4 DISCUSSION

The results presented here suggest that the activity Sir2 serves different purposes at telomeres and the *HML* locus on the one hand, and at rDNA on the other hand: Whereas it controls the initial assembly of the SIR complex at the former, it is required for a step subsequent to assembly at the latter. The activity of Sir2 is also required for the association of Sir3 with rDNA chromatin, suggesting that deacetylation of histones creates a binding site for Sir3 in chromatin. In addition, I show that the intact N-termini of histones H3 and H4 are required for rDNA silencing and furthermore, that the localization of enzymatically inactive Sir2 to rDNA is not sufficient for hypoacetylation of histone H4 associated with rDNA. Based on this and previous data, I will outline models describing the assembly mechanisms of Sir2-containing complexes at silent chromatin regions.

4.1 The sir2-H364Y Point Mutant

The validity of the conclusions drawn in this study requires that the Sir2 point mutation used in the experiments abolishes the enzymatic activity of Sir2 without affecting either its stability or its interaction with other proteins.

Three lines of evidence support a specific role for histidine 364 in substrate binding.

- As shown by Tanny et al. (1999), H364Y has no effect on the protein levels of Sir2 in yeast extracts or its interaction with Sir4 and Net1, two proteins that Sir2 is known to interact with.
- Histidine 364 is conserved in all Sir2-like proteins (Brachmann et al., 1995; Frye, 2000), which suggests that this residue is probably involved in important catalytic or structural functions rather than in species- or locus-specific targeting interactions.

- In the crystal structures of two Sir2-like proteins (the archaeal Sir2Af and the human SIRT2) that were recently solved, the corresponding histidine residue is one of the conserved amino acids that forms the binding site for NAD^+ in these enzymes (Finnin et al., 2001; Min et al., 2001). In the archaeal Sir2Af, histidine 116, which is analogous to H364 in yeast Sir2, makes a hydrogen bond with the 2'-hydroxyl of nicotinamide ribose in NAD^+ (Min et al., 2001). It therefore seems save to assume that the sir2-H364Y mutant protein used in this thesis is unlikely to perturb protein-protein interactions that involve Sir2, but instead has a specific defect in substrate binding or catalysis.

In support of the above arguments, using a different point mutation that converts the conserved glycine 262 within the NAD^+ binding site of Sir2 to alanine, I have obtained similar results (Figures 3.1C and 3.2C). As is the case with the sir2-H364Y mutation, the sir2-G262A mutation abolishes both the *in vitro* NAD^+-dependent deacetylase activity of Sir2 and *in vivo* silencing (Tanny and Moazed, 2001).

The arguments presented above make it extremely unlikely, that the results obtained could be based on perturbed protein-protein interactions or decreased stability caused by the introduced point mutation. It can therefore be safely assumed that all results are due to the abolishment of the enzymatic activity of Sir2.

4.2 The Enzymatic Activity of Sir2 and the Assembly of Silencing Complexes on Chromatin

The enzymatic activity of Sir2 may be required for one or more of several steps in the generation of silenced chromatin: The initial recruitment of silencing complexes to their target DNA region, for their spreading along the chromatin fiber, or for an activity that

directly mediates gene silencing by somehow modifying chromatin after these complexes are already assembled.

My data suggests that at telomeres and the *HML* silent mating type locus, one primary outcome of the enzymatic activity of Sir2 is to allow for the stable assembly of the SIR complex on DNA. The association of Sir2, Sir3, and Sir4 with both telomeric DNA fragments and the *HML* locus is reduced to levels which are similar to background in cells containing an enzymatically inactive Sir2 protein. This suggests that Sir2 acts to create a binding site for the SIR complex on chromatin.

The results presented in this thesis together with previous observations allow me to propose the following model for the initial steps in assembly of the SIR complex at telomeres. I also note that a similar mechanism should apply to the *HM* loci.

4.2.1 A Model for the Assembly of The SIR Complex on Telomeres

The Sir4 protein has been shown to interact with both the Rap1 and yKu70 proteins in two-hybrid assays (Moretti et al., 1994; Tsukamoto et al., 1997), suggesting that the Sir2/Sir4 complex is initially recruited to telomeres by interactions between yKu70, bound to the chromosome end, and possibly Rap1, bound to telomeric repeats (Figure 4.1, step 1). However, stable association with telomeres requires the deacetylation of histones or possibly another event, such as ADPRAc synthesis, which might act as an allosteric effector for other enzymes (Figure 4.1, step 2). This is followed by the stable binding of the Sir2/Sir4 complex and Sir3 to a now deacetylated nucleosome (Figure 4.1, step 3). Through additional rounds of recruitment and deacetylation involving homotypic and heterotypic interactions between the Sir3 and Sir4 proteins, the Sir proteins can spread along the chromatin fiber (Figure 4.1, step 4).

The idea that histone tails are directly involved in the assembly of the SIR complex is also supported by previous studies showing that histone tail mutations disrupt the recruitment

of the Sir proteins to telomeric DNA regions (Hecht et al., 1996). The model presented here implies that histone deacetylation serves as a key step that allows for the regulation of the assembly and spreading of silencing complexes. Although deacetylation may directly promote chromatin condensation *per se*, this model implies that deacetylation also functions to regulate the initial association and step-wise association of histone-binding proteins on silent chromatin domains. This latter function may in fact be the critical outcome of histone deacetylation and other covalent modifications that are coupled to histone binding proteins.

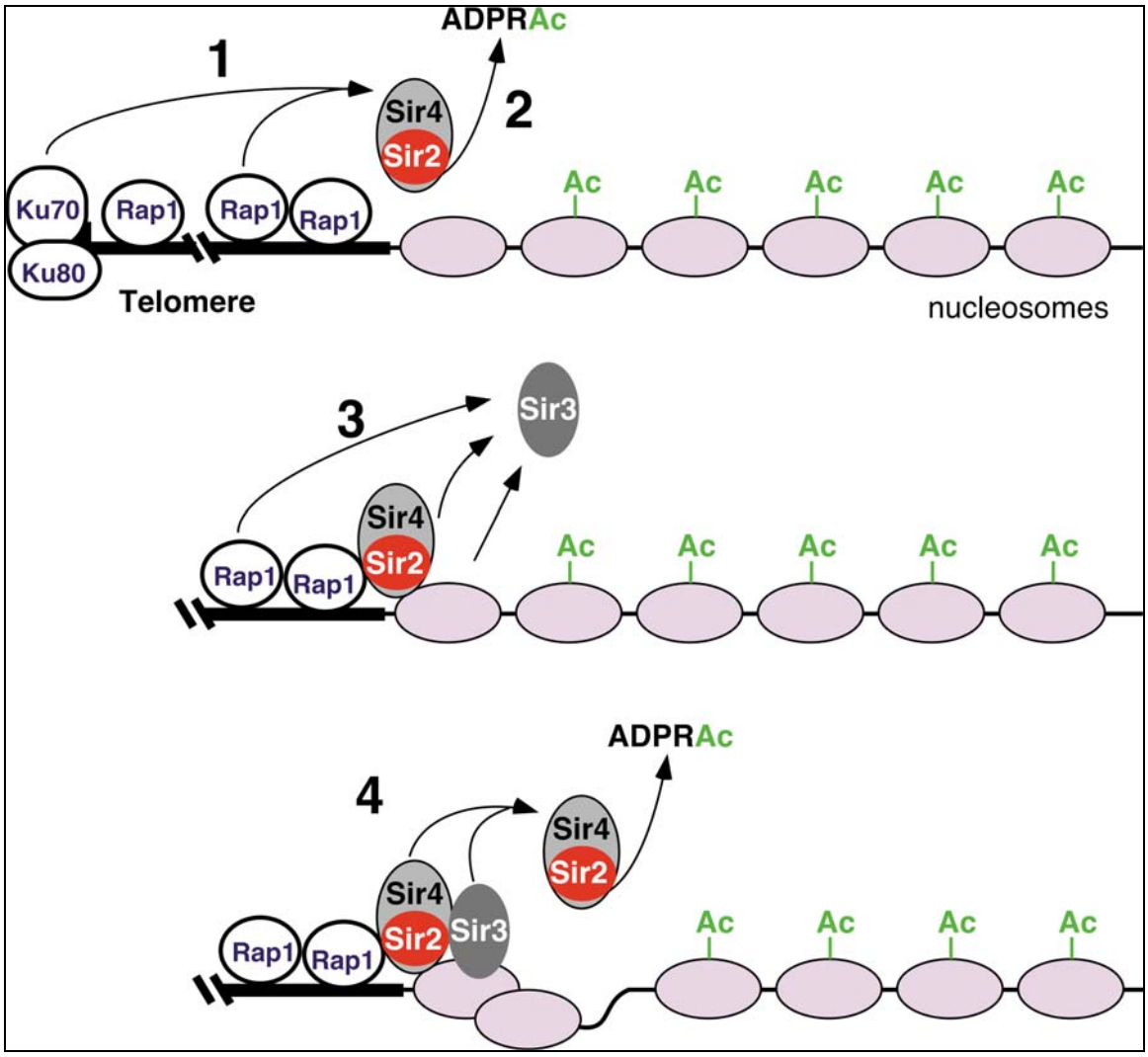

FIGURE 4.1 Model for the initial steps in assembly of the SIR complex at telomeres. Recruitment of the Sir2/Sir4 complex (step 1), deacetylation and ADPRAc synthesis (step 2), binding of Sir3 to the deacetylated nucleosome (step 3), and recruitment of another Sir2/Sir4 complex to initiate the next round of the cycle (step 4) are schematically diagrammed. ADPRAc: O-acetyl-ADP-ribose; Ac: acetyl group on histone N-terminal lysines.

Further studies revealed that the initial binding of the Sir2/Sir4 complex to the telomere is independent of Sir3 (Hoppe et al., 2002). This is therefore likely to be the initial step in assembly of silent chromatin at the telomeres.

4.2.1.1 Common Pathways for the Assembly of Silent Chromatin?

This model is reminiscent of the recently described assembly process for the HP1-based silencing mechanisms in metazoans and the Swi6-based silencing mechanisms in fission yeast.

HP1 is a structural component of heterochromatin and is conserved in a wide range of organisms (Eissenberg and Elgin, 2000; James and Elgin, 1986). Its fission yeast homolog, Swi6, is also a structural component of silent chromatin domains and has been shown to be required for centromeric, mating type, and telomeric silencing (reviewed in Grewal, 2000).

The mammalian HP1 homolog is in a stable complex with another conserved protein, named SUV39H1 (Aagaard et al., 1999). Together with Swi6, Clr4 is required for heterochromatic gene silencing in fission yeast (Ekwall and Ruusala, 1994). Recently, both SUV39H1 and Clr4 were shown to possess methyltransferase activity with specificity for lysine 9 at the amino-terminus of histone H3 (Rea et al., 2000). Methylation of this lysine residue is conserved in many eukaryotes (Strahl et al., 1999), and has been shown to create a binding site for the histone binding proteins HP1 and swi6, which both bind to H3-lysine 9 (Bannister et al., 2001; Lachner et al., 2001). Like Sir3 and Sir4 in budding yeast, HP1 and Swi6 are able to multimerize (Cowell and Austin, 1997; Nielsen et al., 2001). Thus, one can envision that the spreading of the HP1/Swi6 silencing complexes along the chromatin fiber could be generated through cycles of histone binding and modification.

Although the proteins involved in SIR-mediated silencing in budding yeast and in HP1/Swi6-mediated silencing in fission yeast and metazoans share no obvious conserved

sequences (save those for histones), the assembly pathway of silent chromatin of these complexes are similar in that enzymes that modify histone tails bind proteins that specifically recognize and bind those modified histone tails.

Therefore, a conserved aspect of gene silencing mechanisms may involve the direct physical association of histone-modifying enzymes with proteins that recognize and bind to such modified histones.

4.2.2 A Model for the Assembly of the RENT Complex on rDNA

In contrast to the situation at telomeres and the *HML* locus, the enzymatic activity of Sir2 appears to play only a minor role in the stable association of Sir2 with rDNA. I observed levels of cross-linking for wild type Sir2 which were similar to those of sir2-H364Y for DNA fragments throughout most of the rDNA repeat. It has previously been shown that the Net1 subunit of the RENT complex associates with rDNA independently of Sir2 (Straight et al., 1999). Together with the data presented here, these results allow me to propose a mechanism for the assembly of silencing complexes at rDNA (Figure 4.2).

In rDNA, the RENT complex, consisting of at least Net1, Sir2 and Cdc14, assembles on chromatin independently of Sir2 activity (Figure 4.2, step 1). Deacetylation of histones or another outcome of the activity of Sir2 (Figure 4.1, step 2) then results in structural changes of chromatin. These structural changes lead either directly to silencing or they promote a later step required for this process. The activity of Sir2 at rDNA ultimately opens up a binding site for Sir3 (Figure 4.2, step 3). The fact that in *sir2-H364Y* strains the crosslinking of Sir2 to some rDNA fragments within the 18S and 25S rRNA coding regions is diminished (see Figures 3.3 and 3.4), allows one to speculate that Sir2's activity may also regulate the spreading of the RENT complex. Interestingly, however, the amount of Sir2 bound to rDNA regions outside of the non-transcribed

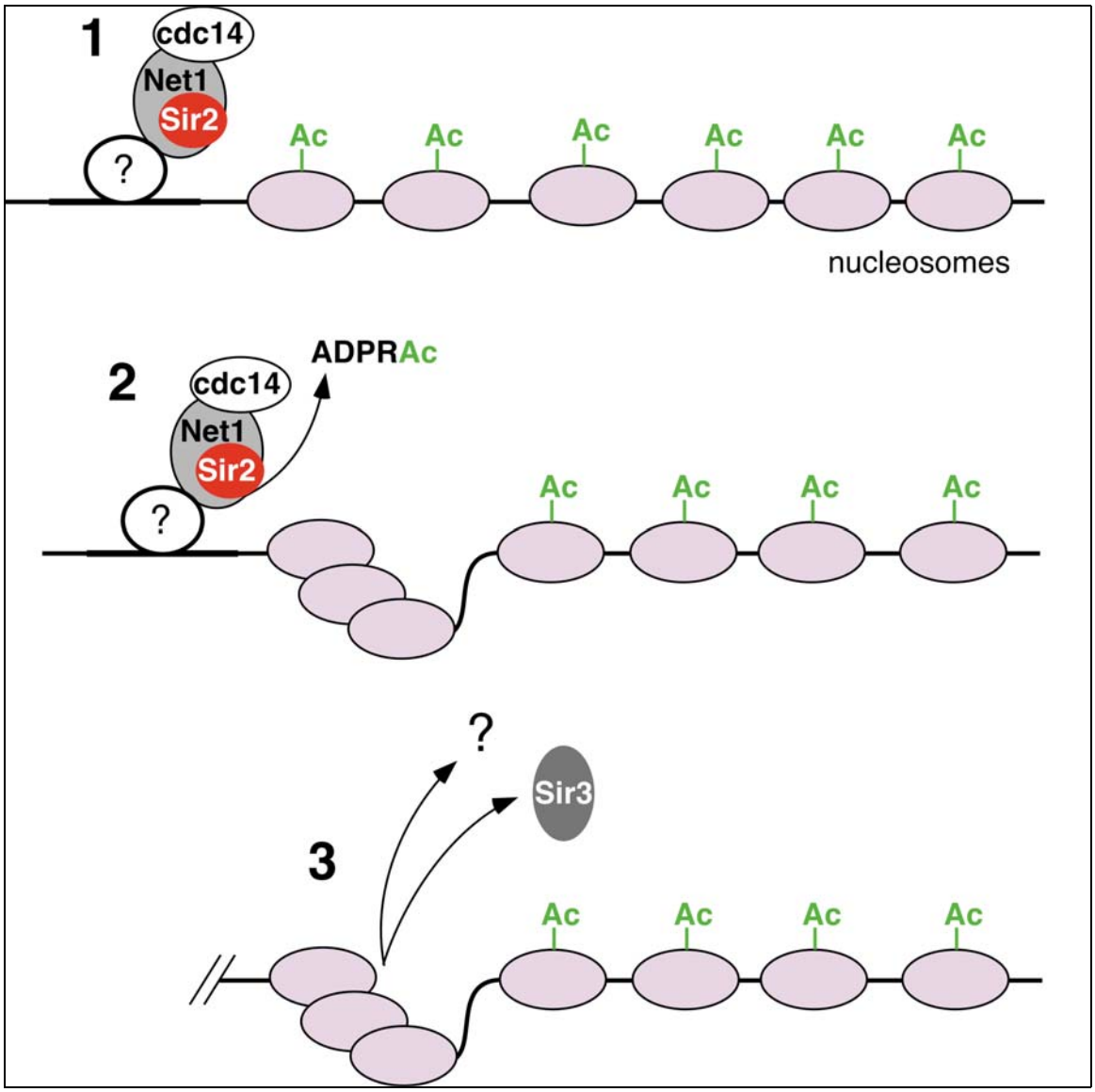

FIGURE 4.2 Model for assembly of the RENT complex and Sir3 at rDNA. The RENT complex contains at least three proteins, Net1, Sir2, and Cdc14 and is proposed to associate with rDNA through interactions with an unknown DNA binding protein(s) independently of Sir2 activity (step 1). Deacetylation of histones, which is coupled to ADPRAc synthesis, then directly or indirectly results in rDNA silencing (step 2). One outcome of Sir2 activity at rDNA is to create a binding site for Sir3 and possibly an unknown histone binding protein that is proposed to act redundantly with Sir3 at rDNA (step 3). ADPRAc: O-acetyl-ADP-ribose; Ac: acetyl group on histone N-terminal lysines.

spacer region (NTS) is only about two fold above background (see Figures 3.3 and 3.4). It therefore seems as if spreading of the RENT complex is rather limited. It also remains to be determined whether Net1 or an unknown component of the complex contains a histone-binding activity that may promote the spreading of Net1 throughout rDNA.

4.3 Recruitment of Sir3 to Silent Chromatin

Several examples exist demonstrating that Sir proteins can display a dynamic localization pattern in the nucleus. The Sir3 protein relocalized from telomeric foci to the nucleolus when telomeric silencing is disrupted (Gotta et al., 1997; Kennedy et al., 1997). In the case of DNA damage, the SIR complex has been shown to relocalize to sites of double stranded DNA breaks (Martin et al., 1999; Mills et al., 1999). The function of Sir3 at the nucleolus is not understood, but knowledge of the mechanism underlying relocalization to rDNA chromatin could help us understand its mode of assembly onto silent chromatin domains. Although it is known that localization of Sir3 to the nucleolus Sir2-dependent, it has not been clear whether Sir2 recruits Sir3 merely through a direct protein-protein interaction or by some other mechanism (Gotta et al., 1997).

My data argues against the possibility that the association of Sir3 with rDNA and its localization to the nucleolus is merely based on direct interactions between Sir2 and Sir3. Also, Sir3 localization to the nucleolus and rDNA is Sir4-independent (Gotta et al., 1997), which makes it seem unlikely that interactions between Sir3 and any of the known silencing proteins are involved (Figure 3.7). Since an enzymatically inactive Sir2 protein that is properly localized to the nucleolus and rDNA fails to recruit Sir3, the association of Sir3 with rDNA is much more likely to be a result of Sir2's enzymatic activity.

These results could be explained by a model in which the deacetylation of histones by Sir2 creates a binding site for the Sir3 protein in rDNA chromatin (Figure 4.2, step 3). In this case, a problem that arises from this hypothesis is how binding specificity to deacetylated regions of chromatin by Sir3 is achieved. One possibility is that the generation of ADPRAc as a product of the deacetylation reaction provides a local signal that stimulates the histone binding activity of Sir3. It is also possible that Sir2 may be a very general deacetylase, which unlike the NAD^+-independent histidine deacetylases (HDACs), displays little substrate specificity and would therefore be able to deacetylate all N–terminal lysines of all four

histones. This could provide a signal that is required for the efficient binding of Sir3 to nucleosomes. In favor of the latter possibility, it has recently been shown that all histone N-terminal residues are hypoacetylated in telomeric and mating type silent chromatin domains (Suka et al., 2001).

Using a silencing growth assay that is based on growth of strains containing the *mURA3* reporter in rDNA, I was able to provide evidence that the localization of Sir3 to rDNA does not lack functional significance. I consistently observed a 5 to10 fold decrease in rDNA silencing in *sir3*Δ strains as compared to *SIR3* wild type strains (Figure 3.8A). This result stands in contrast to previously published results (Smith and Boeke, 1997), where Sir3 was shown not to play a role in rDNA silencing. One might be able to account for these different results by scrutinizing the method applied. Smith and Boeke (1997) used a MET15 colony color assay, which is based on complementation of *met15*Δ cells with a MET15 allele introduced into rDNA. If complementation occurs, colonies appear white on media containing lead ions. If the gene is silenced, colonies have a brown color. Since the effect I observe is rather subtle, it could have easily been missed by this less sensitive assay, which is based on subjective judgment of colony color.

When the marker gene was inserted into the 25S region of rDNA, the silencing defect observed was much more drastic (Figure 3.8B). As discussed above (section 4.2.2), one can assume that Sir2 spreads in rDNA only in a limited fashion, in which case Sir3 could spread along the chromatin further than Sir2, as is the case at the telomeres (Figure 3.1 and Strahl-Bolsinger, 1997). The absence of Sir3 at this more distant location could then account for a more dramatic loss of silencing in that region in *sir3*Δ mutants. Further studies are necessary to elucidate the function of Sir3 at rDNA, possibly together with other yet unknown silencing proteins.

4.4 The Role of Histones in rDNA Silencing

Several lines of evidence suggest that rDNA silencing involves changes in chromatin structure, such as the requirement for the enzymatic activity of Sir2 in rDNA silencing (Imai et al., 2000), the sensitivity of rDNA silencing to a reduction in the dosage of histones H2A and H2B (Bryk et al., 1997), and increased accessibility of rDNA to micrococcal nuclease and *dam* methyltransferase in *sir2*Δ cells (Fritze et al., 1997). Despite their requirement for efficient silencing at telomeres and the *HM* mating type loci, the role of histones H3 and H4 amino termini in rDNA silencing has not been evaluated.

The results presented here show that the amino termini of histones H3 and H4 are required for rDNA silencing. In contrast to what has been reported for telomeres and the *HM* loci regarding the requirement for the N-terminus of histone H3, their role in rDNA silencing appears to be different. In rDNA silencing, the N-terminus of histone H3 plays an essential and non-redundant role (Figure 3.10). But the same H3 N-terminal tail deletion (amino acids 4-30) results only in a modest decrease in telomeric silencing and causes a reduction in silencing at the mating type cassettes only when combined with other mutations (Thompson et al., 1994). Together, these results suggest that the N-terminus of histone H3 plays a more important role in rDNA silencing than in telomeric and HM silencing.

Lysine 16 of histone H4, on the other hand, appears to be similarly important for both rDNA silencing, as shown here, as well as for telomeric and mating type silencing (Johnson et al., 1990; Thompson et al., 1994). This might be unexpected, since this residue is thought to form part of the Sir3 binding site at the telomeres and *HM* loci (Hecht et al., 1996), and although Sir3 can localize to rDNA, its deletion has only a modest effect on rDNA silencing (Figure 3.8), and does not affect rDNA recombination rates (Gottlieb and Esposito, 1989).

My data could be better explained when considering the possibility that Sir2-dependent deacetylation of histones H3 and H4 in rDNA creates a binding site for an as yet unidentified protein with a histone binding activity that is similar to that of Sir3. The

association of Sir3 with rDNA may then result from a competition between Sir3 and a histone binding protein that would perform a redundant function with Sir3. This hypothesis helps explain both the localization of low levels of Sir3 to rDNA and the weak reduction in the efficiency of rDNA silencing in *sir3*Δ strains.

4.5 Histones as *in vivo* Substrates of Sir2

As mentioned above, a large body of previous evidence suggests that histones are among the *in vivo* targets of deacetylation by Sir2 (Braunstein et al., 1993; Guarente, 2000; Imai et al., 2000; Strahl-Bolsinger et al., 1997), and the results presented in this thesis provide additional support for this view. As discussed in section 4.1, the active site mutation used here is unlikely to affect protein-protein interactions that involve Sir2. Although an enzymatically inactive Sir2 protein can bind to rDNA, the extent of hypoacetylation of histone H4 that this binding causes is lower than the hypoacetylation observed in a *SIR2* strain. This supports the view that Sir2-dependent deacetylation of histones is direct.