A General Requirement for the Sin3-Rpd3 Histone Deacetylase Complex in Regulating Silencing in *Saccharomyces cerevisiae*

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Manuscript received January 22, 1999
Accepted for publication April 16, 1999

ABSTRACT

The Sin3-Rpd3 histone deacetylase complex, conserved between human and yeast, represses transcription when targeted by promoter-specific transcription factors. *SIN3* and *RPD3* also affect transcriptional silencing at the *HM* mating loci and at telomeres in yeast. Interestingly, however, deletion of the *SIN3* and *RPD3* genes enhances silencing, implying that the Sin3-Rpd3 complex functions to counteract, rather than to establish or maintain, silencing. Here we demonstrate that Sin3, Rpd3, and Sap30, a novel component of the Sin3-Rpd3 complex, affect silencing not only at the *HMR* and telomeric loci, but also at the rDNA locus. The effects on silencing at all three loci are dependent upon the histone deacetylase activity of Rpd3. Enhanced silencing associated with *sin3*Δ, *rpd3*Δ, and *sap30*Δ is differentially dependent upon Sir2 and Sir4 at the telomeric and rDNA loci and is also dependent upon the ubiquitin-conjugating enzyme Rad6 (Ubc2). We also show that the Cac3 subunit of the CAF-I chromatin assembly factor and Sin3-Rpd3 exert antagonistic effects on silencing. Strikingly, deletion of *GCN5*, which encodes a histone acetyltransferase, enhances silencing in a manner similar to deletion of *RPD3*. A model that integrates the effects of *rpd3*Δ, *gcn5*Δ, and *cac3*Δ on silencing is proposed.

EPIGENETIC effects are heritable, but reversible, changes in gene expression due to alterations in chromatin structure or DNA methylation (reviewed in Henikoff and Matzke 1997). Classic examples of epigenetic phenomena include X-chromosome inactivation in mammals (Panning and Jaenisch 1998), position-effect variegation in Drosophila (Wakimoto 1998), imprinting of specific loci in mammals (Jaenisch 1997), and silencing of the cryptic mating-type loci (*HM*) in *Saccharomyces cerevisiae* (Sherman and Pillus 1997). Epigenetic control usually involves gene silencing, defined as position-dependent, gene-independent transcriptional repression, involving formation of specialized chromatin structures that encompass large regions of the genome (Sherman and Pillus 1997; Grunstein 1998). Despite the importance of silencing in regulating cell growth and development, the molecular mechanisms responsible for establishing and maintaining a particular chromatin structure are not well defined.

In addition to silencing at the cryptic *HM* mating loci, silencing in yeast has been described for reporter genes integrated proximal to telomeres (telomere position effect; Gottschling *et al.* 1990) and within the rDNA array (Bryk *et al.* 1997; Fritze *et al.* 1997; Smith and Boeke 1997). In contrast to stable silencing at the *HM* and rDNA loci, telomeric silencing is variegated, resulting in stochastic patterns of repression for RNA pol II-transcribed genes integrated at telomeres. This effect is comparable to the spread of heterochromatin that accounts for position-effect variegation in flies.

The combination of yeast genetics and biochemistry has led to the discovery of many factors that affect silencing. These include the silent information regulatory (SIR) proteins, the repressor-activator protein Rap1, and the core histones H3 and H4 (Ivy *et al.* 1986; Rine and Herskowitz 1987; Kayne *et al.* 1988; Aparicio *et al.* 1991; Kyrion *et al.* 1993; Thompson *et al.* 1994). Models for silencing suggest that SIR proteins are recruited to DNA by Rap1 and then spread along the DNA by interaction with the N-terminal tails of H3 and H4 (reviewed in Sherman and Pillus 1997; Grunstein 1998). Nonetheless, silencing occurs by distinct mechanisms at each of the silenced loci. For example, telomeric silencing requires Sir2-Sir4, but is independent of Sir1, which is required for the establishment of silencing at the *HM* loci. The only SIR protein required for rDNA silencing is Sir2. Indeed, deletion of the *SIR4* gene enhances rDNA silencing (Bryk *et al.* 1997; Fritze *et al.* 1997; Smith and Boeke 1997; Smith *et al.* 1998), a consequence of redistribution of limiting Sir2 to the nucleolus in the absence of Sir4 (Smith *et al.* 1998).

Other proteins also play important roles in silencing. Rad6 (Ubc2) is an E2 ubiquitin-conjugating enzyme involved in many cellular processes, including DNA repair, UV-induced mutagenesis, N-end rule protein deg-

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radation, sporulation (reviewed in Prakash *et al.* 1993), and Ty1 integration specificity (Picologlou *et al.* 1990; Kang *et al.* 1992; Liebman and Newnam 1993). Recent evidence demonstrated that *RAD6* is required for silencing at telomeres and the *HM* loci and that deletion of *RAD6* derepresses Ty1 transcription and mitotic recombination at the rDNA locus. The ubiquitin-conjugating activity of Rad6, but not Rad6-mediated N-end rule protein degradation, is essential for these processes (Bryk *et al.* 1997; Huang *et al.* 1997). In addition, Rad6 ubiquitinates histones H2A, H2B, and H3 *in vitro* (Sung *et al.* 1988; Haas *et al.* 1990). These results suggest a role for Rad6 as a modifier of localized chromatin structure (Picologlou *et al.* 1990; Kang *et al.* 1992; Liebman and Newnam 1993). Consistent with this hypothesis, Ubp3, a ubiquitin hydrolase, physically interacts with Sir4, and deletion of *UBP3* enhances telomeric and *HML* silencing in yeast (Moazed and Johnson 1996). Another (putative) ubiquitin hydrolase, encoded by the *DOT4* gene, also disrupts silencing when overexpressed (Singer *et al.* 1998).

The chromatin assembly factor I (CAF-I) also affects silencing. Yeast CAF-I is composed of three subunits encoded by the *CAC1*, *CAC2*, and *CAC3* genes (Kaufman *et al.* 1997). The *cac1* and *rlf2* alleles of *CAC1* alter Rap1 localization, perturb telomeric chromatin, and reduce telomeric silencing (Enomoto *et al.* 1997; Kaufman *et al.* 1997; Monson *et al.* 1997). Silencing at the *HM* loci is also reduced in *cac1* and *rlf2* mutants, and similar effects on telomeric and *HM* silencing are conferred by *cac2* and *cac3* mutations (Kaufman *et al.* 1997; Enomoto and Berman 1998). CAF-I affects the maintenance, but not the reestablishment, of silent chromatin (Enomoto and Berman 1998). Cac3 (Msi1) is structurally related to Hat2, the regulatory subunit of the yeast B-type histone acetyltransferase (Parthun *et al.* 1996), to the RbAp48 and RbAp46 subunits of mammalian histone deacetylase complexes (Parthun *et al.* 1996; Taunton *et al.* 1996; Zhang *et al.* 1997), and to the NURF-55 subunit of a Drosophila chromatin remodeling complex (Martinez-Balbas *et al.* 1998). Thus, Cac3 provides a structural link among four distinct complexes that affect histone metabolism.

Silent DNA is packaged into hypoacetylated nucleosomes that exhibit a pattern of histone acetylation reminiscent of metazoan heterochromatin (Turner *et al.* 1992; Braunstein *et al.* 1993, 1996). These results implicate histone acetyltransferases and deacetylases in silencing. Indeed, the Rpd3 histone deacetylase, and its associated protein Sin3, affect silencing at telomeric and *HM* loci (De Rubertis *et al.* 1996; Rundlett *et al.* 1996; Vannier *et al.* 1996). However, in contrast to their role in gene-specific repression, Rpd3 and Sin3 disrupt, rather than establish or maintain, silencing. A similar effect on silencing was reported for Rpd3 in Drosophila (De Rubertis *et al.* 1996).

Mammalian and yeast Sin3 and Rpd3 proteins exist in large multisubunit complexes, estimated to be >2 MD in the case of the yeast Sin3-Rpd3 complex (Kasten *et al.* 1997). In addition to Sin3, which appears to function as a platform for complex assembly, the human Sin3-Rpd3 complex includes the histone deacetylases HDAC1 and HDAC2, the histone-binding proteins RbAp46 and RbAp48, and two novel proteins designated SAP30 and SAP18 (Sin3-associated protein; Zhang *et al.* 1997, 1998). A homolog of human SAP30 has been identified in yeast (Zhang *et al.* 1998). Like *SIN3* and *RPD3*, the yeast *SAP30* gene is not essential for cell viability. However, deletion of *SAP30* confers a set of phenotypes that are shared among *sin3*Δ, *rpd3*Δ, and *sap30*Δ mutants; furthermore, Sap30 coimmunoprecipitates with Rpd3 (Zhang *et al.* 1998). Thus, Sap30 is a novel protein of undefined function, conserved between the yeast and human Sin3-Rpd3 complexes.

It is presently unknown how many proteins affect silencing in yeast. Furthermore, the mechanisms by which these factors mediate silencing are unknown. In this study we have examined the role of the Sin3-Rpd3 complex in silencing at the telomeric, *HMR*, and rDNA loci. Our results demonstrate that the Sin3-Rpd3 complex plays a general role in silencing. Surprisingly, loss of the Gcn5 histone acetyltransferase exerts the same effect on silencing as loss of the Rpd3 histone deacetylase, yet Rpd3 and Gcn5 exert opposite effects on promoter-dependent, position-independent transcription. We propose a model to account for these results.

MATERIALS AND METHODS

Yeast strains and media: The yeast strains used in this study are listed in Table 1. The YMH strains were derived from strain UCC506 (Renauld *et al.* 1993), strains CFY559 or CFY559Δsir2 (Fritze *et al.* 1997), and strain yLP91 (Pemberton and Blobel 1997) by one-step disruption (Rothstein 1991) of the indicated genes. All yeast media were prepared according to standard recipes (Boeke *et al.* 1984; Sherman 1991).

Plasmids: Plasmids used in this study are listed in Table 2. Vectors pRS303 and pRS306 (Sikorski and Hieter 1989) and YCplac33, YEplac112, YIplac128, and YIplac204 (Gietz and Sugino 1988) are described elsewhere. The *rpd3*Δ*::URA3* γ-disruption construct, pM1061, was generated by transferring the *Sal*I-*Sst*I fragment of M1436 (*rpd3*Δ*::LEU2*) to the same sites of pRS306. YEplac112-*RPD3* includes the entire *RPD3* open reading frame inserted between the *ADH1* promoter and *CYC8* terminator in YEplac112. YEplac112-*rpd3* (H188A) is identical to YEplac112-*RPD3*, except that it encodes a form of Rpd3 lacking detectable histone deacetylase activity *in vitro* (Kadosh and Struhl 1998). pM1288 and pM1289 are identical to YEplac112-*RPD3* and YEplac112-*rpd3* (H188A), respectively, except that the vector is YCplac33. pM1176 was constructed by PCR amplification of the *Xba*I-*Not*I N-terminal fragment of *SAP30* (nucleotides 1–590) and ligation into *Spe*I-*Not*I sites of pRS303. The *sap30*Δ*::LEU2* γ-disruption construct, pM1177, was constructed by ligation of the PCR-amplified *Pst*I-*Sal*I C-terminal fragment of *SAP30* (nucleotides 385–1370) and the *Bam*HI-*Sst*I fragment (nucleotides 1–590) from pM1176 into *Pst*I-*Sal*I and *Bam*HI-*Sst*I sites of YIplac128, respectively. The *sap30*Δ*::TRP1* construct, pM1183, was generated by transferring the *Sph*I-*Sst*I fragment of pM1177 (*sap30*Δ*::LEU2*) to the same sites of YIplac204.

Assays for telomeric, *HMR*, and rDNA silencing: Telomeric

TABLE 1

Yeast strains used in this study

Strain	Genotype	Source
UCC506	*MAT***a** *ura3-52 leu2-Δ1 his3-Δ200 trp1-Δ1 lys2-801amber ade2-101ochre URA3-TEL-V-R*	Renauld *et al.* (1993)
YMH267	UCC506 *sin3Δ::LEU2*	This study
YMH272	UCC506 *rpd3Δ::LEU2*	This study
YMH279	UCC506 *sap30Δ::LEU2*	This study
YMH276	UCC506 *cac3Δ::LEU2*	This study
YMH314	UCC506 *cac3Δ::LEU2 sin3Δ::ADE2*	This study
YMH315	UCC506 *cac3Δ::LEU2 rpd3Δ::HIS3*	This study
YMH316	UCC506 *cac3Δ::LEU2 sap30Δ::TRP1*	This study
YMH319	UCC506 *sin3Δ::ADE2*	This study
YMH320	UCC506 *rpd3Δ::HIS3*	This study
YMH321	UCC506 *sap30Δ::TRP1*	This study
YMH358	UCC506 *sir2Δ::TRP1*	This study
YMH360	UCC506 *sir2Δ::TRP1 rpd3Δ::LEU2*	This study
YMH362	UCC506 *sir4Δ::HIS3*	This study
YMH364	UCC506 *sir4Δ::HIS3 rpd3Δ::LEU2*	This study
YMH405	UCC506 *rad6Δ::LEU2*	This study
YMH407	UCC506 *rad6Δ::LEU2 rpd3Δ::HIS3*	This study
YMH366	UCC506 *gen5Δ::HIS3*	This study
YMH370	UCC506 *gen5Δ::HIS3 rpd3Δ::LEU2*	This study
CFY559	*MAT***a** *ura3-52 leu2 lys2 ade2Δ::hisG can1Δ::hisG his4 tyr1-2 css1-1 RDN1-ADE2-CAN1*	Fritze *et al.* (1997)
YMH333	CFY559 *sin3Δ::LEU2*	This study
YMH335	CFY559 *rpd3Δ::LEU2*	This study
YMH337	CFY559 *sap30Δ::LEU2*	This study
CFY559Δsir2	CFY559 *sir2Δ::LEU2*	Fritze *et al.* (1997)
YMH377	CFY559 *sir2Δ::LEU2 rpd3Δ::URA3*	This study
YMH379	CFY559 *sir4Δ::URA3*	This study
YMH381	CFY559 *sir4Δ::URA3 rpd3Δ::LEU2*	This study
YMH413	CFY559 *rad6Δ::URA3*	This study
YMH415	CFY559 *rad6Δ::URA3 rpd3Δ::LEU2*	This study
CY184	*MATα ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 RDN1::ADE2*	Zhu *et al.* (1995)
yLP19	*MAT***a** *ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 hmrΔA::ADE2*	Pemberton and Blobel (1997)
YMH345	yLP19 *sin3Δ::LEU2*	This study
YMH348	yLP19 *rpd3Δ::LEU2*	This study
YMH349	yLP19 *sap30Δ::LEU2*	This study
YMH171	*MATα ura3-52 leu2-3,112 his3 trp1Δ*	Zhang *et al.* (1998)
YMH265	YMH171 *sin3Δ::LEU2*	Zhang *et al.* (1998)
YMH270	YMH171 *rpd3Δ*::LEU2	Zhang *et al.* (1998)
YMH277	YMH171 *sap30Δ*::LEU2	Zhang *et al.* (1998)

silencing was scored as described previously (Aparicio *et al.* 1991). Tenfold serial dilutions of overnight cultures of UCC506 derivatives, containing the *URA3* gene integrated at the right-end telomere of chromosome V (*URA3-TEL-V-R*), were spotted onto 5-fluoroorotic acid (5-FOA) and synthetic complete media and incubated at 30° for 3 days. Silencing at the rDNA and *HMR* loci was scored in the same manner, except that strains containing an *ADE2* reporter at the rDNA or *HMR* loci were spotted onto synthetic complete and −Ade media to monitor the expression of *ADE2*.

The *rpd3Δ rad6Δ* and *rpd3Δ gcn5Δ* double mutants display synthetic slow-growth phenotypes (data not shown). Therefore, silencing at the telomeric *URA3* gene (*URA3-TEL-V-R*) in these strains was scored by measuring cell viability on 5-FOA medium as described previously (Gottschling *et al.* 1990). Cells from overnight cultures were serially diluted and plated onto synthetic complete and 5-FOA media. After 3–4 days of incubation at 30°, the numbers of colonies on each plate were counted. The fraction of 5-FOA-resistant cells in a population was determined from at least three independent experiments and is expressed as the average ratio of colonies formed on 5-FOA medium to those formed on synthetic complete medium. Quantification of rDNA silencing was performed in the same way, except that the fraction of Ade^+ cells is expressed as the average ratio of colonies formed on −Ade medium to those formed on synthetic complete medium.

RESULTS

Deletion of *SAP30* enhances silencing at the *HMR* locus: To determine whether the Sap30 component of the Sin3-Rpd3 complex plays a general role in silencing,

TABLE 2

Plasmids used in this study

Plasmid	Description	Source
YEplac112-*RPD3*	*RPD3* 2μ *TRP1*	Kadosh and Struhl (1998)
YEplac112-*rpd3*	*rpd3* (H188A) 2μ *TRP1*	Kadosh and Struhl (1998)
pM1176	*sap30* (nucleotides 1–590)	This study
pM1177	*sap30*Δ*::LEU2*	This study
pM1183	*sap30*Δ*::TRP1*	This study
pM1288	*RPD3 CEN URA3*	This study
pM1289	*rpd3* (H188A) *CEN URA3*	This study
pM1061	*rpd3*Δ*::URA3*	This study
pMV129 (M1414)	*rpd3*Δ*::HIS3*	R. F. Gaber
M1436	*rpd3*Δ*::LEU2*	D. J. Stillman
M945	*sin3*Δ*::LEU2*	D. J. Stillman
M1142	*sin3*Δ*::ADE2*	D. J. Stillman
C369	*sir2*Δ*::TRP1*	J. R. Broach
pMM7.1	*sir4*Δ*::HIS3*	J. R. Broach
pAR59	*sir4*Δ*::URA3*	D. S. Gross
pJJ211	*rad6*Δ*::LEU2*	L. Prakash
pDG47 (KEp282)	*rad6*Δ*::URA3*	K. Madura
pPK112	*cac3*Δ*::LEU2*	Kaufman *et al.* (1998)

we asked if deletion of *SAP30* affects silencing at the *HMR* locus. Strain yLP19, which contains the *ADE2* gene integrated at the *hmr*Δ*A* locus (*hmr*Δ*A::ADE2*; Pemberton and Blobel 1997), and isogenic *rpd3*Δ (YMH348), *sin3*Δ (YMH345), and *sap30*Δ (YMH349) strains, were used in this analysis. Expression of *ADE2* allows cell growth on medium lacking adenine (−Ade) and results in a white colony phenotype, whereas enhanced silencing impairs cell growth on −Ade medium and confers a pink or red colony phenotype due to accumulation of a red pigment. Tenfold serial dilutions of each strain were spotted onto −Ade and synthetic complete media (+Ade) and incubated at 30° for 3 days. The *sap30*Δ deletion clearly impaired cell growth on −Ade medium, albeit to a lesser extent than either the *rpd3*Δ or *sin3*Δ deletions (Figure 1A). The *sap30*Δ mutant, similar to the *rpd3*Δ and *sin3*Δ mutants, also formed pink colonies on YPD medium, compared to white colonies for the wild-type strain (data not shown). We conclude that *SAP30* counteracts *HMR* silencing in a manner similar to *RPD3* and *SIN3*.

The Sin3-Rpd3 complex affects rDNA silencing: To determine whether the Sin3-Rpd3 complex plays a general role in silencing, we examined the effects of *sin3*Δ, *rpd3*Δ, and *sap30*Δ deletions on rDNA silencing. Strain CFY559 and isogenic *sin3*Δ (YMH333), *rpd3*Δ (YMH335), and *sap30*Δ (YMH337) deletion mutants were used in this study. CFY559 is an *ade2 can1* mutant carrying an *ADE2-CAN1* double marker integrated at the rDNA array (Fritze *et al.* 1997). Expression of the *ADE2* marker within the rDNA array was scored by plating efficiency on −Ade medium and by the colony color phenotype. Compared to the wild-type strain, an ~10^4-fold decrease in colony formation on −Ade medium was observed for the *sin3*Δ and *rpd3*Δ mutants, and a 10^2- to 10^3-fold decrease for the *sap30*Δ mutant (Figure 1B). The diminished *ADE2* expression associated with the *rpd3*Δ, *sin3*Δ, and *sap30*Δ mutations cannot be attributed to loss of the *ADE2-CAN1* marker by recombination between the rDNA repeats because these mutants exhibited a uniform pink colony phenotype rather than the red phenotype associated with deletion of the *ADE2-CAN1* marker by recombination. Furthermore, transformation of pink *rpd3*Δ mutants with plasmid-borne *RPD3* rescued the white colony phenotype, an effect that would not occur if the *ADE2-CAN1* were deleted (data not shown). Also, the *rpd3*Δ, *sin3*Δ, and *sap30*Δ mutations do not cause an Ade^- phenotype when *ADE2* is expressed from its normal chromosomal locus (Figure 1C), demonstrating that the Ade^- phenotypes associated with these mutations are specific for *ADE2* expression from the rDNA locus. Taken together, these results establish that the Sin3-Rpd3 complex also affects silencing at the rDNA locus.

Enhanced silencing associated with *rpd3*Δ is SIR dependent: To determine if the enhanced rDNA silencing associated with loss of components of the Sin3-Rpd3 complex is SIR protein dependent, the *SIR2* and *SIR4* genes were individually deleted in wild-type (CFY559) and isogenic *rpd3*Δ strains containing *ADE2-CAN1* integrated at the rDNA array. Silencing at the rDNA locus was again scored by the efficiency of colony formation on −Ade medium and by colony color. Results are shown in Figure 2A. Whereas the *rpd3*Δ mutation dramatically increased silencing, the *rpd3*Δ *sir2*Δ double mutation restored growth to ~83% of the wild-type strain (*cf.* rows 1–3). In addition, the *rpd3*Δ *sir2*Δ mutant exhibited a white colony phenotype, compared to the

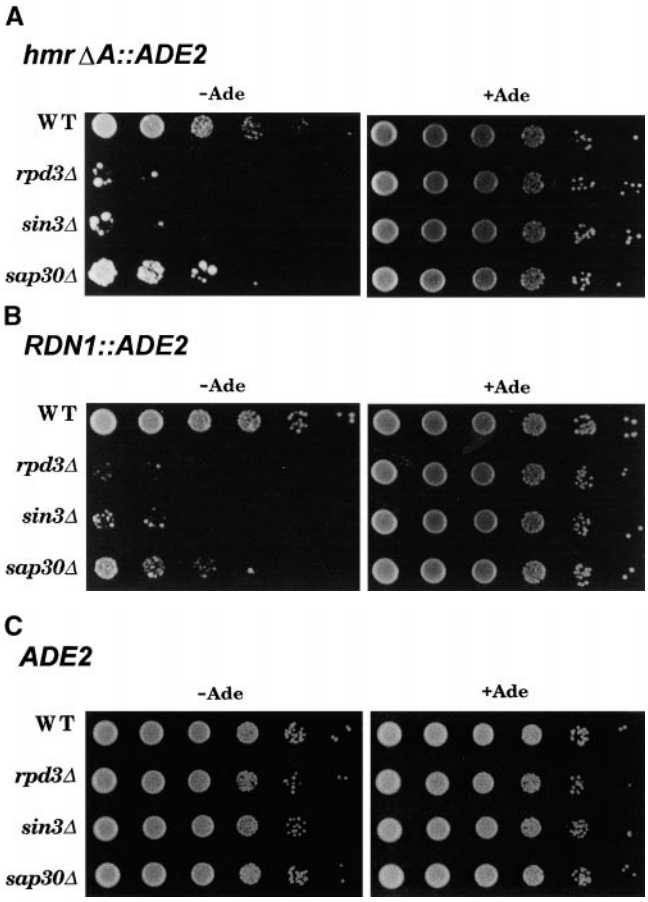

Figure 1.—Deletion of components of the Sin3-Rpd3 complex enhance silencing at the *HMR* and rDNA loci. Tenfold serial dilutions of the indicated strains were spotted onto −Ade and synthetic complete (+Ade) medium, followed by incubation at 30° for 3 days. Impaired growth relative to the wild-type strains is indicative of enhanced silencing. (A) The wild-type strain (yLP19) carries the *ADE2* gene integrated at the *hmr*Δ*A* locus; the *rpd3*Δ (YMH348), *sin3*Δ (YMH345), and *sap30*Δ (YMH349) strains are isogenic derivatives of yLP19. (B) The wild-type strain (CFY559) carries the *ADE2* (and *CAN1*) genes integrated at the rDNA (*RDN1*) locus; the *rpd3*Δ (YMH335), *sin3*Δ (YMH333), and *sap30*Δ (YMH337) strains are isogenic derivatives of CFY559. (C) The wild-type strain (YMH171) carries the *ADE2* gene at its normal chromosomal locus; the *rpd3*Δ (YMH270), *sin3*Δ (YMH265), and *sap30*Δ (YMH277) strains are isogenic derivatives of YMH171.

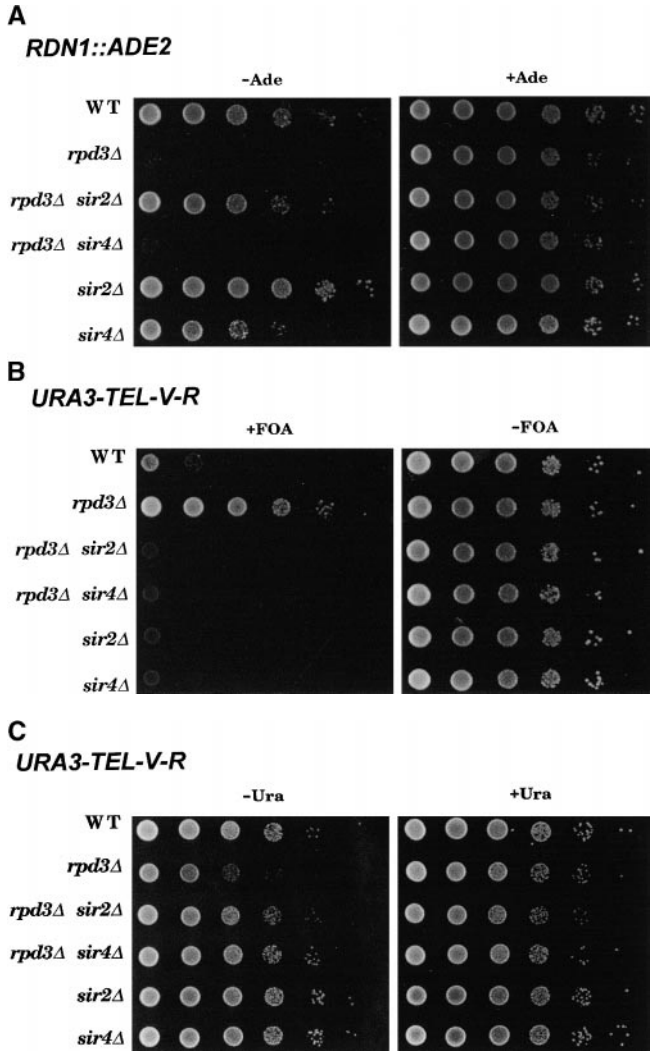

Figure 2.—Enhanced silencing by *rpd3*Δ at the rDNA and telomeric loci is SIR protein dependent. (A) Isogenic wild-type (CFY559), *rpd3*Δ (YMH335), *rpd3*Δ *sir2*Δ (YMH377), *rpd3*Δ *sir4*Δ (YMH381), *sir2*Δ (CFY559Δsir2), and *sir4*Δ (YMH379) strains with *ADE2-CAN1* integrated at the rDNA locus were spotted onto −Ade and synthetic complete (+Ade) medium, followed by incubation at 30° for 3 days. Impaired growth on −Ade medium indicates enhanced silencing of *ADE2*; enhanced growth relative to the *rpd3*Δ mutant indicates loss of silencing. (B) Isogenic wild-type (UCC506), *rpd3*Δ (YMH320), *rpd3*Δ *sir2*Δ (YMH360), *rpd3*Δ *sir4*Δ (YMH364), *sir2*Δ (YMH358), and *sir4*Δ (YMH362) strains carrying the *URA3* gene integrated 2 kb from the right telomere of chromosome V (*TEL-V-R*) were spotted onto 5-FOA (+FOA) and synthetic complete (−FOA) medium, followed by incubation at 30° for 3 days. Enhanced growth on 5-FOA medium indicates enhanced silencing of *URA3*; impaired growth indicates loss of silencing. (C) The same set of strains from B were spotted onto −Ura and synthetic complete (+Ura) medium, followed by incubation at 30° for 3 days. Impaired growth on −Ura medium indicates enhanced silencing of URA3; enhanced growth indicates loss of silencing.

pink phenotype of the *rpd3*Δ single mutant, and this phenotype can be rescued by plasmid-borne *RPD3* (data not shown). However, comparison of the *rpd3*Δ *sir2*Δ double mutant with the *sir2*Δ single mutant revealed increased silencing associated with *rpd3*Δ in the *sir2*Δ background (*cf.* rows 3 and 5). These results demonstrate that enhanced silencing associated with *rpd3*Δ at the rDNA array is *SIR2* dependent, but that *sir2*Δ is not completely epistatic to *rpd3*Δ.

The *sir4*Δ deletion, however, did not counteract the increase in silencing associated with *rpd3*Δ (Figure 2A; *cf.* rows 2 and 4). Also, the *rpd3*Δ single mutant and *rpd3*Δ *sir4*Δ double mutants displayed comparable pink colony phenotypes (data not shown). Consistent with previous results (Fritze *et al.* 1997; Smith and Boeke 1997), deletion of *SIR4* in an *RPD3* wild-type back-

ground enhanced silencing, resulting in an ~10-fold decrease in colony formation on −Ade medium (Figure 2A; *cf.* rows 1 and 6). Therefore, enhanced rDNA silencing associated with the *rpd3*Δ deletion is Sir2 dependent, but Sir4 independent.

In contrast to the requirements for silencing at the rDNA array, both *SIR2* and *SIR4* are essential for silencing at the telomeric and *HM* loci (reviewed in Sherman and Pillus 1997). We therefore asked whether the enhanced telomeric silencing associated with deletion of *RPD3* is Sir2 and Sir4 dependent. Strain UCC506, which contains the *URA3* gene positioned 2.0 kb (2+) from the right-end telomere of chromosome V (*URA3-TEL-V-R*; Renauld *et al.* 1993), and a set of isogenic *rpd3*Δ, *sir2*Δ, and *sir4*Δ derivatives, were used in these experiments. The levels of *URA3* silencing were monitored by cell growth on medium containing 5-FOA, which is toxic to cells expressing *URA3* (Boeke *et al.* 1984). The wild-type *URA3-TEL-V-R* strain grew poorly on 5-FOA medium, whereas the isogenic *rpd3*Δ mutant grew well (Figure 2B; *cf.* rows 1 and 2). However, no 5-FOA-resistant colonies were observed for the *rpd3*Δ *sir2*Δ and *rpd3*Δ *sir4*Δ double mutants or for the *sir2*Δ and *sir4*Δ single mutants (Figure 2B, rows 3–6), indicating that silencing of the telomeric *URA3* gene was disrupted in these strains. We also did the reciprocal assay, scoring growth of the same strains on −Ura medium to determine whether *rpd3*Δ might increase TEL silencing in the *sir*Δ background to an extent that might not be apparent in the FOA assay. No significant growth difference between the *rpd3*Δ *sir2*Δ or *rpd3*Δ *sir4*Δ double mutants and the *sir2*Δ or *sir4*Δ single mutants was observed (Figure 2C). [Growth of the double mutants is slightly impaired relative to the single mutants, but this difference can be accounted for by the weak slow-growth phenotype associated with *rpd3*Δ, which is reflected in the +Ura control (Figure 2C)]. Thus, in contrast to the *SIR4* independence of rDNA silencing, both *SIR2* and *SIR4* are required for the enhanced telomeric silencing associated with *rpd3*Δ.

The Rpd3 effect on silencing is dependent upon histone deacetylase activity: A histone deacetylase motif, containing evolutionarily invariant histidine residues at positions 150, 151, and 188 (H150, H151, and H188), was recently identified in the Rpd3 protein (Kadosh and Struhl 1998). Amino acid replacements of any of these conserved histidine residues abolished enzymatic activity *in vitro* and weakened transcriptional repression of targeted genes *in vivo.* These replacements did not affect either Rpd3 stability or Sin3-Rpd3 interaction.

To determine if the enzymatic activity of Rpd3 is required to counteract silencing, plasmid-borne *RPD3* and *rpd3* (H188A) alleles were introduced into the *rpd3*Δ deletion mutants YMH335 and YMH348, which carry the *ADE2* marker at rDNA and *HMR* loci, respectively. Whereas *RPD3* rescued the growth defect of strain

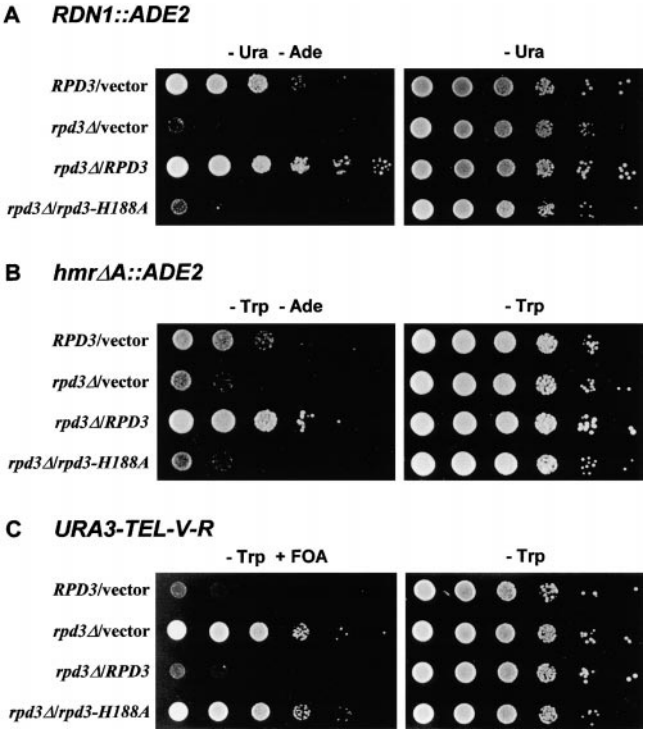

Figure 3.—The *RPD3*-encoded histone deacetylase activity is required for disruption of silencing at the rDNA, *HMR*, and *TEL-V-R* loci. In A–C the *RPD3* cellular genotype is indicated before the slash and the plasmid-borne genotype after the slash. In A and B, disruption of silencing is scored as enhanced growth on −Trp −Ade medium, whereas in C disruption of silencing is scored as diminished growth on −Trp +FOA medium. (A) Strain CFY559 (*RDN1::ADE2 RPD3*) or isogenic strain YMH335 (*RDN1::ADE2 rpd3*Δ) was transformed with vector alone (YCplac33) or its derivatives carrying either *RPD3* or the *rpd3-H188A* allele, which encodes catalytically inactive Rpd3. Tenfold serial dilutions of the resulting Ura^+ transformants were spotted onto −Ura −Ade or −Ura medium and incubated for 3 days at 30°. (B) Strain yLP19 (*hmr*Δ*A::ADE2 RPD3*) or isogenic strain YMH348 (*hmr*Δ*A::ADE2 rpd3*Δ) was transformed with vector alone (YEplac112) or its derivatives carrying either *RPD3* or *rpd3-H188A.* The resulting Trp^+ strains were spotted onto −Trp −Ade or −Trp medium and incubated for 3 days at 30°. (C) Strain UCC506 (*URA3-TEL-V-R RPD3*) or isogenic strain YMH272 (*URA3-TEL-V-R rpd3*Δ) was transformed with the same plasmids defined in B. The resulting Trp^+ strains were spotted onto −Trp +FOA or −Trp medium and incubated for 3 days at 30°.

YMH335 on −Ade medium (Figure 3A, row 3), the *rpd3*-H188A strain remained Ade^- (Figure 3A, row 4). This result demonstrates that *RPD3*, but not *rpd3*-H188A, restores the expression of the *ADE2* gene integrated at the rDNA locus in the *rpd3*Δ mutant. Consistent with this result, strains containing *RPD3* or *rpd3*-H188A formed white and pink colonies, respectively, on YPD medium (data not shown). Similar results were obtained for the strains carrying an *ADE2* reporter inserted in the *HMR* locus (Figure 3B, *cf.* rows 3 and 4; and data not shown). We also note that plasmid-borne expression of *RPD3* results in better growth on selective medium in the

TABLE 3

Effects of *rpd3*Δ, *rad6*Δ, and *gcn5*Δ on telomeric silencing

Strain	Relevant genotype	% viability on FOA
UCC506	WT	$9.5 \times 10^{-3} \pm 0.7 \times 10^{-3}$
YMH320	*rpd3 RAD6*	85 ± 4.8
YMH405	*RPD3 rad6*Δ	$5.6 \times 10^{-4} \pm 1.1 \times 10^{-4}$
YMH407	*rpd3*Δ *rad6*Δ	$6.1 \times 10^{-5} \pm 0.9 \times 10^{-5}$
YMH366	*RPD3 gcn5*Δ	14 ± 1.5
YMH370	*rpd3*Δ *gcn5*Δ	83 ± 3.2

Silencing at the telomeric *URA3* gene (*URA3-TEL-V-R*) in these strains was scored by measuring cell viability on 5-FOA medium as described in materials and methods.

TABLE 4

Effects of *rpd3*Δ and *rad6*Δ on silencing at the rDNA (*RDN1*) locus

Strain	Relevant genotype	% viability on −Ade
CFY559	WT	98 ± 1.4
YMH335	*rpd3*Δ *RAD6*	$6.5 \times 10^{-2} \pm 0.3 \times 10^{-2}$
YMH413	*RPD3 rad6*Δ	94 ± 3.0
YMH415	*rpd3*Δ *rad6*Δ	74 ± 4.0

Silencing at the rDNA locus in these strains was scored by measuring cell viability on −Ade medium as described in materials and methods.

*rpd3*Δ background than does chromosomally expressed *RPD3* for both the *RDN1::ADE2* and *hmr*Δ*::ADE2* strains (Figure 3, A and B, rows 3 *vs.* 1), suggesting that overexpression of *RPD3* might weaken silencing. However, overexpression of *RPD3*, *SIN3*, or *SAP30* in wild-type backgrounds did not weaken silencing at either rDNA or *HMR* (data not shown).

We also examined the requirement for Rpd3 activity in regulating telomeric silencing. In this case strain YMH272 (*URA3-TEL-V-R rpd3*Δ) was used as the host and silencing was scored as enhanced growth (diminished *URA3* expression) on 5-FOA medium. The host *rpd3*Δ strain is 5-FOA-resistant due to enhanced silencing of the *URA3* marker (Figure 3C, row 2). This phenotype is rescued by plasmid-borne *RPD3*, resulting in 5-FOA sensitivity (row 3), but not by the *rpd3*-H188A plasmid (row 4). Taken together, the results in Figure 3 clearly demonstrate that the enzymatic activity of Rpd3 is required to counteract silencing at telomeric, *HMR*, and rDNA loci.

Enhanced silencing associated with *rpd3*Δ is *RAD6* dependent: Several studies have implicated Rad6-mediated ubiquitination as a regulator of silencing in both *S. cerevisiae* and *Schizosaccharomyces pombe* (Bryk *et al.* 1997; Huang *et al.* 1997; Singh *et al.* 1998). To investigate the possible relationship between ubiquitination and deacetylation in regulating silencing, we tested whether the enhanced silencing associated with loss of Rpd3 activity can bypass the requirement for Rad6. Due to the synthetic slow growth defect associated with *rpd3*Δ *rad6*Δ double mutants (data not shown), we assayed telomeric silencing at *URA3-TEL-V-R* by measuring viability of isogenic wild-type (UCC506), *rpd3*Δ (YMH320), *rad6*Δ (YMH405), and *rpd3*Δ *rad6*Δ (YMH407) strains on 5-FOA medium, rather than by the spotting assays described above. Results are presented in Table 3. As expected, *rpd3*Δ enhanced silencing, resulting in a 9000-fold increase in cell viability on 5-FOA medium, whereas *rad6*Δ weakened silencing, causing a 17-fold decrease in cell viability. Strikingly, the *rpd3*Δ *rad6*Δ double deletion further weakened silencing, resulting in a 1.4×10^6-fold decrease in cell viability relative to the *rpd3*Δ single mutant. Similar results were observed for *sin3*Δ *rad6*Δ and *sap30*Δ *rad6*Δ mutants (data not shown). Thus, the enhanced telomeric silencing associated with loss of the Sin3-Rpd3 complex is Rad6 dependent.

Because transcriptional silencing at the rDNA locus is mediated by a novel mechanism that depends on only a single *SIR* gene, *SIR2* (Bryk *et al.* 1997; Fritze *et al.* 1997; Smith and Boeke 1997), and deletion of *SIR4* increases rDNA silencing (Fritze *et al.* 1997; Smith and Boeke 1997; Figure 2A, row 6), we asked if *rad6*Δ exerts an effect on rDNA silencing similar to its effect on telomeric silencing. Deletion mutants comparable to those described above were generated using strain CFY559 (*RDN1-ADE2-CAN1*) and cell viability was scored on −Ade medium. Results are presented in Table 4. Again, *rpd3*Δ (YMH335) enhanced silencing, in this case resulting in a 1500-fold decrease in cell viability. In contrast, *rad6*Δ conferred a negligible effect on its own (YMH413), yet fully suppressed the effect of *rpd3*Δ in the *rpd3*Δ *rad6*Δ double mutant (YMH415), resulting in an 1100-fold increase in cell viability. In addition, wild-type (CFY559), *rad6*Δ (YMH413), and *rpd3*Δ *rad6*Δ (YMH415) strains formed white colonies on YPD medium, indicating efficient *ADE2* expression, whereas the *rpd3*Δ strain was pink (data not shown). Thus, enhanced silencing at the rDNA locus is also Rad6 dependent.

Enhanced silencing associated with loss of Sin3-Rpd3 occurs in the absence of *CAC3*: Components of the CAF-I complex are required for silencing at the *HM* and telomeric loci (Enomoto *et al.* 1997; Kaufman *et al.* 1997; Monson *et al.* 1997; Enomoto and Berman 1998). To determine if components of the Sin3-Rpd3 complex interact with Cac3 to regulate silencing, we tested the ability of *sin3*Δ, *rpd3*Δ, and *sap30*Δ deletions to restore telomeric silencing in a *cac3*Δ strain. Silencing at the *URA3-TEL-V-R* locus was assayed by scoring cell growth on 5-FOA medium, as described above, using an isogenic set of strains with different combinations of *cac3*Δ, *rpd3*Δ, *sin3*Δ, and *sap30*Δ deletions. The results are shown in Figure 4. As expected, *cac3*Δ weakened silencing, scored as enhanced 5-FOA sensitivity (row 2),

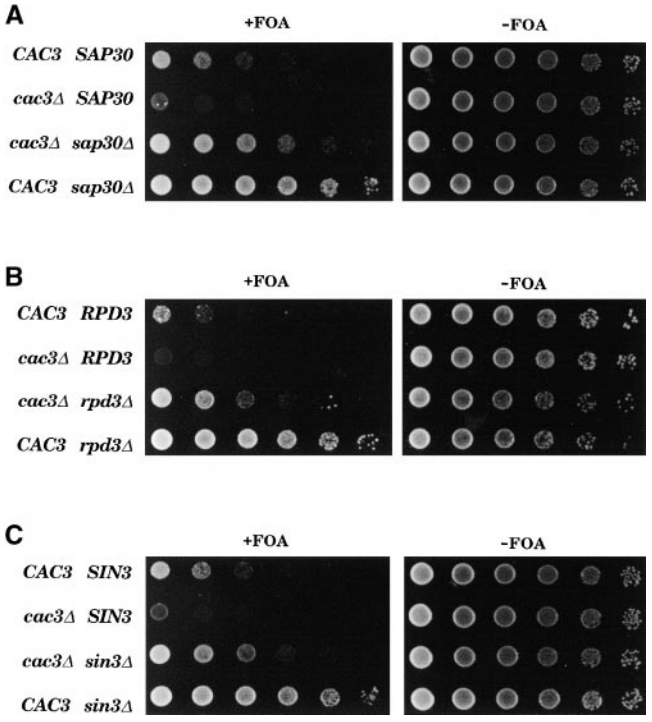

Figure 4.—*CAC3* (*MSI1*) is dispensable for maintenance of telomeric silencing in the absence of an intact Sin3-Rpd3 complex. The effects of *sap30*Δ (A), *rpd3*Δ (B), and *sin3*Δ (C) on silencing at the *URA3-TEL-V-R* locus are shown. All strains are isogenic derivatives of strain UCC506 and were spotted as 10-fold serial dilutions onto medium either containing (+FOA) or lacking (−FOA) 5-FOA. Impaired growth relative to the wild-type control indicates disruption of telomeric *URA3* silencing, whereas enhanced growth indicates enhanced silencing.

whereas *sap30*Δ enhanced silencing (row 4). However, the double mutants (row 3) exhibit intermediate phenotypes corresponding to an ~10-fold increase in silencing relative to the wild-type strain (row 1). These effects were the same for deletion of all three components of the Sin3-Rpd3 complex (A, B, and C). Thus, Cac3 and the Rpd3-Sin3 complex exert opposite effects on silencing in a partially offsetting manner. Interestingly, the human counterpart of Cac3, RbAp46, is found as a component of the Sin3-Rpd3 complex. However, there is no evidence that yeast Cac3 is a component of the yeast Sin3-Rpd3 complex.

Deletion of *GCN5* and *RPD3* exerts similar effects on silencing: Whereas *RPD3* encodes a histone deacetylase that is required for transcriptional repression of targeted genes (Kadosh and Struhl 1998; Rundlett *et al.* 1998), *GCN5* encodes a histone acetyltransferase required for activation of targeted genes (Kuo *et al.* 1998; Wang *et al.* 1998). These activities suggest that Rpd3 and Gcn5 exert opposite effects on transcriptional control of genes targeted by both factors. Indeed, defective activation of the *HO* gene by deletion of *GCN5* can be suppressed by deletion of *RPD3* (Perez-Martin and Johnson 1998).

These results suggested that *gcn5*Δ, in contrast to *rpd3*Δ, might weaken silencing. We tested this possibility by deleting *GCN5* in the *URA3-TEL-V-R* reporter strain UCC506. Surprisingly, *gcn5*Δ dramatically enhanced silencing, resulting in a 1500-fold increase in cell viability on FOA medium (Table 3; *cf.* UCC506 and YMH366). Furthermore, the *rpd3*Δ *gcn5*Δ double mutation (YMH370) did not increase cell viability beyond the effect of *rpd3*Δ alone (YMH320; 9000-fold). A similar effect on silencing was observed at the *hmr*Δ*A::ADE2* locus, where *gcn5*Δ resulted in formation of pink colonies, yet the isogenic wild-type strain remained white (data not shown). These effects are not a consequence of position-independent effects on *URA3* and *ADE2* expression, because neither *rpd3*Δ nor *gcn5*Δ mutations confer uracil auxotrophy, FOA resistance, or the pink colony phenotype associated with impaired *URA3* or *ADE2* expression in an otherwise normal strain. Thus, Gcn5 histone acetyltransferase, like Rpd3 histone deacetylase, counteracts silencing.

DISCUSSION

The Sin3-Rpd3 complex plays a general role in silencing: A role for Sin3 and Rpd3 in silencing at telomeric and *HM* cryptic mating loci has been shown previously (De Rubertis *et al.* 1996; Rundlett *et al.* 1996; Vannier *et al.* 1996). The results presented here confirm and extend those results by establishing that disruption of silencing at the rDNA array also requires Sin3 and Rpd3, as well as Sap30, a recently defined subunit of the Sin3-Rpd3 complex. Recently, the *RPD3*, *SIN3*, and *SAP30* genes were also identified as *IRS* genes in a genetic screen for mutations that increase rDNA silencing (Smith *et al.* 1999). Furthermore, disruption of silencing at telomeric, *HMR*, and rDNA loci is dependent upon the enzymatic activity of Rpd3. These results establish a general requirement for the Rpd3 histone deacetylase in epigenetic control of gene expression.

Relationship of Rpd3 to Rad6: Recent studies demonstrated that deletion of *RAD6* counteracts silencing at telomeric, *HM*, and rDNA loci in *S. cerevisiae*, and at the silent mating loci in *S. pombe* (Bryk *et al.* 1997; Huang *et al.* 1997; Singh *et al.* 1998). These effects are dependent upon the ubiquitin-conjugating activity of Rad6, but not its N-end rule protein-degrading activity. Conversely, the Ubp3 and (putative) Dot4 ubiquitin hydrolases counteract silencing (Moazed and Johnson 1996; Singer *et al.* 1998). Several models have been proposed to account for these results (Huang *et al.* 1997). One suggests that repression is dependent upon ubiquitination of silencing regulators. Consistent with this idea, histones H2A, H2B, and H3 are ubiquitinated by Rad6 *in vitro* (Sung *et al.* 1988; Haas *et al.* 1990) and Ubp3 physically interacts with Sir4 *in vivo* (Moazed and Johnson 1996). Ubiquitination has also been linked to silencing in Drosophila (Henchoz *et al.* 1996), and inactivation

of a ubiquitin-conjugating enzyme has been associated with chromatin defects in mice (Roest *et al.* 1996).

A striking result presented here is that *rad6*Δ is epistatic to the effect of *rpd3*Δ on silencing at the telomeric and rDNA loci (Tables 3 and 4). One possible explanation for this result is that the Rpd3 histone acetyltransferase regulates expression of *RAD6*, which in turn is required for silencing. However, Western blot analysis showed that Rad6 protein levels are essentially unchanged in isogenic wild-type, *rpd3*Δ, and *gcn5*Δ strains (data not shown). Therefore, neither Rpd3 nor Gcn5 affects silencing indirectly through *RAD6*. The more direct effect of Rad6 on silencing is consistent with the possibility that Rad6 affects silencing by ubiquitination of direct effectors of silencing.

Effects of Rpd3 and Gcn5 on silencing: The Sin3-Rpd3 complex facilitates transcriptional repression as a consequence of targeted recruitment by DNA-binding transcriptional repressors (Kadosh and Struhl 1997; Rundlett *et al.* 1998). Yet the Sin3-Rpd3 complex exerts the opposite effect on silenced loci, enhancing silencing in the absence of Sin3, Rpd3, and Sap30. How does the Sin3-Rpd3 complex counteract silencing, yet repress transcription at promoter-specific targets?

A distinct possibility is that the effect of the Sin3-Rpd3 complex is indirect. For example, Sin3-Rpd3 might repress expression of genes generally required for silencing such that in the absence of Sin3-Rpd3 these genes are derepressed, leading to increased silencing. This scenario seems likely given the general role of Rpd3 in transcriptional repression. Nonetheless, the effect of *rpd3*Δ on silencing cannot be accounted for by increased expression of either *RAD6* (above) or SIR genes. Overexpression of *SIR4* does not enhance silencing, but instead weakens silencing at *HMR*, telomeric, and rDNA loci (Sussel and Shore 1991; Renauld *et al.* 1993; Smith *et al.* 1998). Also, the negative effect of *SIR4* on *HM* silencing can be compensated by co-overexpression of *SIR3* (Marshall *et al.* 1987). Sir2 is the only SIR protein required for silencing at all three loci. However, increased *SIR2* expression slightly weakens silencing at the *HM* loci (M. Cockell and S. M. Gasser, personal communication), yet enhances rDNA silencing (Fritze *et al.* 1997; Smith *et al.* 1998; Z.-W. Sun, unpublished results). Thus, enhanced silencing associated with loss of Sin3-Rpd3 function cannot be accounted for by overexpression of *SIR* genes.

A notable and unexpected result presented here is that deletion of *GCN5* enhances silencing (Table 3). Accordingly, loss of Gcn5 histone acetyltransferase activity has the same effect on silencing as loss of Rpd3 histone deacetylase activity. This result was surprising because *GCN5* and *RPD3* exert opposite effects on transcriptional control of genes targeted by both factors. Indeed, defective activation associated with deletion of *GCN5* can be suppressed by deletion of *RPD3* (Perez-Martin and Johnson 1998).

The similar effects of *rpd3*Δ and *gcn5*Δ on silencing might be an important clue toward understanding how Rpd3 and Gcn5 influence silencing. Even though *rpd3*Δ and *gcn5*Δ affect the expression of a broad range of genes, *rpd3*Δ generally enhances transcription, whereas *gcn5*Δ impairs transcription. A stimulatory effect of *gcn5*Δ on expression of silencing factors would be opposite to its effect on most genes. This possibility seems especially unlikely if the same factors are also affected by *rpd3*Δ. An alternative possibility, described below, is that Rpd3 and Gcn5 affect silencing directly by generating the histone acetylation pattern specific to silent chromatin.

A model for the role of Sin3-Rpd3 in silencing: A substantial body of evidence indicates that silencing is a consequence of modified chromatin structure (Kayne *et al.* 1988; Megee *et al.* 1990; Park and Szostak 1990; Gottschling 1992; Singh and Klar 1992; Chen-Cleland *et al.* 1993). Analysis of the patterns of histone acetylation at the *HM* loci revealed that histones H3 and H4 are hypoacetylated relative to their counterparts in transcriptionally active regions of the genome (Braunstein *et al.* 1993). Moreover, the acetylation pattern of H4—hypoacetylation of lysines at positions 5, 8, and 16 (K5, K8, and K16) and hyperacetylation of lysine at position 12 (K12)—is identical to the H4 acetylation pattern in Drosophila heterochromatin (Turner *et al.* 1992; Braunstein *et al.* 1996). These results underscore the importance of acetylation and deacetylation of specific histone residues in regulating silencing.

Perhaps Rpd3 and Gcn5 affect silencing by catalyzing formation of the histone acetylation pattern resident in silent chromatin. Newly synthesized histone H4 is acetylated at K5 and K12, which are conserved modifications among humans, Drosophila, and Tetrahymena, and this pattern is thought to be important for assembly of H4 onto replicating DNA (Allis *et al.* 1985; Sobel *et al.* 1994, 1995). The CAF-I chromatin assembly factor deposits newly synthesized histones H3 and H4, but not those from bulk chromatin, onto DNA (Smith and Stillman 1991). Indeed, human CAF-I exhibits substrate specificity for H4 acetylated at K5, K8, and/or K12 (Verreault *et al.* 1996). A similar chromatin assembly complex has also been identified in yeast (Kaufman *et al.* 1997). Furthermore, acetylation of one or more K5, K8, and K12 residues of H4 provides the recognition signal for chromatin assembly (Ma *et al.* 1998), suggesting that the substrate specificities of the human and yeast CAF-I complexes are similar. Mutations in the *CAC1*, *CAC2*, and *CAC3* genes, which encode yeast CAF-I (Kaufman *et al.* 1997), decrease telomeric, *HM*, and rDNA silencing (Enomoto *et al.* 1997; Kaufman *et al.* 1997; Monson *et al.* 1997; Enomoto and Berman 1998; Smith *et al.* 1999). These observations led to the proposal that CAF-I provides the substrate specificity to ensure that nucleosomes are assembled from appropri-

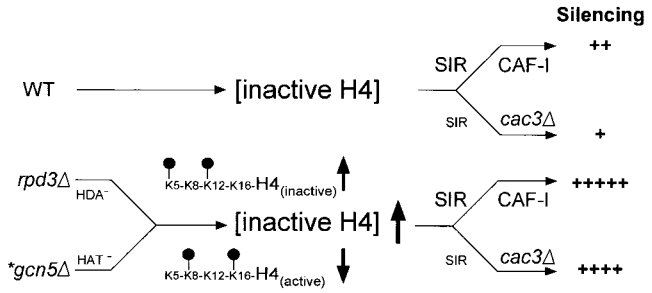

Figure 5.—A model to account for the general effects of *rpd3*Δ, *gcn5*Δ, and *cac3*Δ on silencing. The *rpd3*Δ (*sin3*Δ and *sap30*Δ) mutation eliminates Sin3-Rpd3 histone deacetylase (HDA) activity. Because Rpd3 has specificity for K5 and K12 of histone H4 (Rundlett *et al.* 1996), *rpd3*Δ would increase the level of the "inactive" or "prosilencing" form of H4. The *gcn5*Δ mutation eliminates Gcn5 histone acetyltransferase (HAT) activity. Because Gcn5 has specificity for K8 and K16 of H4 (Kuo *et al.* 1996), *gcn5*Δ would decrease the level of the "active" or "antisilencing" form of H4. Consequently, both *rpd3*Δ and *gcn5*Δ would enhance silencing by increasing the relative level of the inactive form of H4. The *cac3*Δ deletion would inactivate the CAF-I chromatin assembly complex, which has been proposed to ensure that only inactive histones are assembled into silent chromatin and that local SIR protein concentrations are elevated to form a "wall" of silent chromatin (Monson *et al.* 1997; Enomoto and Berman 1998). Consequently, *cac3*Δ would weaken silencing in both the wild-type (++ *vs.* +) and *rpd3*Δ background (+++++ *vs.* ++++) due to loss of substrate specificity for the inactive form of H4, and to decreased local SIR protein concentrations (denoted by large and small SIR fonts). The asterisk preceding *gcn5*Δ denotes that the effect of *cac3*Δ has not been tested in a *gcn5*Δ background. The black circles denote acetylation at the indicated lysine residues (K) of histone H4. The vertical arrows denote increased levels of the indicated forms of H4. This model is also applicable for histone H3, because H3 can be a substrate for Rpd3 (Rundlett *et al.* 1996), Gcn5 (Kuo *et al.* 1996), and CAF-I (Verreault *et al.* 1996), and is a structural component of silent chromatin (Grunstein 1998). However, the specific acetylation pattern of H3 in silent chromatin has yet to be defined.

ately acetylated histones (Monson *et al.* 1997; Enomoto and Berman 1998).

The following model is proposed to explain the role of Rpd3 in silencing (Figure 5). Accordingly, Rpd3 would play a direct role in silencing by affecting the relative levels of the "inactive" (heterochromatin) and "active" (euchromatin) forms of histone H4. This ratio would affect the efficiency of formation of silent chromatin in much the same way that components of silent chromatin and a transcriptional activator compete to establish either the silent or active state of gene expression at telomeres following the disassembly of silent chromatin during DNA replication. This effect was proposed to account for the random nature of phenotypic switching in variegated gene expression (Aparicio and Gottschling 1994). Our model is consistent with the recent identification of multiple genes associated with DNA replication and chromatin modification in a genetic screen for rDNA silencing defects (Smith *et al.* 1999). In the *sin3*Δ, *rpd3*Δ, or *sap30*Δ strains, the relative levels of H4 acetylated at K5 and K12 would increase due to loss of histone deacetylase activity. H4 acetylated at K12 is the inactive form, thereby accounting for enhanced silencing associated with loss of Sin3-Rpd3 function. This scenario is dependent upon substrate specificity of Rpd3 for H4 K12. Indeed, *rpd3*Δ enhances acetylation of H4 residues K5 and K12 (Rundlett *et al.* 1996).

This model would also account for the enhanced silencing associated with *gcn5*Δ (Table 3). Accordingly, the Gcn5 histone acetyltransferase would directly affect silencing by catalyzing acetylation of H4 residues K8 and K16. Consistent with this premise, an H4 K16Q replacement, which simulates acetylated K16, disrupts the interaction between H4 and Sir3 (Hecht *et al.* 1995). This result led to the proposal that K16 hypoacetylation might be important for H4 interaction with Sir3 in heterochromatin (Grunstein 1998). In the *gcn5*Δ strain, the levels of H4 acetylated at K8 and K16 would decrease, thereby increasing the relative levels of the inactive form of H4 acetylated at K5 and K12. Again, this proposal is consistent with the specificity of Gcn5 for H4 residues K8 and K16 (Kuo *et al.* 1996). This model is also applicable for histone H3, because H3 can be a substrate for Rpd3 (Rundlett *et al.* 1996), Gcn5 (Kuo *et al.* 1996), and CAF-I (Verreault *et al.* 1996), and is a structural component of silent chromatin (Grunstein 1998). However, the specific acetylation pattern of H3 in silent chromatin has yet to be defined.

To facilitate inheritance of silencing, CAF-I would ensure that only appropriately acetylated inactive histones (both newly synthesized and recycled from the previous cell cycle) are assembled into silent chromatin (Monson *et al.* 1997; Enomoto and Berman 1998). CAF-I might also exclude histones with the active acetylation pattern from being recycled into silent chromatin. In the case of a derepressed silent locus from the previous cell cycle, this function would be especially relevant (Enomoto and Berman 1998). In the *cac*Δ mutants, new nucleosomes must be assembled by an alternative pathway (Monson *et al.* 1997; Kaufman *et al.* 1998; Qian *et al.* 1998). If the alternative assembly complex lacks the substrate specificity of CAF-I, then the increased level of inactive histones associated with the absence of either Rpd3 or Gcn5 would facilitate silent chromatin assembly. This would account for the offsetting effects of *cac3*Δ and either *rpd3*Δ, *sin3*Δ, or *sap30*Δ (Figure 4).

A second function of CAF-I would be to ensure that local Sir2, Sir3, and Sir4 protein concentrations are sufficiently elevated to permit assembly of a strong silencer. This conclusion is based on improved silencing associated with elevated levels of Sir2, Sir3, or Sir4 in *cac1*Δ mutants, and on disruption of silencing associated with limiting amounts of Sir2 or Sir3 in an otherwise wild-type background (Enomoto and Berman 1998). Therefore, the decreased local SIR protein concentra-

tions associated with *cac3*Δ would partially weaken the enhanced silencing caused by *sin3*Δ, *rpd3*Δ, and *sap30*Δ. This is consistent with the observation that loss of the Sin3-Rpd3 complex does not bypass the SIR protein requirement for maintaining silencing (Figure 2 and Vannier *et al.* 1996).

A key feature of this model is that the acetylation state of histones affects the efficiency of assembly of silent chromatin. The model does not propose that the acetylation pattern at silent loci would necessarily change upon deletion of *RPD3* or *GCN5*. Indeed, chromatin immunoprecipitation experiments, demonstrating that *rpd3*Δ and *sin3*Δ alter the acetylation pattern of histone H4 at Ume6-regulated promoters, showed that the H4 acetylation pattern at a telomeric locus is unchanged by *rpd3*Δ and *sin3*Δ (Rundlett *et al.* 1998), despite the dramatic effects of these mutations on telomeric silencing.

We are grateful to Jeff Smith, Jef Boeke, Susan Gasser, Ken Robzyk, and Mary Ann Osley for communicating results prior to publication. We also thank Mike Christman, Shelley Esposito, Dan Gottschling, and Lucy Pemberton for yeast strains; Kevin Struhl for *rpd3* alleles; and Jim Broach, Leonard Guarente, Kiran Madura, David McNabb, Ines Pinto, Louise Prakash, David Stillman, and Fred Winston for plasmids. We also acknowledge David Gross, Yi Zhang, and Danny Reinberg for fruitful discussions and critical review of the manuscript. Research in M.H.'s laboratory is supported by National Institutes of Health grant GM-39484.

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Communicating editor: F. Winston