1 INTRODUCTION

1.1 Gene Silencing: An Introduction

The packaging of DNA into chromatin appears to play a central role in the regulation of nearly every aspect of gene transcription. During recent years, an increasing number of complexes associated with the activation or repression of transcription have been shown to contain chromatin remodeling activity or enzymes that covalently modify histones (Jenuwein and Allis, 2001; Kingston and Narlikar, 1999). Studies of gene silencing in the budding yeast, *Saccharomyces cerevisiae*, provided one of the first examples for the direct role of chromatin structure in the regulation of gene activity (Kayne et al., 1988; reviewed in Grunstein, 1997).

During the development of multicellular organisms, the expression of certain genes is often permanently shut off after a certain developmental stage has been reached. Gene silencing contributes to the maintenance of gene expression patterns, allowing only for the expression of the appropriate subset of genes in each cell type. Evidence from a number of experimental systems suggests that eukaryotic cells maintain the gene expression programs that were specified during embryonic development through modifications in the structure of their chromosomes. These modifications are heritable; that is to say, they are passed on to the daughter cell during cell division.

Silencing entails the inactivation of chromosome domains by packaging them into a specialized chromatin structure, rendering them inaccessible to DNA binding proteins (Grewal, 2000; Paro, 1993). Such inaccessible DNA domains are assembled by silencing proteins acting directly on nucleosome structure. The nucleosome, shown in figure 1.1, is the basic unit of folding in eukaryotic chromosomes. Here, 146 base pairs of DNA are wrapped around a histone core octamer for almost two turns. This octamer consists of two H2A-H2B dimers flanking a centrally located $(H3\text{-}H4)_2$ tetramer (Luger et al., 1997, reviewed in

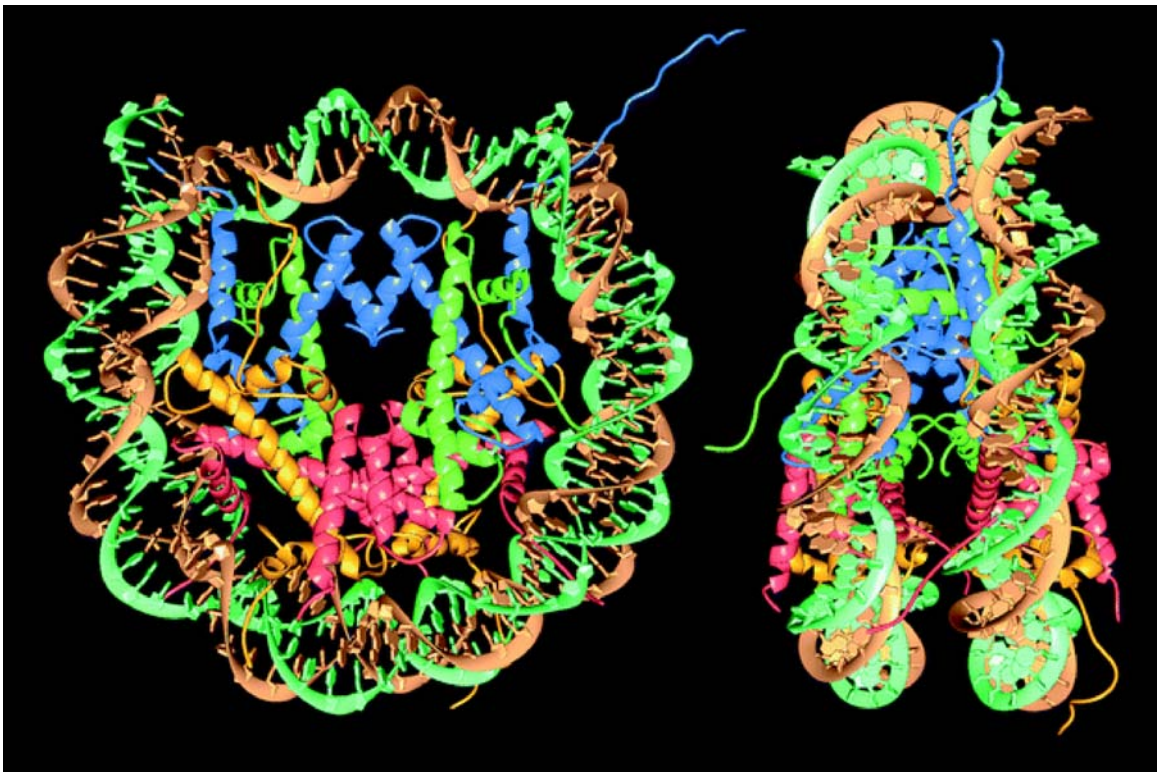

FIGURE 1.1 Nucleosome core particle: ribbon traces for the 146 base pairs DNA phosphodiester backbones (brown and turquoise) and eight histone protein main chains (blue: H3; green: H4; yellow: H2A; red: H2B). The views are down the DNA superhelix axis for the left particle and perpendicular to it for the right particle. For both particles, the pseudo-twofold axis is aligned vertically with the DNA center at the top (From Luger et al., 1997).

Kornberg and Lorch, 1999). The folding of genes into higher order chromatin structures is an event that contributes to transcriptional repression. It has recently become clear that the compaction of DNA is a key regulatory event that many of the processes controlling gene expression act upon. This, for example, is true for various ATP-dependent chromatin remodeling complexes, which have been found to modulate histone-DNA interactions. Thereby, they enable the binding of regulatory proteins as well as the transcription machinery to DNA (reviewed in Kingston and Narlikar, 1999; Peterson and Workman, 2000; Struhl, 1996; Tamkun, 1995). In silencing, nucleosomes are rendered resistant to the activity of remodeling factors, leading to negative regulation of transcription (reviewed in Francis and Kingston, 2001).

1.2 Characteristics of Gene Silencing

Perhaps the most prominent feature of silencing is that it acts in a regional rather than promoter-specific manner to generate large repressed chromatin domains, that are inaccessible to DNA binding proteins (Rine, 1999). In yeast, promoters specific for RNA polymerase II and III, which are inserted into silent chromatin domains, are repressed (Huang et al., 1997; Schnell and Rine, 1986). Silent DNA regions are also less accessible to the cellular recombination machinery and to exogenous nucleases, such as the *dam* methyltransferase and restriction endonucleases (Gottschling, 1992; Loo and Rine, 1994; Singh and Klar, 1992; Wallrath and Elgin, 1995). Another important property of silent chromatin is that it is stable throughout mitotic and meiotic cell divisions. During chromosome duplication, the entire structure of the chromatin domain that has been silenced, consisting of DNA and its associated, transcription-repressing proteins, is replicated (reviewed in Grewal, 2000). This epigenetic mechanism is believed to allow for the maintenance of cell identity and stable gene expression patterns in eukaryotes.

Interestingly, central characteristics of gene silencing, such as general inaccessibility and epigenetic inheritance, are also features of heterochromatin. Therefore, silent chromatin domains are often referred to as "heterochromatic". Unlike most chromosome regions that undergo cycles of condensation and decondensation as cells pass through the cell cycle, heterochromatin appears condensed throughout the cell cycle. Studies in *Drosophila melanogaster* showed that inactivation of genes translocated into the vicinity of heterochromatin correlated with the spreading of heterochromatin over these genes (reviewed in Weiler and Wakimoto, 1995). This discovery established that the heterochromatic state is associated with gene inactivity.

1.3 The Silencing Machinery in *Saccharomyces cerevisiae*

Silencing in budding yeast is present in two mechanistically related forms: In one form it occurs at the homothallic, or silent mating type locus (*HM* loci, *HML* and *HMR*), and the telomeric DNA regions. The second form can be found within the highly repetitive ribosomal DNA gene cluster (rDNA). Through a number of genetic and biochemical studies, the main regulatory sites and proteins that collaborate to assemble silenced DNA in budding yeast have been identified (reviewed in Gartenberg, 2000) (Figure 1.2).

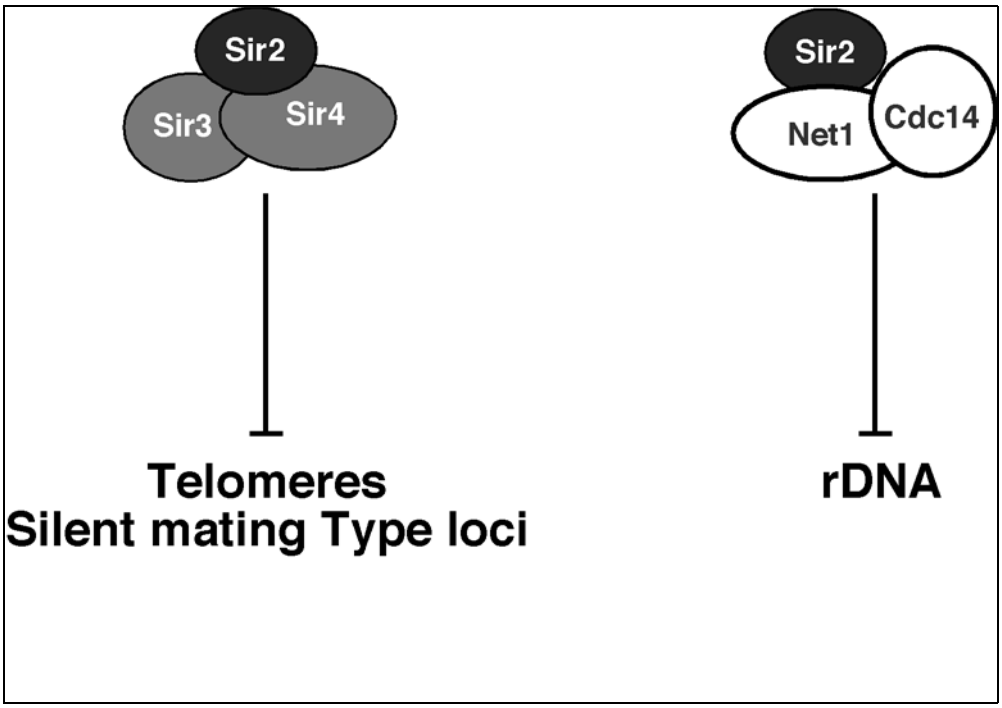

FIGURE 1.2 Two distinct silencing complexes exist in budding yeast. The SIR complex contains Sir2, Sir3, and Sir4 and can be found at the telomeres and the silent mating type cassettes. The RENT complex contains Sir2, Cdc14 and Net1 and acts at rDNA.

The Silent information regulator 2 (Sir2) protein is the common component of the two distinct multi-protein complexes, shown in figure 1.2 (Ghidelli et al., 2001; Moazed and Johnson, 1996; Moazed et al., 1997; Moretti et al., 1994, Straight et al., 1999). In one complex, Sir2 is tightly associated with the Sir4 protein and this Sir2/Sir4 complex, together with Sir3, assembles at the silent mating type and telomeric DNA regions into a nucleosome

binding complex called the SIR complex (Hecht, et al. 1995; Moazed et al., 1997). In the RENT complex, Sir2 interacts with Net1 and Cdc14 and localizes to the nucleolus.

1.3.1 SILENCING AT THE MATING TYPE CASSETTES AND TELOMERES

Gene silencing in yeast was first discovered at the silent mating type locus, and later at telomeric DNA regions (Nasmyth et al., 1981; Gottschling et al., 1990; Klar et al., 1981). Silencing at the *HM* loci, which is required for the replacement of the *MAT* alleles in genotype switching, is essential for maintenance of haploid cell identity (Herskowitz et al., 1992). Here, the *MAT*α information at the *HML* locus, 12 kb from the left telomere of chromosome III, and the *MAT**a*** transcript at the *HMR* locus, 20 kb away from the right arm of the same chromosome, are silenced (Loo and Rine, 1995). Telomeres are specialized functional complexes that protect the ends of eukaryotic chromosomes. Telomeres can repress reporter genes, which are inserted in their proximity by a telomere position effect (TPE) (Gottschling et al., 1990). In addition, silencing has been shown to contribute to maintaining the integrity of telomeric repeats (Palladino et al., 1993). Therefore, besides its role in regulating cell identity, silencing is also associated with the maintenance of chromosome structures.

Silencing at the above loci is mediated by a nucleosome-binding complex, called the SIR complex, shown in figure 1.2 (Aparicio et al., 1991; Hecht et al., 1995; Klar et al., 1979; Moazed et al., 1997; Rine and Herskowitz, 1987; Strahl-Bolsinger et al., 1997). This complex contains the Sir2, Sir3, and Sir4 proteins. It is recruited through interactions with DNA-binding proteins that bind to chromosome ends or specific *cis*-acting regulatory elements, called silencers. The Rap1 protein, for example, binds to multiple sites within the yeast telomeric repeats. Together with the chromosome end-binding proteins, yKu70 and yKu80, it recruits the SIR complex via interactions with Sir3 and Sir4 (Boulton and Jackson, 1998;

Kyrion et al., 1993; Laroche et al., 1998; Moretti et al., 1994). Sir2 then gets recruited via Sir4. Both have been shown to form a tightly associated complex *in vitro* and *in vivo* (Moazed et al., 1997; Strahl-Bolsinger et al., 1997). Chromatin immunoprecipitation experiments show that the Sir proteins are associated with DNA throughout silent chromatin domains, which supports a model of Sir silencing complexes spreading along the chromatin fiber (Hecht et al., 1996).

A similar situation applies to the silent mating type cassettes. Both *MHL* and *HMR* are surrounded by a pair of related yet distinct silencer sequences, called *HML-E*, *HML-I*, *HMR-E*, and *HMR-I*. The silencers consist of specific arrays of binding sites for *trans*-acting factors, namely the ARS-binding factor 1 (Abf1), the origin recognition complex (ORC), and Rap1, which in turn allows for the recruitment of the SIR complex (Nasmyth, 1982).

1.3.2 rDNA Silencing

The ribosomal RNA genes in S. cerevisiae exist as tandem arrays of approximately 150 copies, which make up 10 % of the entire genome. They are sequestered into a nuclear subdomain called the nucleolus, which is the center for ribosomal RNA synthesis and ribosome assembly (reviewed in Carmo-Fonseca et al., 2000; Shaw and Jordan, 1995).

Silencing at the rRNA gene array (rDNA) may seem paradoxical. On the one hand, rDNA genes are very highly transcribed. It has been estimated that transcription by RNA polymerase I (PolI) represents nearly 60 % of the total transcription of the yeast cell (Warner, 1999). On the other hand, rDNA chromatin structure is regulated by silencing, a mechanism that suppresses transcription (Bryk et al., 1997; Fritze et al., 1997; Smith and Boeke, 1997; Thon and Verhein-Hansen, 2000). At least two possible explanations come to mind that can resolve the apparent contradiction between rDNA silencing and high transcription rates: One could envision that rDNA may be packaged into a type of silent chromatin that leaves it

somehow accessible to RNA polymerase I, while limiting access to the RNA polymerase II transcription machinery and recombination enzymes. Alternatively, since only about half of the rRNA genes are transcribed at any given time, rDNA silencing could act only on those repeats that are transcriptionally inactive.

At the rDNA region, silencing requires a distinct multiprotein complex, called RENT (REgulator of Nucleolar silencing and Telophase exit), which is shown in figure 1.2 (Shou et al., 1999; Straight et al., 1999). In addition to Sir2, RENT contains at least two other proteins, Net1 and Cdc14, but not Sir3 and Sir4 (Shou et al., 1999; Straight et al., 1999).

Cdc14 is a dual specificity protein phosphatase (Taylor et al., 1997), and part of the mitotic exit network, a group of proteins that form a signaling cascade. This cascade leads to the release of Cdc14, allowing it to dephosphorylate various protein targets, which in turn allows the cell to exit from mitosis (Visintin et al., 1998).

Net1 (also known as Cfi1), the core subunit of RENT, is required for the localization of Sir2 to rDNA and for rDNA silencing (Straight et al., 1999). In addition to its role in rDNA silencing, Net1 is required for the regulation of exit from mitosis and regulates the activity of the Cdc14 protein phosphatase by sequestering it in the nucleolus until telophase (Shou et al., 1999; Visintin et al., 1999). Net1 is also required to maintain nucleolar localization of Nop1 (Straight et al., 1999), a protein that has been implicated in pre-rRNA processing, methylation, and ribosome assembly (Tollervey et al., 1993). As we were able to show, Net1 also has two other functions in the nucleolus: It directly activates synthesis of rRNA by RNA Pol I, and plays a general role in regulating nucleolar structure (Shou et al., 2000).

Chromatin immunoprecipitation experiments show that both Net1 and Sir2 are associated with DNA fragments throughout the length of rDNA repeats (Gotta et al., 1997; Straight et al., 1999). This suggests that the Net1/Sir2 complex can spread along chromatin. Since genetic evidence shows the association of Net1 with rDNA fragments does not require

Sir2 (Straight et al., 1999), the mechanism of this kind of spreading is likely to be different from that of the SIR complex.

1.3.2.1 The role of rDNA silencing in life-span control

Beside its role in stabilizing the repetitive nature of rDNA, Sinclair and Guarente (1997) showed that rDNA silencing is a determinant of life span in yeast. In *sir2* mutant cells, for example, where silencing is abolished, increased hyper-recombination rates at rDNA result in the excision of rDNA repeat units, leading to the creation of extra-chromosomal circles. The authors proposed that the retention and amplification of these circles in mother cells results in eventual senescence. In addition, it has recently been shown that a homolog of Sir2 is involved in regulation of life span in *Caenorhabditis elegans*, suggesting that this role of Sir2-like proteins is conserved in eukaryotes (Tissenbaum and Guarente, 2001).

1.4 The role of histones in silencing

There is a long-standing correlation between gene inactivation and histone hypoacetylation (Grunstein, 1997), suggesting that deacetylation is the important function of Sir2 in silencing.

Silencing involves specific alteration in chromatin structure. This includes changes in the acetylation state of histone amino termini, as well as association of histone-binding proteins with chromatin domains. The amino termini of histones H3 and H4 play an essential role in silencing at the mating type loci and telomeres in budding yeast, although and quite surprisingly, they are not required for growth (Aparicio et al. 1991; Kayne et al., 1988).

Two lines of evidence suggest that the H3 and H4 tails appear to play a direct role in the assembly of silent chromatin domains at these loci through interactions with the Sir3 and Sir4 components of the SIR complex: First, certain *sir3* alleles can suppress the silencing

defect of H4 tail mutations. Second, both Sir3 and Sir4 can bind to the amino-termini of H3 and H4 in vitro (Hecht et al., 1995; Johnson et al., 1990).

A number of studies suggest that the hypoacetylated state of specific lysine residues in these tails (for example, at positions 5, 8, 12, and 16 in H4) is important for their ability to interact with Sir3 and Sir4:

- Mutation experiments were used to test for the importance of each histone tail residue in silencing. The positively charged lysine residues were mutated either to glycine or glutamine, which are not charged and therefore mimic the acetylated state. Mutations to arginine, which is positively charged, mimic the deacetylated state. It was shown that mutations which change histone tail lysine residues to arginine have less severe silencing defects than mutations that change lysines to glycine or glutamine (Braunstein et al., 1996; Johnson et al., 1990).
- Histone tails within silent chromatin domains are generally found to be hypo-acetylated (Braunstein et al., 1993; Suka et al., 2001).
- Overexpression of the Sir2 protein results in general histone hypo-acetylation (Braunstein et al., 1993).
- Sir2 has been shown to be an NAD^+-dependent deacetylase with *in vitro* preference for histone H4 lysine 16, an amino acid that plays a critical role in silencing (Imai et al., 2000; Landry et al., 2000; Smith et al., 2000).

Together, these observations suggest a model for the assembly of silent chromatin that involves the recruitment of the SIR complex to DNA and its subsequent spreading along the chromosome fiber through interactions involving the binding of Sir3 and Sir4 to deacetylated histone tails at telomeres and the *HM* loci, thereby enabling the Sir3 and Sir4 proteins to bind. Consistent with this model, chromatin immunoprecipitation experiments show that the Sir proteins are associated with DNA throughout silent chromatin domains (Hecht et al., 1996). However, the idea that Sir2 deacetylates histones directly *in vivo* has yet to be tested.

Furthermore, while a strong genetic link exists between silencing and histone hypoacetylation at *HM* loci and telomeres, the role of histones tails in rDNA silencing is unknown and none of the components of RENT have yet been shown to bind to histones or DNA directly. Thus, it is unclear whether Sir2 activity at rDNA is required to deacetylate histones or some other protein.

1.5 The Enzymatic Activity of Sir2

All forms of silencing in yeast require Sir2, a member of an ancient family of proteins which occur in organisms ranging from bacteria to complex eukaryotes (Frye, 2000). Members of this family share a 250 amino acid core domain that is between 25 % to 60 % identical (Figure 1.3).

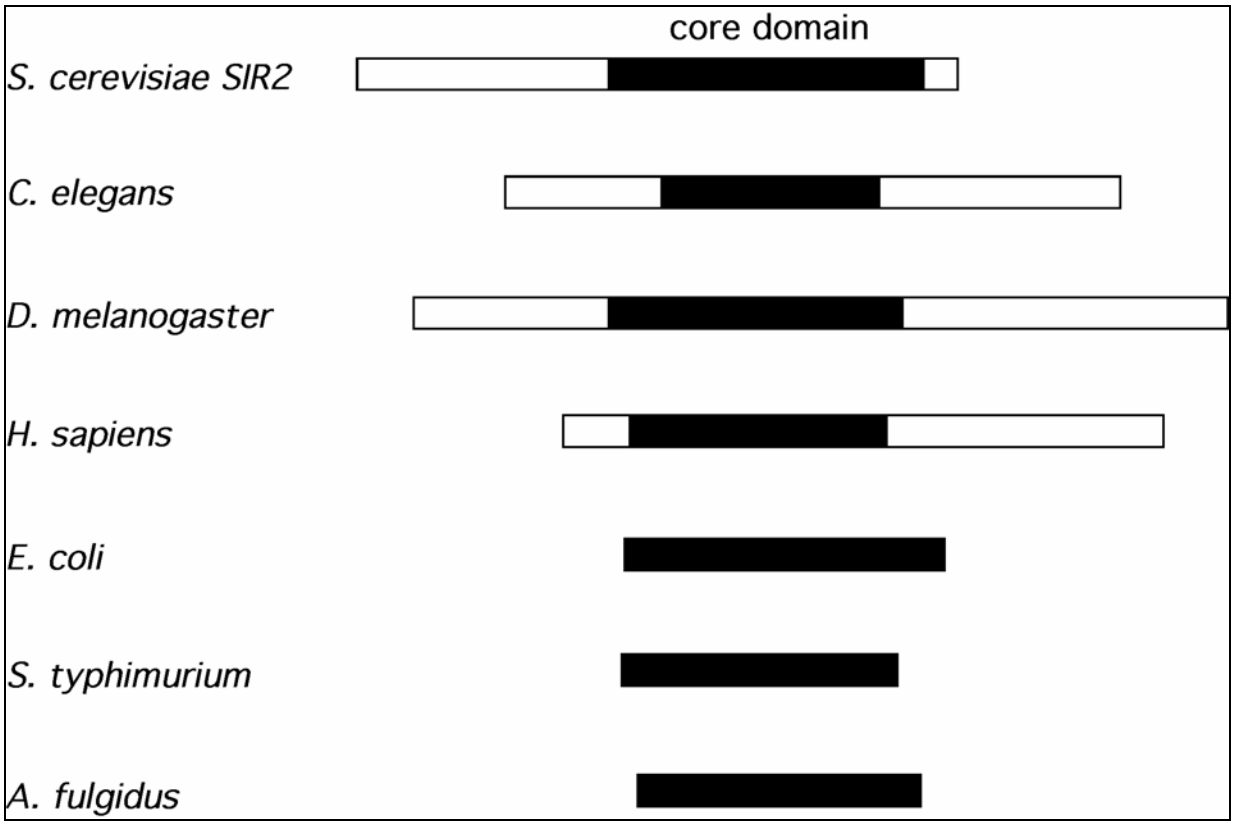

FIGURE 1.3 Members of the Sir2 family of protein contain a highly conserved core domain (dark boxes).

Sir2 has recently been shown to be an unusual deacetylase: It possess NAD^+-dependent protein and histone deacetylation activity *in vitro* (Tanner et al., 2000; Tanny and Moazed, 2001). Unlike the Rpd3 class of histone deacetylases, which catalyzes conventional amide hydrolysis reactions, Sir2 couples deacetylation to the hydrolysis of NAD^+, transferring the acetyl group from its protein substrate to ADP-ribose, thereby generating a novel compound, O-acetyl-ADP-ribose (ADPRAc) (Tanner et al., 2000; Tanny and Moazed, 2001) (Figure 1.4).

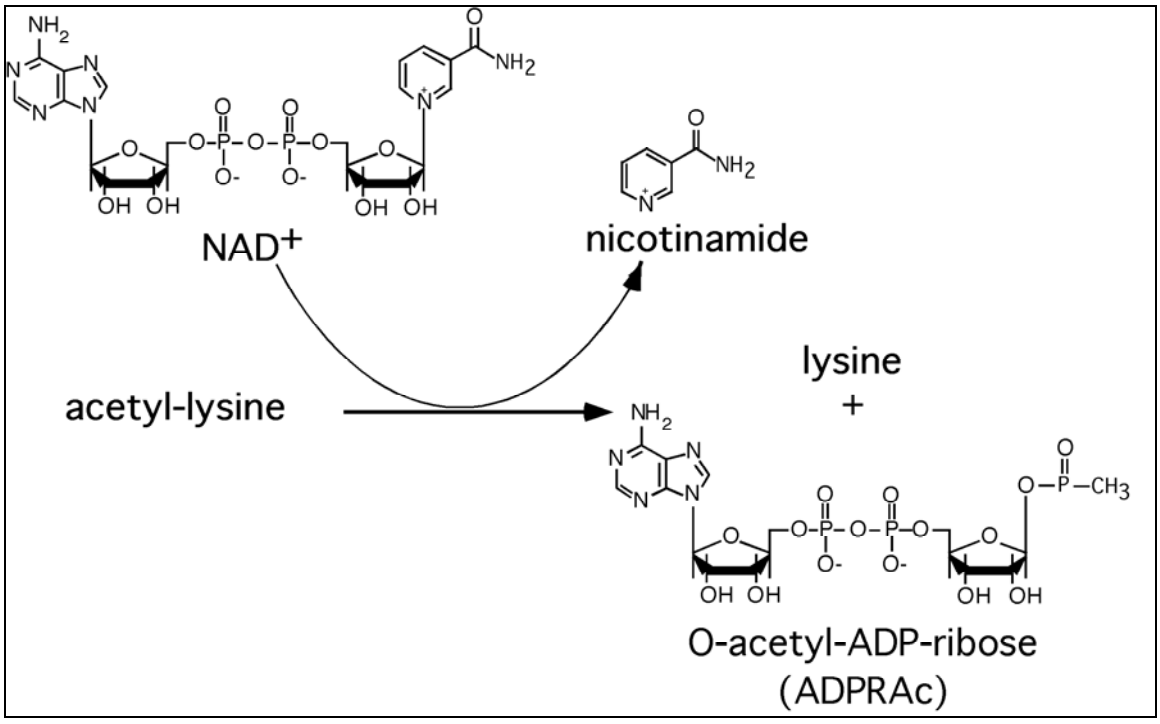

FIGURE 1.4 Sir2 is an NAD^+-dependent deacetylase. The breaking of a high energy bond, which frees nicotinamide, is utilized to deacetylate the substrate. A novel compound, O-acetyl-ADP-ribose (ADPRAc), is formed.

Mutations that abolish the *in vitro* activity of Sir2 result in a complete loss of silencing *in vivo*, suggesting that direct deacetylation is required for silencing (Imai et al., 2000; Tanny et al., 1999; Tanny and Moazed, 2001). To investigate the possible role of Sir2‘s enzymatic activity in the assembly of silencing complexes and histone deacetylation in silent loci, I

made use of two inactive Sir2 proteins (Figure 1.5). Each contains a point mutation in the conserved catalytic core domain (Tanny and Moazed, 2001). It has previously been shown that the *sir2-H364Y* allele encodes a protein that forms stable complexes with other silencing proteins but is completely defective for silencing *in vivo* (Ghidelli et al., 2001; Tanny et al., 1999). The *sir2-G262A* allele has also been shown to be completely defective for silencing *in vivo* (J. C. Tanny, personal communication).

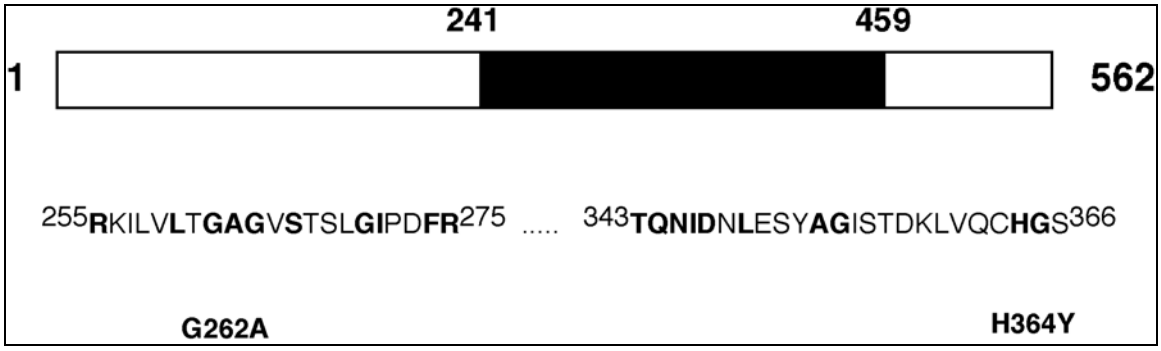

FIGURE 1.5 Two Sir2 point mutants were used in this study. The Sir2 protein is depicted schematically, with its catalytic core domain in black. Conserved amino acids are written in bold letters and mutations are indicated below.

1.6 Thesis Goals

Gene silencing is a process by which large regions of DNA get assembled into a compact structure that is inaccessible to the transcription machinery. In budding yeast, it occurs at the silent mating type loci, telomeres, and the ribosomal DNA repeats. The key protein seems to be Sir2, which is required at all of these sites. Recently, Sir2 has been shown to be an NAD^+-dependent deacetylase.

The aim of my thesis was to elucidate the role of Sir2's enzymatic activity in the formation of silencing complexes. Various possibilities for such a role can be envisioned: One can rationalize that Sir2 might play a role in either the initial assembly of silencing complexes, the spreading of silencing complexes, or a step subsequent to spreading.

Using point mutants of Sir2, which abolish its enzymatic activity, I wanted to compare the formation of silencing complexes at a telomere, rDNA and a silent mating type cassette in the *sir2* mutant background with a *SIR2* strain. To address this question, a comparative chromatin immunoprecipitation assay had to be established.

It has been hypothesized that Sir2 might deacetylate the N-terminal tails of histones H3 and H4 *in vivo*, leading ultimately to chromatin compaction. I attempted to test this hypothesis by studying the effect of histone tail mutations on silencing at the rDNA locus.

The results of these experiments would provide insight into Sir2's role in the formation of silencing complexes and would then enable me to propose a mechanism for the assembly of silencing complexes at silenced loci.