S is not required for the activation of *RNR3*. Set2-dependent methylation of histone H3 is required for the recruitment of Rpd3S to the ORFs of genes (7, 23). We found that deleting *SET2* individually or in the Δ*hos2* mutant did not reduce transcription to a level any lower than that of the Δ*hos2* single mutant. In contrast, deleting *SDS3* led to a reduction in transcription equal to that of the Δ*rpd3* single mutant and transcription in the Δ*sds3*/Δ*hos2* double mutant was severely impaired. *HUG1* responded the same to these mutations (data not shown). The defect in the activation of the Δ*sds3*/Δ*hos2* double mutant was very similar to that for the Δ*rpd3*/Δ*hos2* mutant. Rpd3L contains two sequence-specific DNA binding proteins, Ash1 and Ume6, but deleting either gene does not affect the activation of *RNR3* or *HUG1* (data not shown).

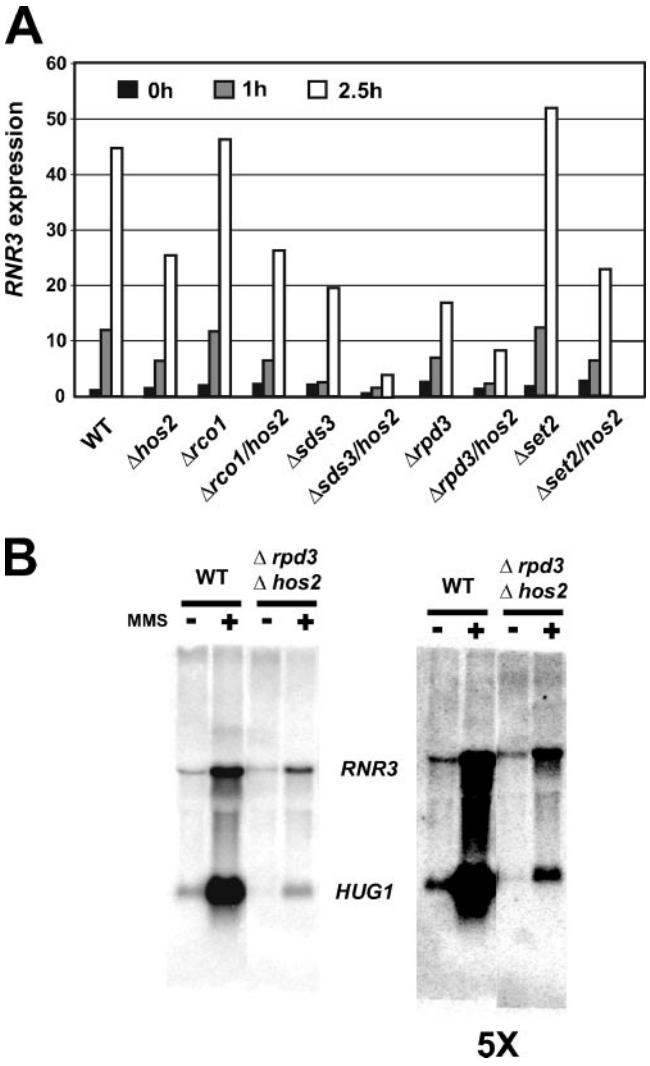

FIG. 5. Rpd3L regulates *RNR3* expression. (A) Quantification of of *RNR3* expression detected by Northern blotting in untreated (−) cells (black bars) and in cells treated (+) with 0.03% MMS for 1 h (gray bars) and 2.5 h (white bars). Data are expressed relative to the signal from the untreated wild-type (WT) cells, which was arbitrarily set to 1.0. Rco1 and Sds3 are unique to Rpd3S and Rpd3L, respectively. (B) Presentation of the Northern blotting of the entire gel. The panel on the right (5×) shows an overexposure of the same blot.

Rpd3S is required to suppress transcription from cryptic promoters within the coding sequence of genes, and the mutation of subunits in the complex causes RNA transcripts to be produced from within the ORF (7). To further demonstrate that Rpd3S does not regulate *RNR3*, we looked for the pres-

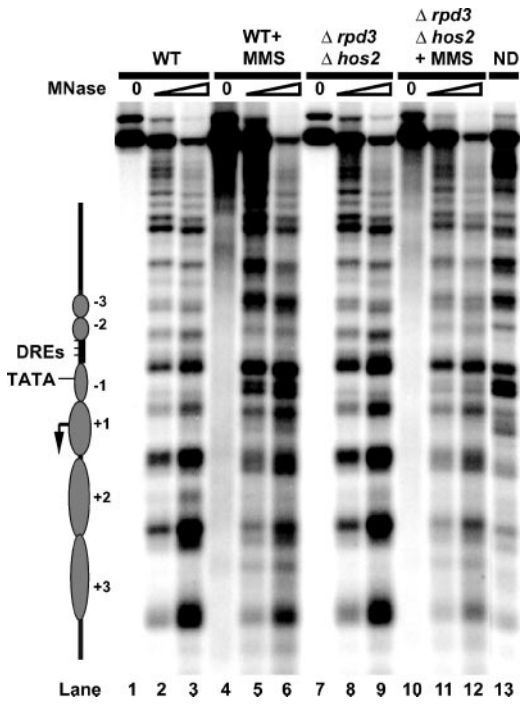

FIG. 6. Rpd3 and Hos2 are not required for nucleosome positioning or disruption at *RNR3.* MNase mapping of nucleosome positioning at the *RNR3* promoter in wild-type (WT) and Δ*rpd3*/Δ*hos2* cells. Cells were untreated (−) or treated (+) with 0.03% MMS for 2.5 h prior to harvesting of the cells for nucleus isolation. The positions of nucleosomes are illustrated on the left side of the panel and are supported by detailed mapping studies (28). DRE, damage response element; ND, naked DNA digest.

ence of alternative transcripts in the Δ*rpd3*/Δ*hos2* mutant. The examination of the Northern blot, even upon overexposure, did not reveal the presence of alternative transcripts (Fig. 5B). Collectively, the data strongly suggest that the Rpd3L complex is responsible for regulating *RNR3* transcription by targeting deacetylation to the promoter.

Chromatin remodeling and histone modifications at the *RNR3* promoter. Previous work from our lab has shown that the *RNR3* promoter undergoes extensive remodeling, which requires TAF_{II}s, components of the general transcription machinery, and SWI/SNF (27, 28, 40). Defects in the activation of *RNR3* in the Δ*rpd3*/Δ*hos2* double mutant prompted us to examine whether the remodeling of *RNR3* requires these two HDACs. MNase mapping was performed, and we found that deleting *RPD3* and *HOS2* had no effect on nucleosome positioning in the absence of DNA damage (Fig. 6). An examination of the pattern obtained from the Δ*rpd3*/Δ*hos2* mutant treated with MMS did not reveal any obvious qualitative defects in the remodeling of the *RNR3* promoter. Thus, Rpd3 and Hos2 are not required for maintaining nucleosome positioning or the DNA damage-induced disruption in positioning, suggesting that HDACs are required for a step after nucleosome disruption.

It is difficult to obtain quantitative measurements of chromatin remodeling by using MNase mapping techniques. Furthermore, the same pattern can be obtained if nucleosome positioning is disrupted (randomization) or if nucleosomes are evicted from the promoter. Another method used to assess the type and extent of chromatin remodeling is to measure the cross-linking of histone H3 to the promoter (1, 5, 35). The activation and remodeling of *RNR3* coincide with a reduction in the cross-linking of histone H4 over the promoter, presumably the result of nucleosome eviction (50). The ChIP assay was conducted using antibodies to the core domain of histone H3 in wild-type and mutant cells (Fig. 7A, left panel). The results show that even in the absence of DNA damage, the level of cross-linking of H3 to *RNR3* was somewhat reduced in the double mutant. This suggests that there may be subtle differences in chromatin structure that were not revealed by MNase mapping. Importantly, H3 cross-linking was reduced in both the wild type and the mutant when the cells were treated with MMS. The overall level of cross-linking of H3 in MMS-treated mutant cells was also slightly lower than that observed in wild-type cells; thus, the overall reductions in H3 cross-linking (nucleosome eviction) were similar between the two strains.

Gcn5-dependent acetylation of histone H3 is required for maximal levels of *RNR3* transcription (40). We next examined the levels of histone H3 and H4 acetylation at the *RNR3* promoter in the wild-type and mutant cells. Since histone H3 cross-linking is reduced by MMS treatment, the levels of acetylation were corrected for the amount of histone H3 cross-linking. As described previously, histone H3 acetylation is dramatically increased upon MMS treatment (Fig. 7A, middle panel). In addition, the activation of the gene correlates with an increase in histone H4 acetylation (Fig. 7A, right panel). An examination of the levels of histone H3 acetylation in the double mutant revealed that deleting *RPD3* and *HOS2* had only a small effect on H3 acetylation, indicating that HDACs are not required for SAGA-dependent acetylation (Fig. 7A, middle panel). Deleting both HDACs caused constitutive histone H4 acetylation, which could be increased somewhat by MMS treatment. Thus, while *RPD3* and *HOS2* are required to suppress H4 acetylation in the absence of DNA damage, they are not required for the increased histone acetylation in response to DNA damage. Collectively, the data indicate that HDACs are required for a step after chromatin remodeling, histone acetylation, and eviction.

HDACs are required for a late stage of PIC assembly. Previous work from our lab has shown that the derepression of *RNR3* requires the recruitment of TAF_{II}s, general transcription factors, and SWI/SNF (27, 40). We next identified the steps in activation that require Rpd3 and Hos2. We examined the recruitment of the TFIID subunits TBP and TAF1, Swi2, and RNA polymerase II in wild-type and Δ*rpd3*/Δ*hos2* cells. No significant difference in the cross-linking of TBP, TAF1, SWI2, or Pol II was observed in untreated Δ*rpd3*/Δ*hos2* and wild-type cells; all were recruited to a low, background level (Fig. 7B). Treatment of wild-type cells with MMS resulted in a significant increase in the recruitment of TFIID, SWI/SNF, and RNA Pol II to *RNR3*. Consistent with the low levels of transcription in the double mutant, the recruitment of RNA polymerase II was severely compromised in these cells. Comparatively, the reduction in the cross-linking of TFIID and SWI/SNF was much less severe than that of RNA polymerase II. In the HDAC mutant, the recruitment of TBP, TAF1, and Swi2 was reduced to about 60, 70, and 80% of the levels in wild-type cells, respectively. The recruitment of SWI/SNF is consistent with the nucleo-

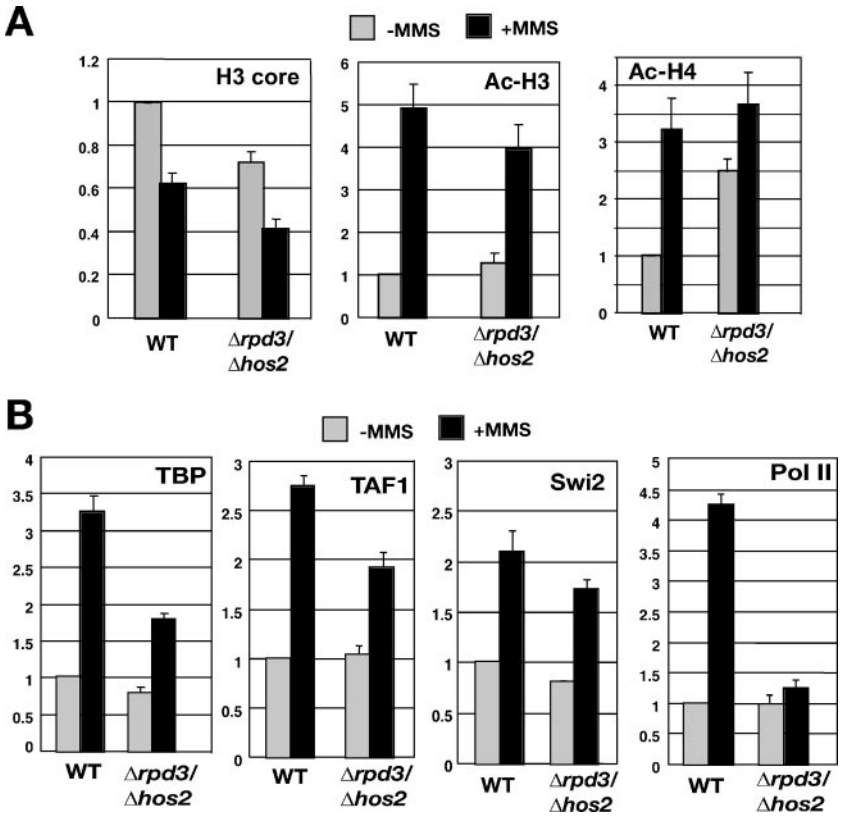

FIG. 7. Rpd3 and Hos2 are required for Pol II recruitment. (A) Examination of H3 cross-linking and histone modifications in wild-type (WT) and Δ*rpd3*/Δ*hos2* cells. Polyclonal antisera recognizing the core domain of H3 (left), diacetylated H3 (middle), and H4 acetylated at K5 (right) were used. The levels of H3 and H4 acetylation were normalized to the level of H3 cross-linking. Results from untreated cells (gray bars) and cells treated with 0.03% MMS (black bars) are displayed. (B) Analysis of PIC formation and SWI/SNF recruitment. Immunoprecipitations were carried out using polyclonal antibodies against TBP, TAF1, and Swi2 and a monoclonal antibody against RNA polymerase II (8WG16). Occupancy was measured at the *RNR3* promoter. Error bars indicate standard deviations.

some mapping and histone cross-linking data, suggesting that chromatin remodeling of *RNR3* is only weakly, if at all, affected in the mutant (Fig. 6 and 7A). While it is difficult to assess whether the recruitment of SWI/SNF and that of TFIID in the mutant are different from each other, clearly the recruitment of RNA Pol II is much more strongly affected than that of either SWI/SNF or TFIID. These results suggest that Rpd3 and Hos2 act downstream of chromatin remodeling and TFIID recruitment and regulate a late step in transcription initiation. Furthermore, the data also argue that the remodeling of the promoter and the recruitment of SWI/SNF can occur in the absence of RNA polymerase II, separating the functions of TAF_{II}s and Pol II in SWI/SNF recruitment (40). Our previous work could not separate the functions of Pol II and TFIID in the recruitment of SWI/SNF because inactivating a temperature-sensitive mutant of any general transcription factor caused the complete disruption of PIC formation. Analysis of the Δ*rpd3*/Δ*hos2* mutant has identified a novel intermediate in the *RNR3* activation pathway.

HDAC activity is required for Rpd3 and Hos2 function. The catalytic activities of Rpd3 and Hos2 are required for the activation of the *GAL* locus (45). To investigate whether the HDAC activities of Rpd3 and Hos2 are required for the activation of *RNR3* and *HUG1*, constructs in which conserved histidine residues in Rpd3 (H150 and H151) and Hos2 (H195 and H196) were substituted with alanine residues were made. These mutations have been shown to disrupt the deacetylase activities of Rpd3 and Hos2 (21, 45). The mutant proteins are expressed at similar levels in vivo (21, 45; data not shown). The mutants were integrated at the natural genomic loci, and we analyzed the expression of *RNR3* and *HUG1* and the cross-linking of PIC components to *RNR3* in these strains (Fig. 8). Mutating the catalytic histidine residues in Rpd3 and Hos2 phenocopied the deletion mutants, strongly arguing that HDAC activity is required for the activation of DNA damage-inducible genes (Fig. 8A and B).

Regulation of DNA damage-inducible genes by redundant histone acetylation. *GCN5* is required for acetylating histone H3 at *RNR3*. Deleting *GCN5* completely eliminates the DNA damage-induced H3 histone acetylation; however, expression is only partially affected (40). This suggests either that the acetylation of H3 only facilitates high levels of expression or that the acetylation of other histones partially compensates for the loss of histone H3 acetylation. Figure 7A indicates that histone H4 acetylation at *RNR3* is increased in MMS-treated cells, suggesting that the acetylation of both histone H3 and histone H4 is required for the activation of *RNR3*. We next examined the expression of *RNR3* and *HUG1* in an *ESA1* mutant, *esa1-414* (8), and in a strain containing Δ*gcn5*/*esa1-414* mutations. As reported previously, the deletion of *GCN5* alone

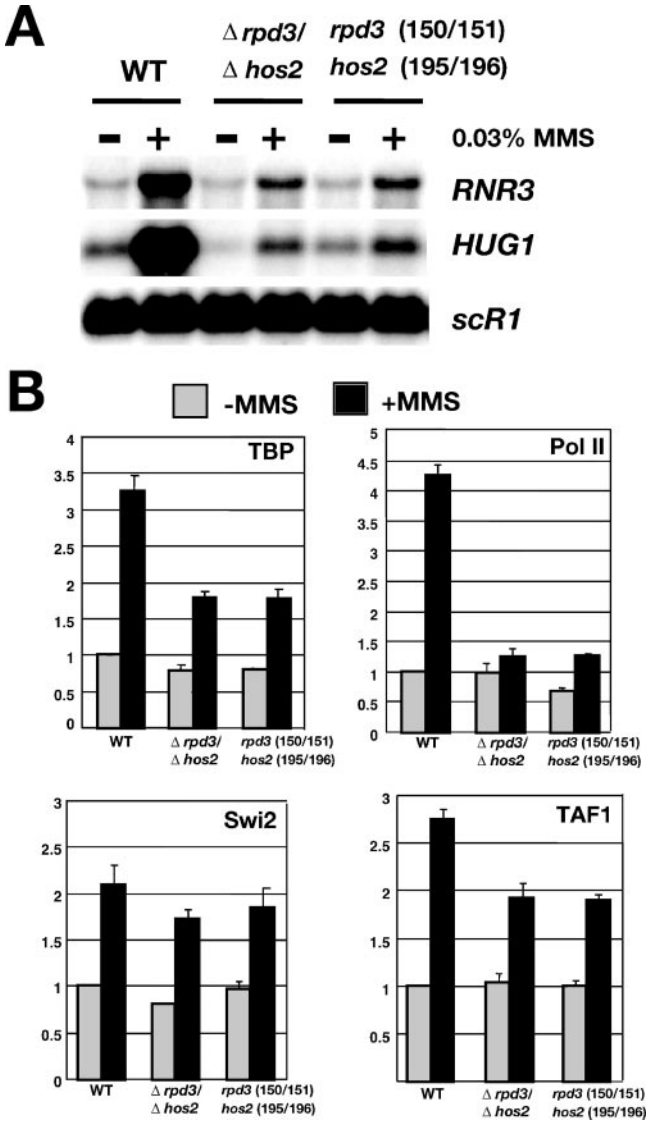

FIG. 8. HDAC activities of *RPD3* and *HOS2* are required for gene activation. Strains containing alanine substitutions within conserved histidines in Rpd3 (H150 and H151) and Hos2 (H195 and H196) were constructed. Cells were untreated (−) or treated (+) with 0.03% MMS for 2.5 h at 30°C. (A) Northern blot analysis of *RNR3* and *HUG1* in the wild type (WT), the Δ*rpd3*/Δ*hos2* deletion mutant, and the *rpd3*/*hos2* catalytic double mutant. *scR1* is used as a loading control. (B) ChIP assay monitoring the level of TBP, TAF1, RNA Pol II, and Swi2p cross-linking in wild-type, Δ*rpd3*/Δ*hos2*, and *rpd3*/*hos2* catalytic mutant cells. Error bars indicate standard deviations.

caused a partial defect in *RNR3* expression, a reduction of about 50% (Fig. 9A). Likewise, the activation of *RNR3* was reduced similarly in an *esa1-414* single mutant. Strikingly, the expression of *RNR3* was essentially abolished in the double mutant, indicating that *GCN5* and *ESA1* play redundant functions in the regulation of DNA damage-inducible genes. It is likely that redundancy between Gcn5 and Esa1 occurs at many genes, as synthetic lethal interactions between Δ*gcn5* and multiple alleles of *ESA1* mutants were observed (Fig. 8B). The effect is somewhat selective for *GCN5* because *SAS3*, which plays a redundant role with *GCN5* in global histone H3 acetylation (17), is not required for *RNR3* expression and deleting *SAS3* does not significantly worsen the transcriptional defect of the *esa1-414* mutant (A. E. Dempsey and J. C. Reese, unpublished data). Redundancy between *GCN5* and *ESA1* was also observed at *HUG1* (Fig. 9A). Interestingly, when we compared the expression of *HUG1* to that of *RNR3* in these mutants, we found that *HUG1* is much less sensitive to the *esa1-414* mutation but significantly more sensitive to the Δ*gcn5* mutation. This difference may explain, at least in part, why *HUG1* responds differently to single HDAC mutations (Fig. 2A) and to deletions in genes targeted by Tup1 for repression (49). *RNR3* and *HUG1* require different patterns of histone H3 and H4 modification.

The lysine residues within the H4 tail are required for activation. The data thus far suggest that the activation of *RNR3* requires stringent regulation of the modification of histone H4. We next asked whether the lysine residues within the tail of histone H4 are required for the activation of *RNR3*. The H4 tail, and the lysine residues specifically, have been shown to be important for the activation of *GAL1* and the expression of many genes in vivo (13, 14, 39). The activation of *RNR3* was examined in strains containing lysines 5, 8, 12, and 16 substituted with either arginine (K/R) or glutamine (K/Q) residues. Glutamine neutralizes the charge of the lysine residues and is believed to mimic the charge neutralization effects of acetylation, and arginine maintains the positive charge at each position within the tail. The Northern blot presented in Fig. 10 shows that mutating the lysine residues to either glutamine or arginine significantly impaired the activation of *RNR3*. As noted at the *GAL1*, *PHO5*, and *CUP1* genes, arginine substitutions had a stronger effect on activated transcription than glutamine substitutions (14). Thus, the overall charge of the tail may not be as important as the structure of the modified lysine side chains.

DISCUSSION

An emerging picture in transcription is that factors once thought to play a role in repression exclusively have been discovered to be required for gene activation too. The best examples to date are the HDACs. Long known to repress transcription, their role in activation has been appreciated only recently. However, the mechanism and whether HDACs directly affect initiation were unclear. Here we show that HDACs can affect the activation of genes by acting at the promoter and provide evidence that they do so by regulating a specific step in transcription initiation, the recruitment and maintenance of RNA polymerase II.

Gene-specific integration of HDAC activities at Tup1 targets. The concept that Rpd3 and Hos2 cooperate in the process of gene activation has been documented at the *GAL* genes and osmotic stress genes (12, 45). Unlike with the *GAL*-induced genes, Rpd3 associates specifically with the URS region of *RNR3* as part of the Rpd3L complex and disrupting the elongation-associated Rpd3S complex had no effect on *RNR3* activation. The activation of GAL genes requires the recruitment of HDACs and the deacetylation of histones within the ORF (45) and may require the Rpd3S complex. The action of Rpd3L at the promoter of *RNR3*, versus that within the ORF, is fully consistent with the observation that the recruitment of

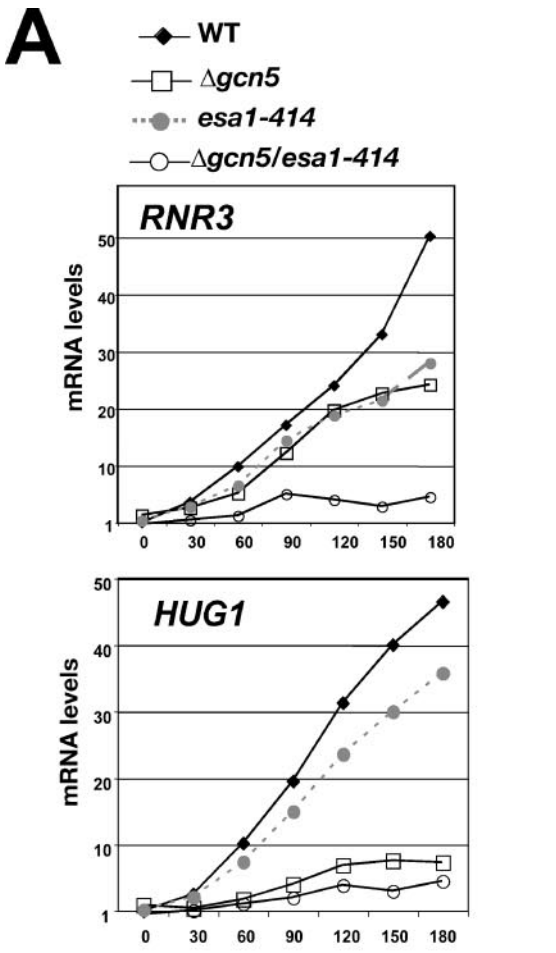

FIG. 9. Redundant H3 and H4 acetylation regulates *RNR3* expression. (A) Graphs of *RNR3* and *HUG1* expression in wild-type (WT), Δ*gcn5*, *esa1-414*, and Δ*gcn5*/*esa1-414* cells after treatment with 0.03% MMS. Cells were grown to early log phase at room temperature, shifted to 30°C for 2 h and then treated with MMS for the times indicated on the *x* axis (minutes). Data are expressed relative to corresponding levels in untreated wild-type cells and were corrected for that of the loading control (*scR1*). (B) Growth of *ESA1* and *GCN5* mutants on YPAD. Plates were scanned after 2 (30, 33, and 37°C) and 3 days (24°C). *ESA1* mutants have been described previously (8).

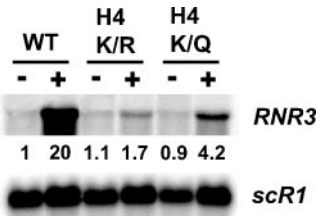

FIG. 10. *RNR3* activation requires the lysine residues in the histone H4 tail. The wild type (WT) (PKY501) and strains containing K-to-R (K/R) (LDY722) or K-to-Q (K/Q) (LDY107) substitutions (14) in each of the four acetylated lysine residues in the tail of histone H4 were subjected to Northern blot analysis to measure *RNR3* transcription. The values under the *RNR3* panel are the relative levels of mRNA normalized to the *scR1* loading control. −, absence of MMS; +, presence of MMS.

RNA polymerase II and the formation of PIC are strongly reduced. This result suggests that the modification of chromatin or nonchromatin proteins close to the promoter regulates the formation of the PIC. It is not clear how Rpd3L is recruited to the URS of *RNR3*, but surprisingly, it is not dependent upon Crt1 or Ssn6/Tup1.

Hda1 is required for full repression of *RNR3* and *HUG1*, and in fact, Δ*hda1* is the only HDAC mutation that causes derepression (Fig. 1) (47). On the other hand, Rpd3 and Hos2 are required for the activation of *RNR3*. Thus, Ssn6-Tup1 represses transcription by recruiting certain HDACs to promoters, but it also blocks the recruitment of others that play a role in activation, such as Rpd3. This suggests that a novel function of Ssn6-Tup1 is to block the recruitment of HDACs that are required for the activation of transcription. We propose that Ssn6-Tup1 acts as an integrator of both positive and negative signals through selective HDAC recruitment. Furthermore, this model may explain, at least in part, the requirement for Tup1 in gene activation. It is interesting to note that the requirement for Ssn6-Tup1 for the activation of *GAL1* transcription requires Cti6, a verified subunit of the Rpd3L complex (7, 32), and that both Tup1 and Rpd3-Sin3 are required for the activation of osmotic stress genes (12, 33). Direct interactions between Tup1, Cti6, Rpd3, and Hos2 have been observed (10, 32, 46), and Tup1 recruits Cti6 (Rpd3L?) to the *GAL1* promoter (32). It is not known whether Tup1 is required to recruit Cti6 or other components of the Rpd3L complex to osmotic stress genes. Therefore, there is a significant amount of evidence to support our model. Although there are similarities in the ways Tup1 and the Rpd3L complex regulate *GAL1* and osmotic stress genes and *RNR3*, the exact mechanisms are different. Tup1 remains associated with active

galactose- and stress-induced genes (32, 33), and Tup1 and the Rpd3L subunit Cti6 are required for SAGA recruitment and histone H3 acetylation at *GAL1* (32). Furthermore, the recruitment of SWI/SNF to osmotic stress genes requires Tup1 (33). In contrast, Tup1 leaves the *RNR3* promoter (49, 50, 51), Ssn6-Tup1 blocks the association of Rpd3L with the promoter, and Rpd3 is dispensable for SAGA-dependent histone H3 acetylation and SWI/SNF recruitment. It is likely that another DNA binding protein fulfils the function of recruiting Rpd3L and the chromatin remodeling machinery to *RNR3* after Tup1 disassociates. Thus, the mechanism of how Ssn6-Tup1 orchestrates HDAC recruitment is gene specific and may be dependent upon the sequence-specific DNA binding proteins that coordinate the activation and repression functions.

Rpd3L and Hos2 regulate Pol II occupancy. Exactly how Rpd3 and Hos2 activate transcription is not clear; however, their enzymatic activities are required. Interestingly, we have shown that constitutive histone H3 acetylation does not cause the same effect. *RNR3* and *HUG1* are fully induced in Δ*hda1* cells and show high levels of H3 acetylation (Fig. 1 and 2) (50), suggesting specificity for the H4 tail. It is clear that the lysine residues within the H4 tail are important for *RNR3* activation. In addition, since genetically disrupting chromatin structure by deleting *CRT1* suppresses the activation defect of the Δ*rpd3*/Δ*hos2* mutant (data not shown), Rpd3 and Hos2 are likely acting on the chromatin at the promoter of *RNR3*. While these observations suggest that H4 may be the target of Rpd3 and Hos2, it is difficult to conclude definitively that disrupting Rpd3/Hos2 function accounts for the reduced activation of *RNR3* in the H4 tail mutants. These mutations could equally affect other stages of gene expression. Additional characterization of histone tail mutants will be required to resolve this. We cannot rule out that these two HDACs have targets other than histones. Acetylation and deacetylation of transcription factors have been described for mammalian cells, although they have not been described for yeast (19, 41).

The observation that TFIID and SWI/SNF recruitment and chromatin remodeling are largely intact in the Δ*rpd3*/Δ*hos2* mutant, yet RNA polymerase II is strongly affected, indicates that Rpd3 and Hos2 are required for "later stages" of PIC formation or to orchestrate multiple rounds of transcription. It may involve cycles of acetylation and deacetylation at the promoter. There are a number of examples where dynamic histone modifications have been linked to gene activity (16, 30, 31). We have not observed "waves" of histone acetylation/deacetylation of histone H4 at *RNR3* on a consistent basis (data not shown). Nor do we observe waves of PIC formation as observed at the pS2 promoter (30). The failure to detect transient or cyclic changes at *RNR3* may be explained by difficulties in synchronizing the promoters in the population during the activation process, but they may occur nonetheless. Reducing acetylation by mutating *ESA1* or causing constitutive acetylation by deleting HDACs blocks activation, suggesting that "short circuiting" the normal regulatory mechanisms leads to uncoordinated events at the promoter and possibly dead-end pathways. Thus, the role of HDACs in activation may be to prevent unproductive pathways from forming by resetting the state of the promoter, analogous to the way chaperones function in protein folding, resulting in additional rounds of Pol II recruitment and transcription. A possibility is that the activities of nucleosome assembly and disassembly factors are regulated by changes in histone modifications. Acetylation marks may need to be erased to allow for histone reassembly or modification after the passage of polymerase. This may explain why histone H3 cross-linking is reproducibly reduced at the promoter of *RNR3* in the Δ*rpd3*/Δ*hos2* mutant (Fig. 7A). Genetic interactions between histone tail mutants, HATs and HDACs and the histone chaperone/nucleosome assembly complex FACT have been described previously (15). Moreover, the mutation or depletion of FACT subunits strongly reduces TBP and polymerase binding to promoters (4, 29), indicating that FACT regulates more than transcription elongation. Given that polymerase recruitment is strongly reduced at *RNR3*, ChIP assays for FACT components will not be particularly enlightening. Histone deacetylation has been linked to restoring chromatin structure within the ORFs of genes to allow for efficient elongation of transcription in vivo. Our analysis suggests that a mechanism is in place to regulate the recruitment of Pol II to promoters as well.

ACKNOWLEDGMENTS

We thank members of the Reese lab and the Penn State gene regulation groups for advice and comments on this work. Lorraine Pillus and Michael Grunstein are thanked for contributing strains used in this work.

This research was supported by funds provided by the National Institutes of Health (GM58672) and by an Established Investigator Grant from the American Heart Association to J.C.R.

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