3 RESULTS

3.1 The Role of the Enzymatic Activity of Sir2 for Efficient Association of the SIR Complex with DNA

The Sir proteins are recruited to DNA by site-specific DNA-binding proteins and subsequently spread along the chromatin fiber. I wanted o investigate this spreading mechanism, which is currently poorly understood. To address this question, I employed the chromatin immunoprecipitation (ChIP) assay.

On the basis that the enzymatic activity of Sir2 is an absolute requirement for all silencing in yeast, it can be rationalized that mutations specifically disrupting Sir2's activity may interfere with any of several steps: The initial recruitment of Sir2 and other silencing proteins to DNA, the spreading of these proteins along the chromatin fiber, or a step subsequent to spreading of silencing proteins. The ChIP assay allowed me to analyze the association of each Sir protein with DNA fragments to distinguish between these possibilities. In these experiments, specific DNA products were amplified by PCR in the presence of a radioactively labeled nucleotide (Hecht et al., 1996; Kuras and Struhl, 1999). The association of each protein with the DNA fragments was then assessed by quantitation of the phosphoimager data.

In these experiments, a strain expressing a wild type Sir2 protein was compared to a strain which expresses the sir2-H364Y allele, which encodes an enzymatically inactive protein. As a control, a strain in which the *SIR2* locus is deleted (*sir2*Δ strain) was used to assess the intensity of the background signal. It should be noted that previous data shows that recruitment of both Sir3 and Sir4 is Sir2-dependent (Strahl-Bolsinger et al., 1997). In addition, a strain expressing a sir2-G262Y mutant was used to confirm the results obtained with the sir2-H364Y mutation.

3.1.1 The Association of the SIR Complex with Telomeres

To analyzed the recruitment of the SIR complex to telomeres, four target sequences were chosen, 0.6, 1.4, 1.8 and 4.7 kb away from the telomeric repeats on the right arm of chromosome VI (Figure 3.1A).

In a wild type background, Sir2, Sir3, and Sir4 were associated with DNA at distances of 0.6, 1.4, and 2.8 kb from the telomere, and Sir3 was still associated with DNA 4.7 kb away from the telomere (Figure 3.1B), which was also reported previously (Hecht et al., 1996; Strahl-Bolsinger et al., 1997). In a strain containing the *sir2-H364Y* allele, the association of Sir2 with a DNA fragment at 0.6 kb from the telomere was greatly reduced (Figure 3.1B), which is also consistent with previous observations (Tanny et al., 1999). Moreover, in the *H364Y* mutant strain, the association of Sir3 and Sir4 with this telomeric DNA fragment was diminished to levels that were the same or only slightly higher than that observed in a *sir2*Δ strain. The association of Sir2, Sir3, and Sir4 with DNA fragments at 1.4, 2.8, and 4.7 kb from the telomere was diminished to levels that were similar to those observed in a *sir2*Δ strain (Figure 3.1B). As shown in figure 3.4, quantitation of the ChIP data from two independent experiments showed that the Sir2, Sir3, and Sir4 proteins bound 3 to 5 times more DNA at 0.6 to 1.4 kb away from the telomere in *SIR2* strains as compared to *sir2*Δ and *sir2-H364Y* strains.

To confirm the above results, I used a different Sir2 catalytic site mutation, that converts the conserved glycine 262 to an alanine residue (sir2-G262A). Like sir2- H364Y, this mutation abolished the enzymatic activity of Sir2 *in vitro* and all silencing *in vivo* (Tanny and Moazed, 2001; J. C. Tanny, personal communication). As shown in figure 3.1C, similar results were obtained. That is, the signals for Sir2 and Sir3 were greatly diminished over those obtained from *SIR2* strains. In both *sir2-H364Y* and *sir2-G262A* strains, the levels of Sir2 associated with DNA fragments at distances of 0.6 and 1.4 kb away from the telomere

were higher than background, suggesting that some binding to DNA could occur independently of Sir2's enzymatic activity (Figure 3.1B and C).

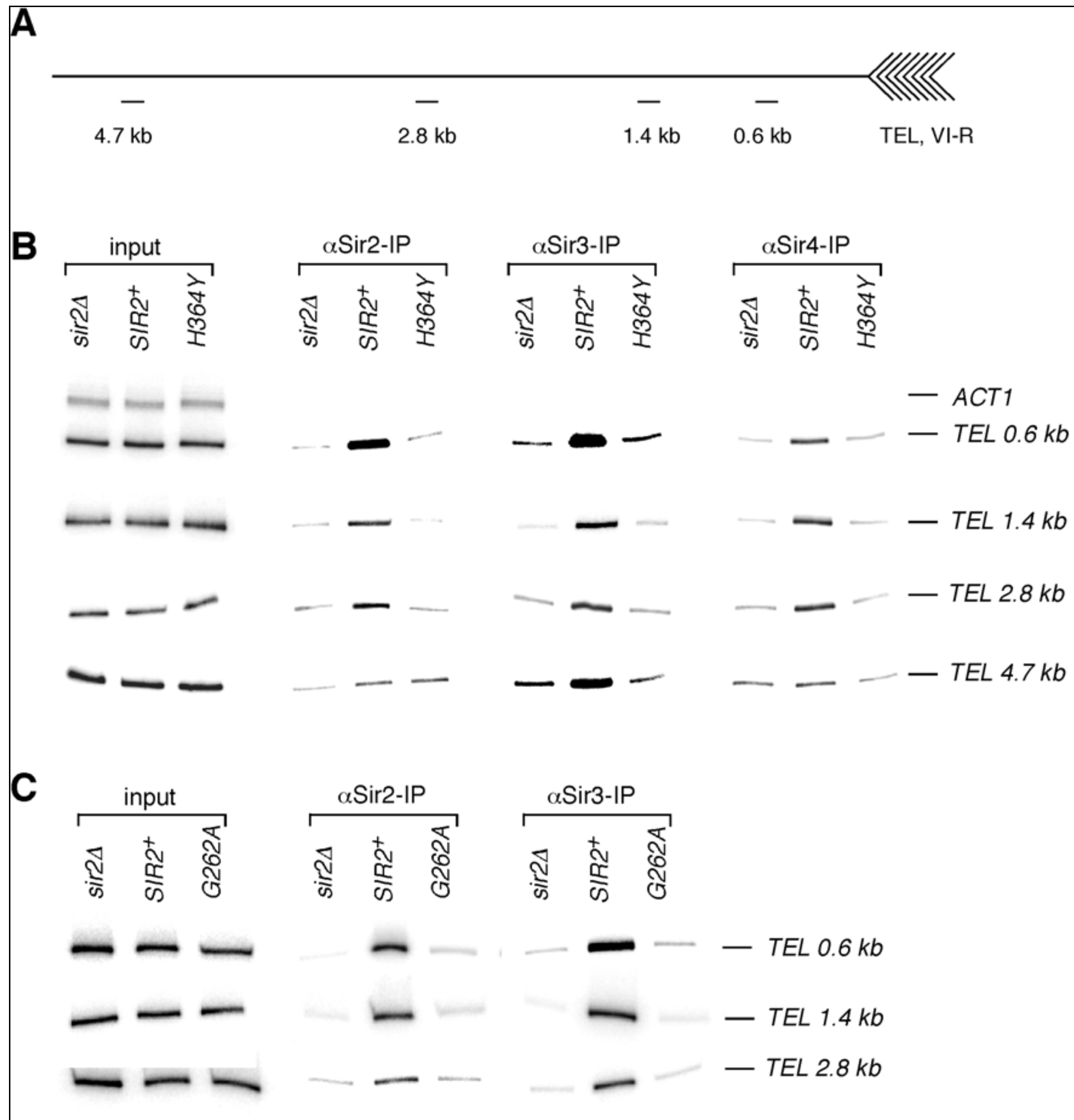

FIGURE 3.1 Chromatin immunoprecipitation (ChIP) experiments showing the association of the Sir2, Sir3, and Sir4 proteins with telomeric DNA regions in *sir2*Δ, *SIR2* wild type, and *sir2*-mutant strains. **(A)** Schematic diagram showing the location of PCR primers, corresponding to the subtelomeric region on the right arm of chromosome VI (TEL, VI-R), used in ChIP experiments. The telomeric primers amplify DNA fragments from 0.6, 1.4, 2.8, and 4.7 kb from the telomeric repeats.
(B and C) Chromatin immunoprecipitation was carried out from a *sir2* deletion strain (*sir2*Δ), a *SIR2* wild type strain (*SIR2*$^{+}$), and strains containing *sir2* alleles that encode enzymatically inactive Sir2 proteins (*sir2-H364Y* in panel B and *sir2-G262A* in panel C) with anti-Sir2, anti-Sir3, and anti-Sir4 antibodies. Panels show phosphoimager data of PCR amplifications corresponding to chromatin input and immunoprecipitated chromatin for the indicated regions of telomere VI-R.

Taken together, these results suggest that the enzymatic activity of Sir2 is required for the efficient association of the Sir2, Sir3, and Sir4 proteins with telomeric DNA regions as well as for their spreading along the chromatin fiber.

3.1.2 The Association of the SIR Complex with the *HML-E* Silencer

The mechanism of SIR complex recruitment at the silent mating type loci is different than that acting at the telomeres in several ways. For one, silencing initiates at well-defined non-repetitive regulatory sites, called silencers. This offers the unique opportunity to investigate the role of Sir2's enzymatic activity at a unique initiator element, whereas silencing at telomeres is initiated at repetitive sites. Also, the recruitment of the SIR complex is mediated by a different set of proteins: Whereas the recruitment at telomeres is mediated by yKu70/yKu80 and Rap1, silencing at the silent mating type cassettes requires the Origin Recognition Complex (ORC), Abf1, and also Sir1 (reviewed in Gartenberg, 2000). The latter is believed to contribute to the establishment of silencing by recruiting the Sir2/Sir4 complex to the silencer (Fox et al., 1997; Pillus and Rine, 1989; Triolo and Sternglanz, 1996). This may of course result in different requirements for the activity of Sir2. I therefore wanted to investigate the role of Sir2' s enzymatic activity at a silencer of a silent mating type cassette.

In order to differentiate whether Sir2 activity is required for the initial assembly of the SIR complex on DNA or whether it is required for its spreading along the chromatin fiber, I investigated the role for the enzymatic activity of Sir2 in the assembly of the SIR complex at the *HML-E* silencer, located on the left arm of chromosome III (see Figure 3.2A). Here, the effect of the *H364Y* mutation on crosslinking of Sir proteins to the silencer is tested.

The association of Sir2, Sir3, and Sir4 with the *HML-E* silencer was greatly diminished in the *sir2-H364Y* strains. This is also true for a DNA fragment in the α2 open reading frame, located 0.6 kb away from the silencer (Figure 3.2B). However, in both the *sir2-H364Y* and *sir2-G262A* mutant strains, the anti-Sir2 antibody precipitated the *HML-E*

DNA fragment at levels above background. This suggests that the mutant protein could weakly assemble onto the silencer (Figure 3.2B-C and Figure 3.4).

Taken together, these results suggest that the enzymatic activity of Sir2 is required for the stable initial assembly of the SIR complex on a silencer.

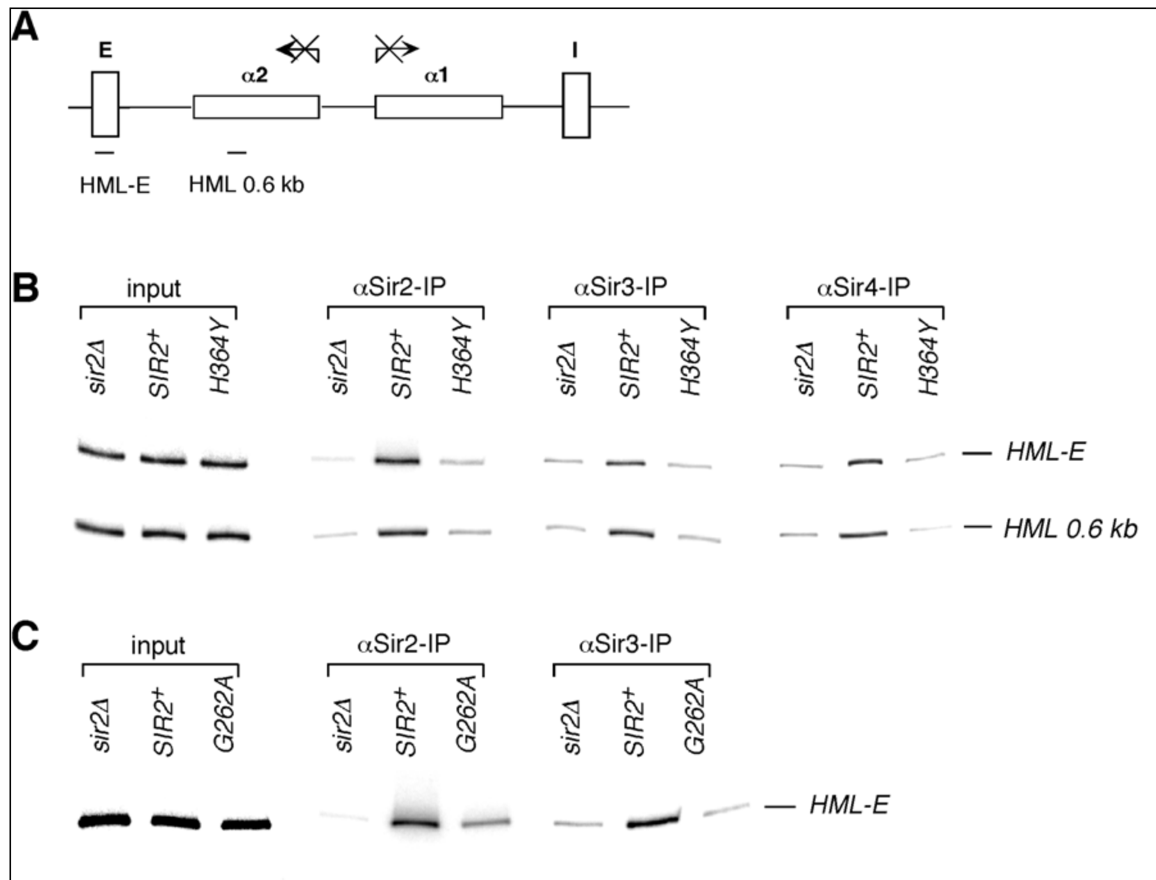

FIGURE 3.2 Chromatin immunoprecipitation (ChIP) experiments showing the association of the Sir2, Sir3, and Sir4 proteins with the *HML* mating type locus in *sir2*Δ, *SIR2* wild type, and *sir2-H364Y* strains.
(A) Schematic diagram showing the location of PCR primers, corresponding to the *HML* locus, used in ChIP experiments. The HML primers flank the E silencer (*HML-E*) or are located 0.6 kb from the silencer within the α2 coding region.
(B and C) Chromatin immunoprecipitation was carried out from a *sir2* deletion strain (*sir2*Δ), a *SIR2* wild type strain (*SIR2*$^{+}$), and strains containing *sir2* alleles that encode enzymatically inactive Sir2 proteins (*sir2-H364Y* in panel B and *sir2-G262A* in panel C) with anti-Sir2, anti-Sir3, and anti-Sir4 antibodies.

3.1.3 The Association of the RENT complex with rDNA

Sir2 localizes to the rDNA repeats as a component of the RENT complex. This multi-protein complex also contains the Net1 and Cdc14 proteins (Gotta et al., 1997; Shou et al.,

1999; Straight et al., 1999). Interestingly, the association of Net1 with rDNA fragments does not require Sir2, as deduced from genetic evidence (Straight et al., 1999). It therefore seems likely that the mechanism of this kind of spreading is different from that of the SIR complex.

To investigate the requirement for the enzymatic activity of Sir2 for its association with rDNA fragments, I tested the association of Sir2 and Sir2-H364Y with DNA fragments throughout the rDNA repeat (Figure 3.3A).

In contrast to what I observed for both the telomeres and the *HML* locus (Figures 3.1 and 3.2), association of the sir2-H364Y protein with rDNA was similar to that observed with wild type Sir2 (Figure 3.3). Quatitation of the phosphoimager data in figure 3.3 showed that the efficiency of crosslinking for sir2-H364Y to various rDNA fragments was between 69 to 88 % of wild type Sir2 (Figure 3.4). As was reported previously by Gotta et al. (1997), Sir2 localized to the rDNA non-transcribed spacer with the highest efficiency. Its association was weakest with 25S and 18S DNA fragments, which was less than two fold above background (Figure 3.3B-C and Figure 3.4).

Based on these and previous observations by Straight et al. (1999) demonstrating that the Net1 and Cdc14 proteins localize to rDNA independently of Sir2, I conclude that the RENT complex localizes to rDNA independently of interactions involving Sir2 or its enzymatic activity.

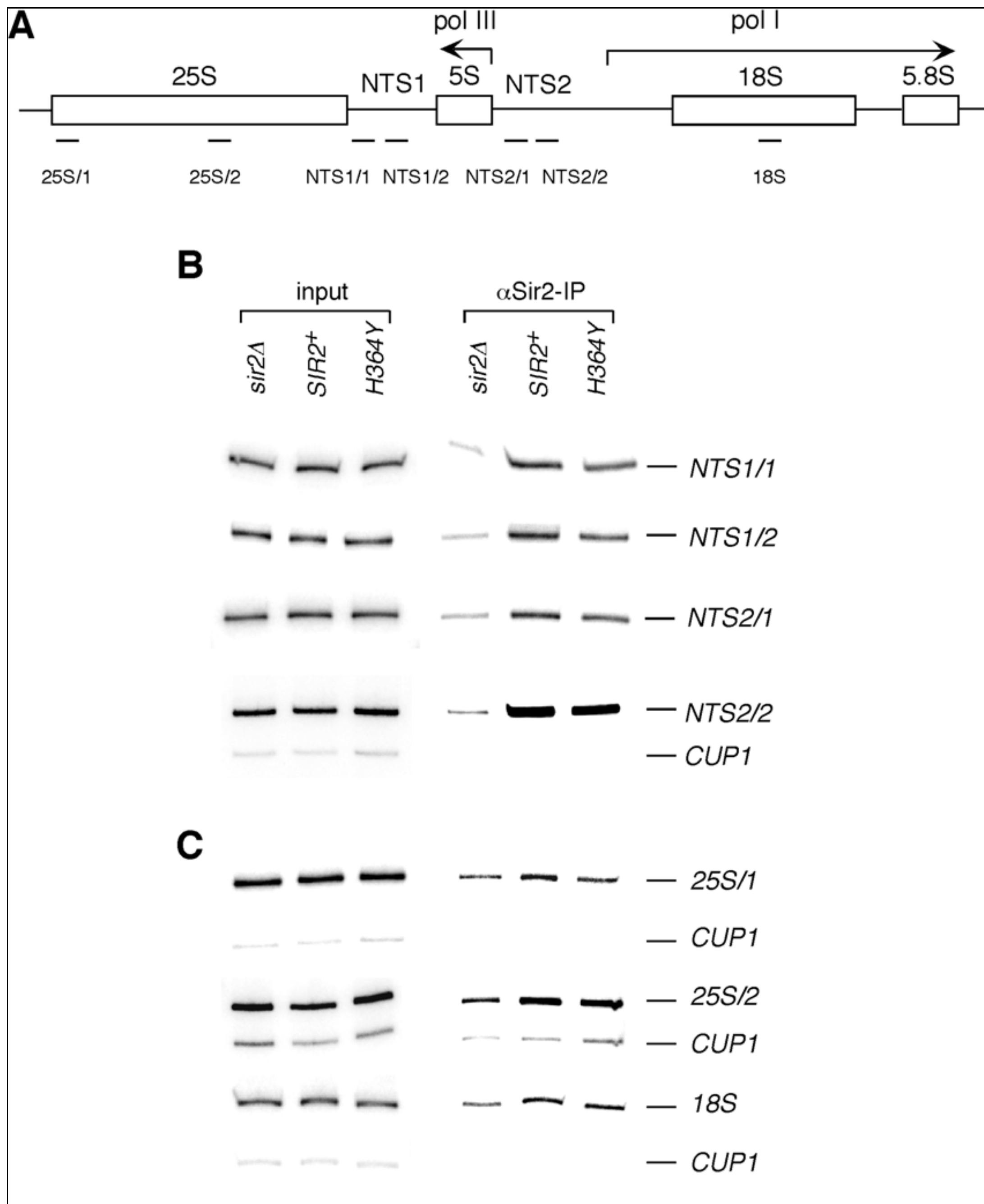

FIGURE 3.3 Chromatin immunoprecipitation experiments showing that the enzymatic activity of Sir2 is not required for the efficient association of Sir2 with the rDNA locus.
(A) Schematic diagram of a single rDNA repeat unit showing the position of the polI-transcribed 18S, 5.8S, and 25S genes within the 35S rRNA precursor as well as the polIII-transcribed 5S RNA. The location of DNA fragments amplified in the ChIP experiments is indicated under the rDNA repeat.
(B and C) Panels show phosphoimager data of PCR amplifications corresponding to input and immunoprecipitated chromatin for the indicated regions of rDNA.

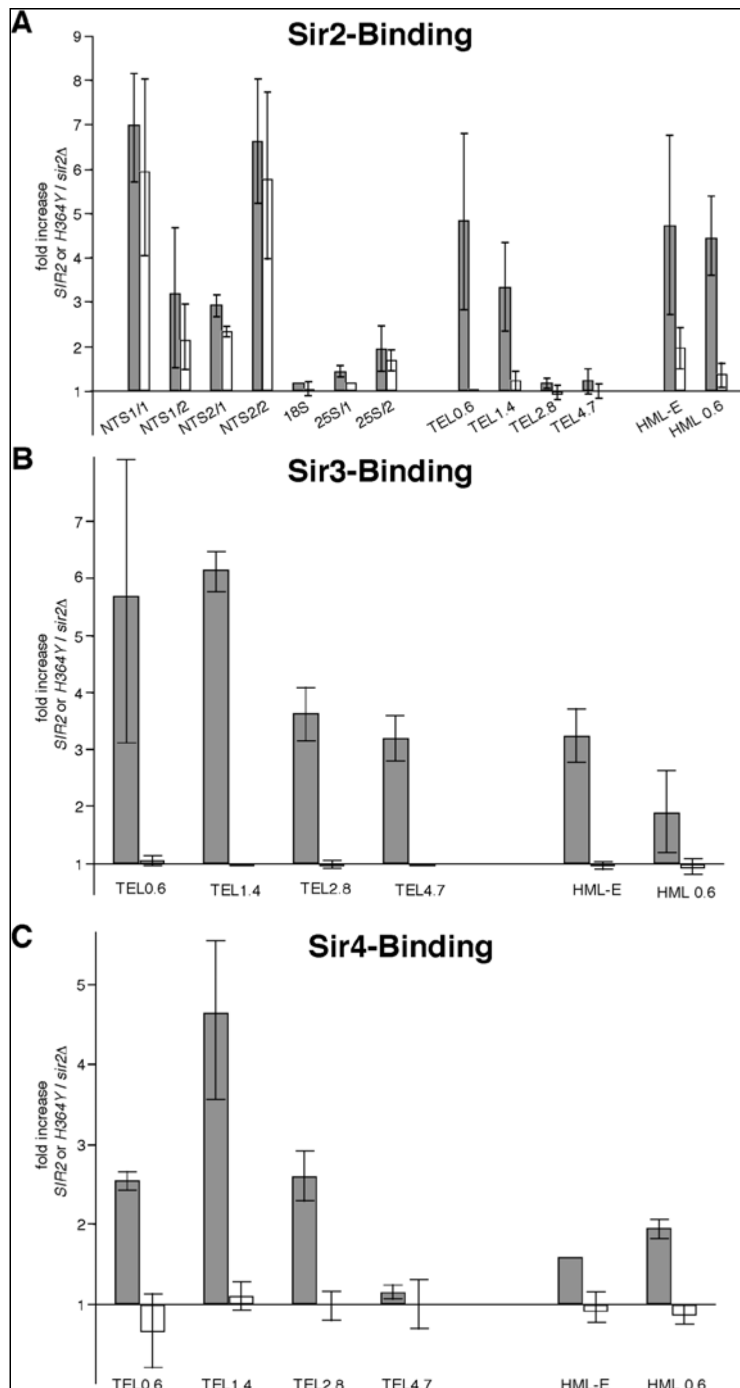

FIGURE 3.4 Quantitation of the chromatin immunoprecipitation data.
Phosphoimager data from two independent ChIP experiments (one of which is shown in Figures 3.1, 3.2, and 3.3) were quantitated and average values are shown in each panel. In each case parallel immunoprecipitations were carried out from crosslinked chromatin fractions prepared from *SIR2*, *sir2*Δ, and *sir2-H364Y* backgrounds. Vertical bars denote the fold enrichment for DNA fragments precipitated with anti-Sir2 **(A)**, anti-Sir3 **(B)**, and anti-Sir4 **(C)** antibodies from *SIR2* wild type (shaded bars) or *sir2-H364Y* (open bars) chromatin relative to *sir2*Δ chromatin.

3.1.4 The Enzymatic Activity of Sir2 and Subnuclear Localization of the Sir Proteins

Previously, the Sir2, Sir3, and Sir4 proteins have been shown to localize near the nuclear periphery, which is thought to stem from their association with cluster-forming telomeres (Palladino et al., 1993). In addition, it was shown that Sir2 also localizes to the nucleolus, revealing a crescent-shaped structure (Gotta et al., 1997). To obtain an independent line of evidence for the requirement of the enzymatic activity of Sir2, the localization patterns of Sir2, Sir3, and Sir4 as GFP-fusion proteins in cells with *SIR2* and *sir2-H364Y* background were investigated.

In strains containing the enzymatically inactive Sir2-H364Y-GFP protein, the characteristic telomeric foci corresponding to Sir2-GFP were lost (compare Figure 3.5A and B). Similarly, the telomeric foci of Sir3-GFP and GFP-Sir4 observed in *SIR2* cells (Figure 3.5C and E) became dispersed throughout the nucleus in cells containing the *sir2-H364Y* allele. In contrast, Sir2-H364Y-GFP remains localized to a nuclear subdomain that resembles the nucleolus (compare Figure 3.5A and B).

These results show that the enzymatic activity of Sir2 is dispensable for its localization to the nucleolus, but is required for the localization of the SIR complex to telomeres, corroborating the ChIP data in figures 3.1, 3.2, and 3.3.

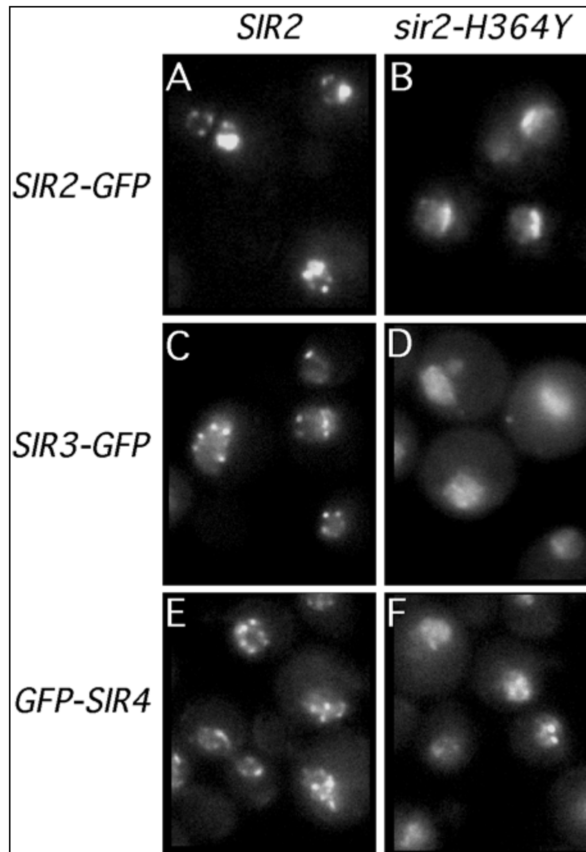

FIGURE 3.5 The enzymatic activity of Sir2 is required for the localization of the SIR complex to telomeric foci but not for the localization of Sir2 to the nucleolus.
The localization of each Sir2-GFP, Sir3-GFP, and Sir4-GFP in cells containing either wild type Sir2 **(A, C, and E)** or the enzymatically inactive sir2-H364Y **(B, D, and F)** is shown. Panels **A** and **B** show the localization of Sir2-GFP and sir2-H364Y-GFP, respectively. (In cooperation with J. C. Tanny)

3.2 The Role of Sir2 in the Recruitment of Sir3 to rDNA

The Sir3 protein is normally not required for efficient rDNA silencing (Smith and Boeke, 1997). However, it relocalizes to the nucleolus in strains containing mutations that disrupt silencing at the telomeres. Sir3 may also play a role in the extension of life span as observed in the *sir4-42* allele, a truncation mutation of SIR4 (Kennedy et al., 1997). In *sir4-42* cells, Sir3 is relocated from telomeric foci and is relocalized to the nucleolus in a Sir2-dependent fashion (Gotta et al., 1997; Kennedy et al., 1997). Two explanations seem possible, namely that the mere presence of Sir2 in the nucleolus was sufficient for recruitment of Sir3 to rDNA and the nucleolus, or that the recruitment of Sir3 is facilitated by Sir2's activity, providing for another step which is required for this event.

The fact that enzymatically inactive Sir2 associates with rDNA with nearly wild type efficiency, as shown in Figure 3.3, enabled me to test which of these two possibilities is the cause for Sir3 recruitment to the nucleolus. Two different methods were used for an independent assessment: First, the localization of Sir3-GFP was observed in *SIR2* or *sir2-H364Y*, and *SIR4* or *sir4*Δ strains. Second, I used the ChIP assay to analyze the association of Sir3 with rDNA fragments in both *SIR4* and *sir4*Δ strains containing either wild type Sir2 or the enzymatically inactive sir2-H364Y mutant.

The deletion of *SIR4* in a *SIR2* wild type strain resulted in the redistribution of Sir3-GFP from telomeric foci to a subnuclear region resembling the nucleolus, such as previously reported by Gotta et al. (1997) (compare Figure 3.6B and D). In contrast, in the *sir2-H364Y* strains, Sir3-GFP was dispersed throughout the nucleus in the *SIR4* (Figure 3.6H) as well as the *sir4*Δ strains (Figure 3.6F).

These results suggest that the enzymatic activity of Sir2, rather than the presence of Sir2 in the nucleolus, is required for the efficient recruitment of Sir3.

Consistent with the localization data in Figure 3.6, I found Sir3 bound to rDNA only in the presence of wild type Sir2. Its localization to rDNA in a *sir2-H364Y* background was similar to that in a *sir2*Δ background (Figure 3.7A). The deletion of *SIR4* resulted in an increase in the amount of Sir3 associated with rDNA fragments. This association required both Sir2 and its enzymatic activity (Figure 3.7B).

Surprisingly, I found that low levels of Sir3 are normally associated with rDNA even in a *SIR4* wild type background and that this association is Sir2-dependent. I also found that the Sir4 protein associated with rDNA at levels above background in a fashion that requires the enzymatic activity of Sir2 (Figure 3.7B).

Taken together, these results suggest that the enzymatic activity of Sir2 is required for the association of Sir3 with DNA fragments throughout rDNA.

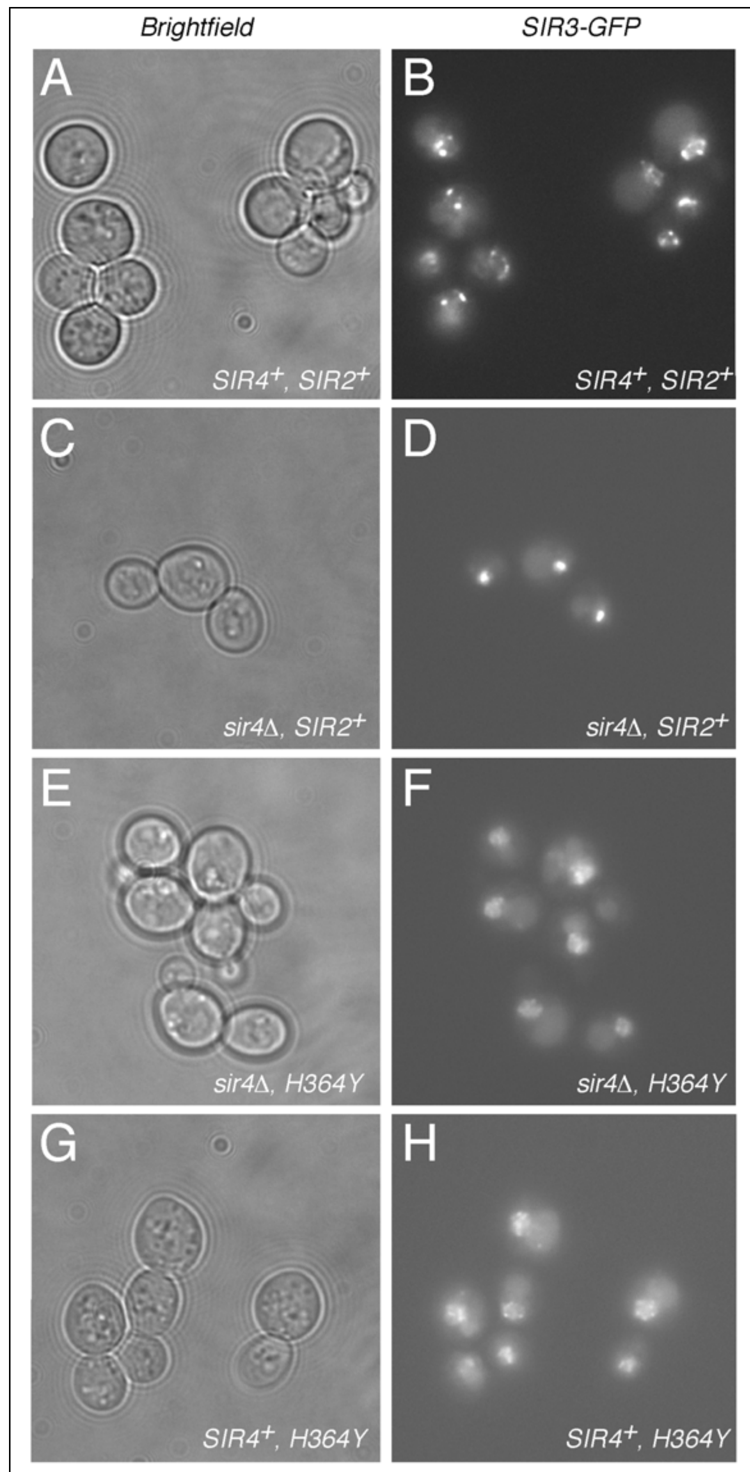

FIGURE 3.6 The relocalization of Sir3 to the nucleolus in *sir4*Δ cells requires the enzymatic activity of Sir2. Sir3-GFP localization in *SIR4*, *SIR2* **(B)**, *sir4*Δ, *SIR2* **(D)**, *sir4*Δ, *sir2-H364Y* **(F)**, and *SIR4*, *sir2-H364Y* **(H)** cells is shown. **A, C, E,** and **G** are brightfield images of the corresponding GFP fluorescent images in **B, D, F,** and **H,** respectively.

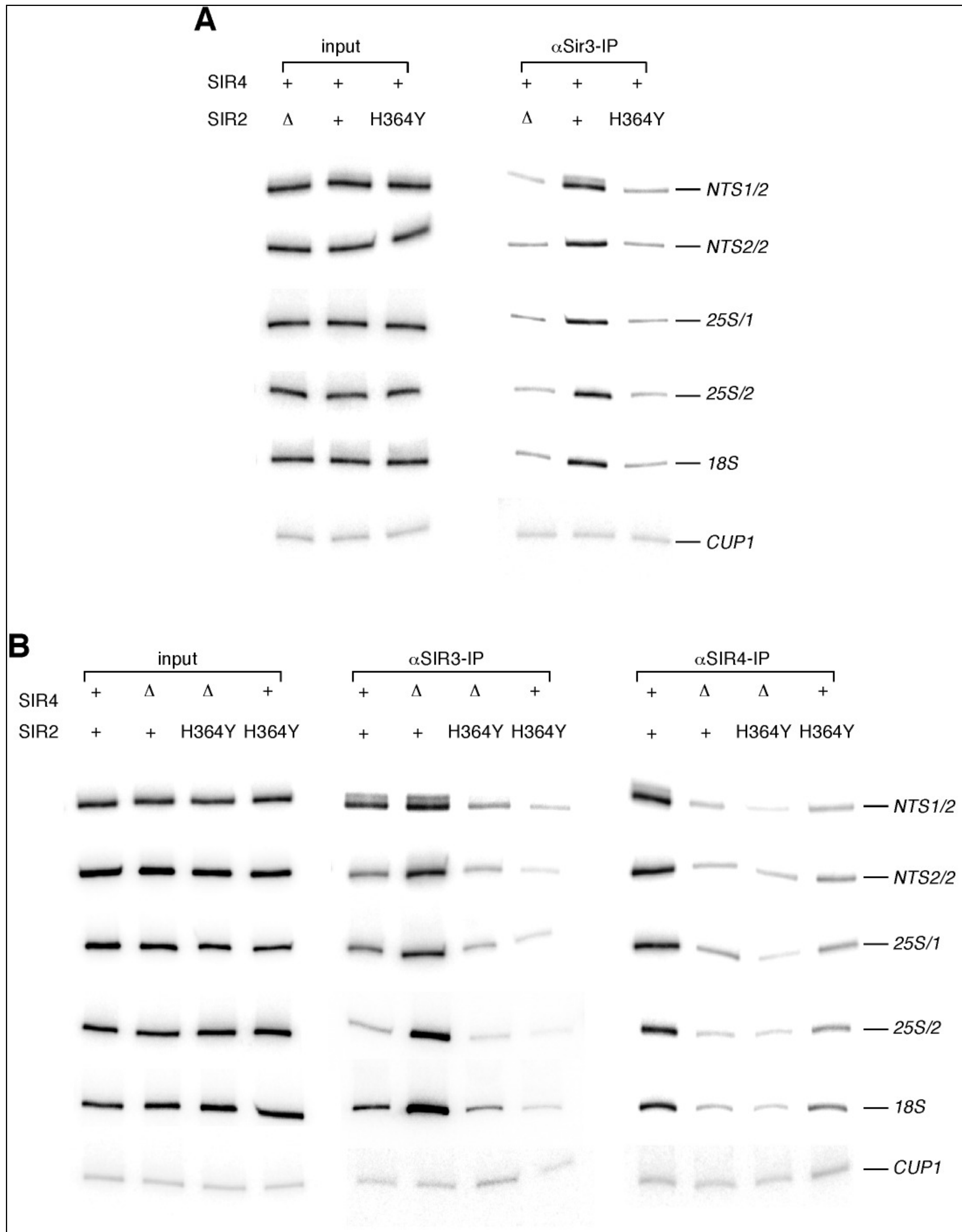

FIGURE 3.7 Chromatin immunoprecipitation experiments showing that the association of Sir3 with rDNA requires the enzymatic activity of Sir2.
(A) Association of Sir3 with rDNA fragments in sir2Δ, SIR2 wild type, and sir2-H364Y strains containing wild type SIR4. (B) Association of Sir3 and Sir4 proteins with rDNA fragments in strains with the indicated genotype.

3.3 A Role For Sir3 in rDNA Silencing?

It has previously been shown that rDNA silencing is independent of Sir3 silencing (Smith and Boeke, 1997). Since my results obtained by ChIP assay (Figure 3.7) suggest that Sir3 might indeed play a role in rDNA silencing, I wanted to test this idea using a silencing assay. A mURA3 marker gene was introduced either into the NTS region (Figure 3.8A), or the 25S region of a rDNA unit (Figure 3.8B).

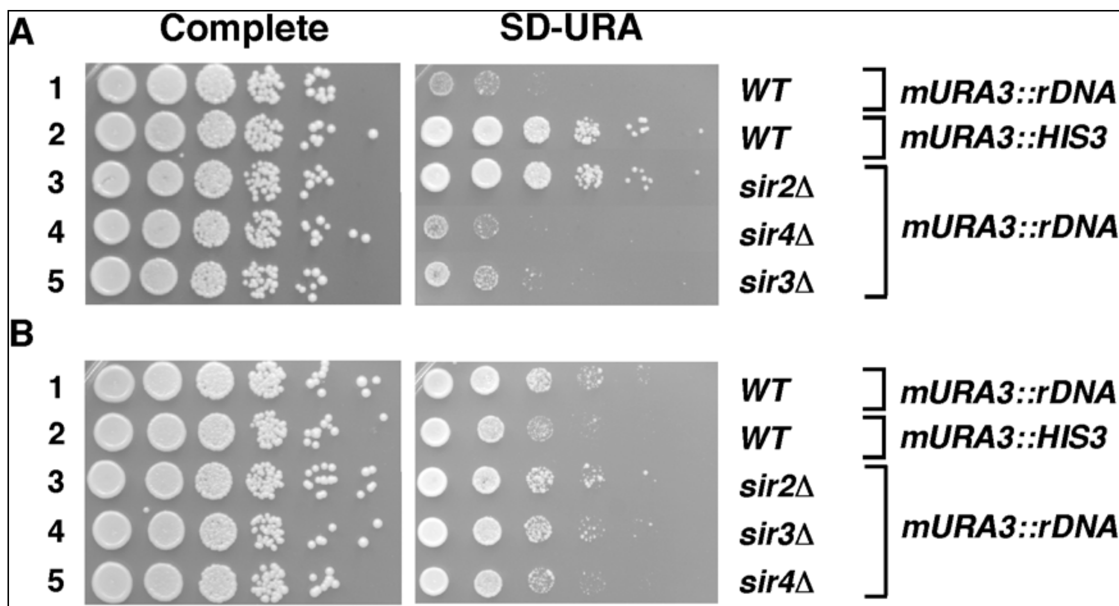

FIGURE 3.8 rDNA silencing is reduced in sir3 mutants.
Ten fold serial dilutions of wild type or the indicated sir mutant strains were plated on complete medium or medium lacking uracil (SD-URA) and plates were photographed after 2-3 days of growth at 30 °C. The mURA3 marker was introduced either into the NTS **(A)**, or the 25S region **(B)** of the rDNA unit.

As shown in figure 3.8A, I observed a 5 to10 fold decrease in rDNA silencing in *sir3*Δ strains as compared to *SIR3* wild type strains (Figure 3.8A, compare lanes 1 and 5). This effect was more drastic if the marker gene was introduced into the 25S region, (Figure 3.8B, compare lanes 1 and 4). As controls, the marker gene was inserted into a non-silenced region

(lanes 2) and silencing was also measured in a *sir4*Δ strain (Figure 3.8A, lane 4, and Figure 3.8, lane 5). To ensure equal cell numbers, cells were plated on complete media (left panels).

3.4 The NAD$^+$-dependent Deacetylase Activity of Sir2 and Histone H4 Deacetylation

Within silent chromatin domains, histones are hypoacetylated (Braunstein et al., 1993; Suka et al., 2001). Since Sir2 is able to deacetylate histones *in vitro*, together with the fact that Sir2 is associated with silent chromatin domains and with the histone-binding proteins Sir3 and Sir4, one is tempted to speculate that histones may be *in vivo* substrates for deacetylation by Sir2. To test whether the enzymatic activity of Sir2 is required for H4 hypoacetylation, I used an antibody that recognizes the tetra-acetylated tail of histone H4 to perform a ChIP assay.

3.4.1 Deacetylation of Histone H4 at the Telomeres and *HML-E*

I observed higher levels of *HML-E* and subtelomeric DNA (0.6 and 1.4 kb from telomeres) associated with the anti-tetra-acetyl-H4 immunoprecipitates from *sir2*Δ and *sir2-H364Y* strains compared to immunoprecipitates from the isogenic *SIR2* wild type strain. This indicates that histone H4 associated with these silent chromatin regions was hypoacetylated in a *SIR2*- and Sir2 activity-dependent fashion (Figure 3.9A). It should be noted, however, that the acetylation of H4 in the *sir2-H364Y* strain (1.4 fold increase) is lower than in a *sir2*Δ strain (2.5 fold increase) when compared to the wild type strain (Figure 3.9A). This difference could indicate that the low amounts of enzymatically inactive Sir2 bound to telomeres and the *HML* locus may be able to inhibit acetylation of histone H4.

These results suggest that the enzymatic activity of Sir2 is responsible for the hypoacetylation of histone H4 at distances of up to 0.6 and 1.4 kb away from the telomere.

3.4.1 Deacetylation of Histone H4 at rDNA

The fact that enzymatically inactive Sir2 localizes to rDNA with nearly wild type efficiency (Figures 3.3 and 3.4) allows for the evaluation of the possible role of Sir2 in hypoacetylation of histones associated with rDNA independently of the localization of the Sir2 protein itself to DNA.

Similar to what I observed for telomeric DNA fragments, 1.5 to 1.9 fold higher levels of rDNA/NTS fragments were precipitated with an anti-tetra-acetyl-H4 antibody using chromatin from *sir2*Δ and *sir2-H364Y* strains, as compared to a *SIR2* wild type strain. This indicates that histone H4 is hypoacetylated in rDNA, and that this hypoacetylation is dependent on both Sir2 and its activity (Figure 3.9B).

As a control for all experiments addressing the acetylation state of histone H4, *ACT1* was used, which encodes a non-silenced, actively transcribed gene. As expected, the anti-acetyl-H4 immunoprecipitates from the *sir2*Δ and *sir2-H364Y* strains did not contain higher levels of acetylated histone H4 than those from the wild type strain (Figure 3.9C).

These results lead one to conclude that it is the enzymatic activity of Sir2, rather than the localization of Sir2 to rDNA, that is required for hypoacetylation of histone H4 associated with rDNA chromatin.

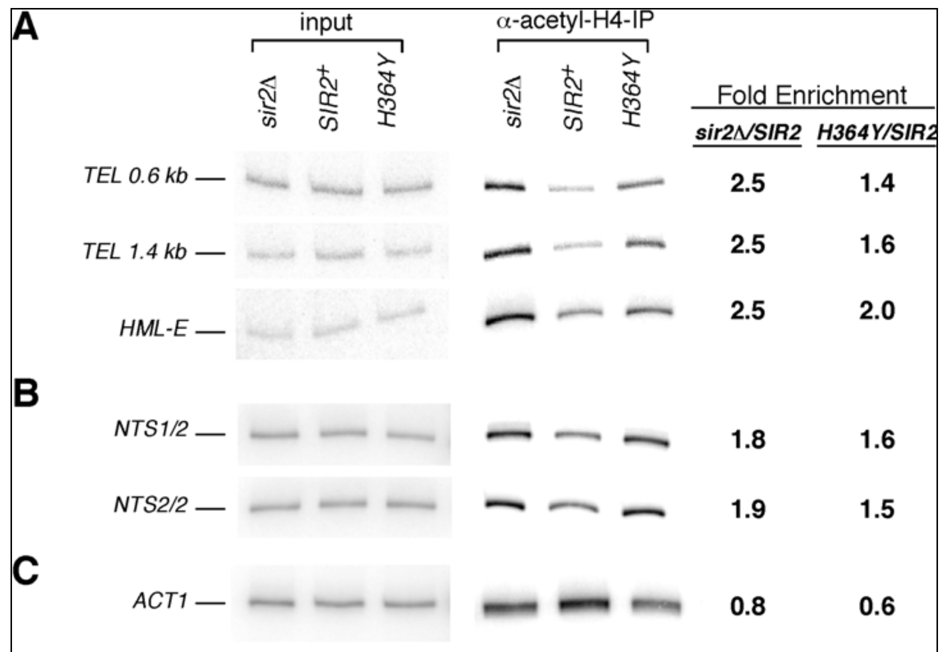

FIGURE 3.9 Sir2-dependent hypoacetylation histone H4 in telomeric, HML, and rDNA regions. Chromatin immunoprecipitations were carried out using an anti-acetylated H4 antibody from *sir2Δ*, *SIR2* wild type, and *sir2-H364Y* strains. PCR amplifications of input and immunoprecipitated DNA for telomeric **(A)**, HML **(B)**, rDNA **(C)**, and the *ACT1* locus **(D)** are shown.

3.5 The N-termini of Histones H3 and H4 in rDNA Silencing

At both the silent mating type loci and telomeres, the highly conserved N-termini of histone H3 and histone H4 play a central role in the assembly of silent chromatin domains (Kayne et al., 1988), but their role in rDNA silencing has not yet been assessed.

The requirement for the enzymatic activity of Sir2 in rDNA silencing, and the Sir2-dependent hypoacetylation of histone H4 associated with rDNA (Figure 3.9B) suggests that histones and changes in their acetylation state may also be involved in rDNA silencing. In order to investigate the possible role of histones H3 and H4 in rDNA silencing, I tested the efficiency of rDNA silencing in strains carrying a mutant allele for histone H3 or H4. In these strains, the endogenous copies of the genes encoding for the H3 and H4 histones have been deleted and replaced with H3 and H4 mutants. One of these mutants, *h3Δ4-30*, contains an H3 N-terminal deletion removing amino acids 4 to 30. The other mutant carries a lysine 16 to asparagine mutation in the gene for histone H4 (*h4K16Q*). rDNA silencing was assessed

using strains constructed with the reporter system described by Smith and Boeke (1997), whereby a modified *URA3* reporter gene (*mURA3*) is inserted into the rDNA array. Growth on selective media (without uracil) then allows for the measurement of silencing.

As reported by Smith and Boeke (1997), insertion of the *mURA3* reporter gene within the rDNA locus resulted in a great loss of growth on medium lacking uracil, due to silencing of the reporter (Figure 3.10, row 2). In contrast, when the same reporter gene was inserted at the *HIS3* gene as an example for a non-silenced locus, robust growth on medium lacking uracil was observed (Figure 3.10, rows 1 and 5). Deletion of the H3 tail (*h3*Δ*4-30*) or the lysine 16 to glutamine (*h4K16Q*) mutation in histone H4 each lead to loss of rDNA silencing by about 1000 fold, as judged by increased growth on medium lacking uracil (Figure 3.10, compare rows 4 and 5 with row 2). Loss of rDNA silencing in these strains was similar to the loss observed in a *sir2*Δ strain (Figure 3.10, compare rows 4 and 5 with row 3) (Smith and Boeke, 1997). As additional controls, shown on the left of figure 3.10, all strains grew well on complete medium and the histone mutations had no effect on growth rate on medium lacking uracil when the reporter gene was inserted at the non-silenced *HIS3* locus (Figure 3.10, rows 8 through 10).

These results indicate that histone H4-lysine 16 and the N-terminus of histone H3 are absolutely required for rDNA silencing.

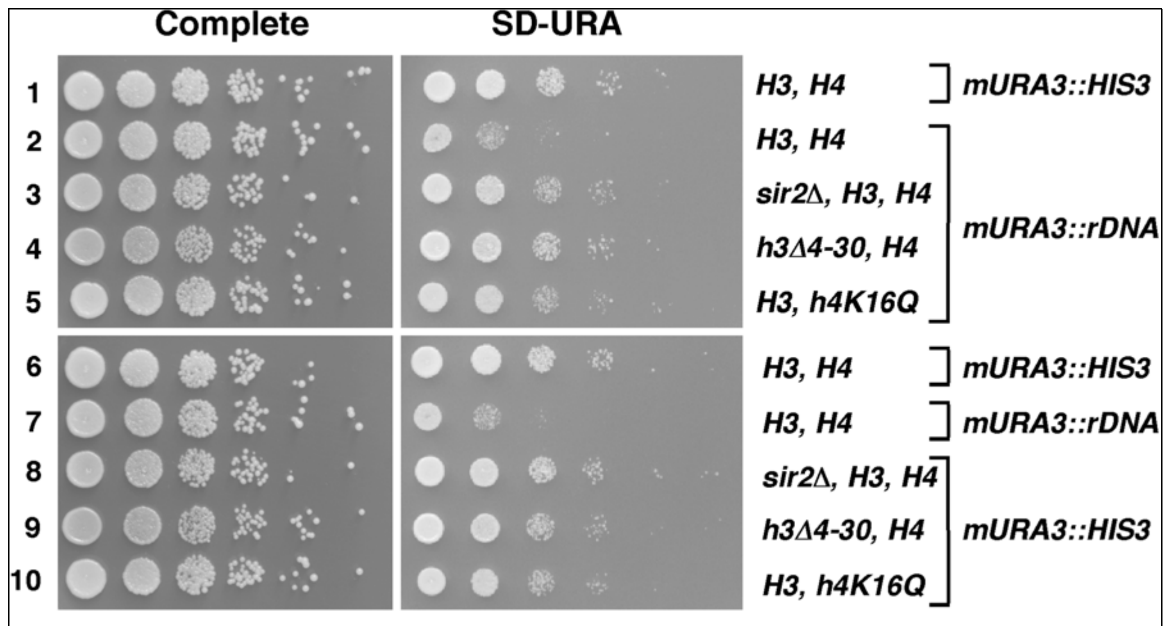

FIGURE 3.10 Loss of rDNA silencing in histone H3 and H4 mutants.
Ten fold serial dilutions of wild type or the indicated histone mutant strains were plated on complete medium or medium lacking uracil (SD-URA) and plates were photographed after 2-3 days of growth at