THE ROLE OF HISTONES AND HISTONE MODIFYING ENZYMES IN RIBOSOMAL DNA SILENCING IN *Saccharomyces cerevisiae*

A Dissertation

by

CHONGHUA LI

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2008

Major Subject: Biochemistry

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Approved by:

Chair of Committee,	Mary Bryk
Committee Members,	Timothy Hall
	Michael Polymenis
	Martin Scholtz
Head of Department,	Gregory Reinhart

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ABSTRACT

The Role of Histones and Histone Modifying Enzymes in Ribosomal DNA Silencing in *Saccharomyces cerevisiae*. (December 2008)

Chonghua Li, B.S., Tsinghua University; M.S., Tsinghua University

Chair of Advisory Committee: Dr. Mary Bryk

In *S. cerevisiae*, the ribosomal DNA locus is silent for RNA polymerase II (Pol II) transcription and recombination (rDNA silencing). Our goal is to understand how histones and histone-modifying enzymes regulate the silent chromatin at the rDNA locus.

Sir2, a NAD^+-dependent histone deacetylase, is required for rDNA silencing. To understand how Sir2 regulates rDNA silencing, we performed chromatin immunoprecipitation to measure the association of modified histones across the rDNA repeat in wild-type and *sir2Δ* cells. We found that in *sir2Δ* cells, histone H3 at the rDNA became hyperacetylated and hypermethylated. High levels of K4-methylated H3 correlate with Pol II transcription. Consistent with this, we found that the nontranscribed spacer (NTS) region was transcribed by Pol II in *sir2Δ* cells. To investigate if transcription of the NTS region regulates rDNA silencing, we overexpressed this region both *in trans* and *in cis*. Our data showed that overexpression of the NTS region *in cis* caused Pol II silencing defect and hyperrecombination at the rDNA. These data suggest that Sir2 contributes to maintain the silent chromatin at the rDNA by repressing Pol II

transcription in the NTS region. We also found that the NTS transcripts could be translated *in vitro* and that they copurified with polysomes, suggesting that the transcripts may encode proteins or that the transcripts are somehow involved in the process of translation.

Additionally, we examined the role of linker histone H1 in regulating rDNA silencing. We found that, unlike Sir2 that represses both Pol II transcription and recombination, histone H1 only represses recombination at the rDNA. The hyperrecombination defect at the rDNA is more severe in *sir2Δ hho1Δ* double mutant than in either single mutant, suggesting histone H1 and Sir2 act independently. Consistently, *hho1Δ* cells did not accumulate extrachromosomal rDNA circles (ERCs) or the Holliday junction intermediates, which accumulate in *sir2Δ* cells. These data suggest that histone H1 and Sir2 regulate different recombination pathways.

In summary, my research has provided insight into the mechanism of how silent chromatin at the rDNA locus is regulated, which will help us understand how fundamental components of chromosomes affect gene expression and genome stability.

DEDICATION

I would like to dedicate this dissertation to my parents, Shumei Zhang and Mingfeng Li, and my husband, Rui Xiao. Without your love and support, none of this would have been possible. Thank you.

ACKNOWLEDGEMENTS

First of all, I would like to thank my advisor, Dr. Mary Bryk, for all the guidance and support. She is the one who opened the door to science to me. She has taught me how to think in a scientific way, design and develop experiments carefully, and analyze data critically. She always inspired me with great ideas and encouraged me when I was down. I am very grateful to have her as my mentor. I would also like to acknowledge my committee members, Dr. Michael Polymenis, Dr. Tim Hall, Dr. Martin Sholtz, and my previous committee member, Dr. Patti Liwang, for all the valuable discussion, advice and help.

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CHAPTER I

INTRODUCTION

Eukaryotic cells contain nuclear DNA filaments known as chromosomes that carry genetic information. Combined end-to-end, the DNA in a human cell stretches to ~2 meters. In order to fit within the nucleus with a diameter about 5 μm, DNA molecules are compacted into a nucleoprotein structure named chromatin. Chromatin compaction starts with nucleosome, the fundamental subunit of chromatin with DNA wrapping around a histone octamer, forming a "beads-on-a-string" structure (Figure 1-1) (Luger *et al.*, 1997; White *et al.*, 2001; Davey *et al.*, 2002; Richmond and Davey, 2003). Nucleosomal chromatin is packed into a 30 nm fiber and later folded into 300 nm giant loops (Finch and Klug, 1976). The loops are then condensed into 700 nm fibers and finally form chromosomes with a diameter of 1400 nm (Figure 1-2).

The compaction and folding of chromatin is not uniform. There are loosely assembled regions known as euchromatin, and highly condensed regions known as heterochromatin. The degree of chromatin folding influences the level of various cellular processes, such as transcription, recombination, replication, and DNA repair (Aguilera *et al.*, 2000; Morales *et al.*, 2001; Verger and Crossley, 2004; Razin *et al.*, 2007). For example, genes located in the euchromatin are more likely to be transcribed due to the

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This dissertation follows the style and format of *Molecular Biology of the Cell*.

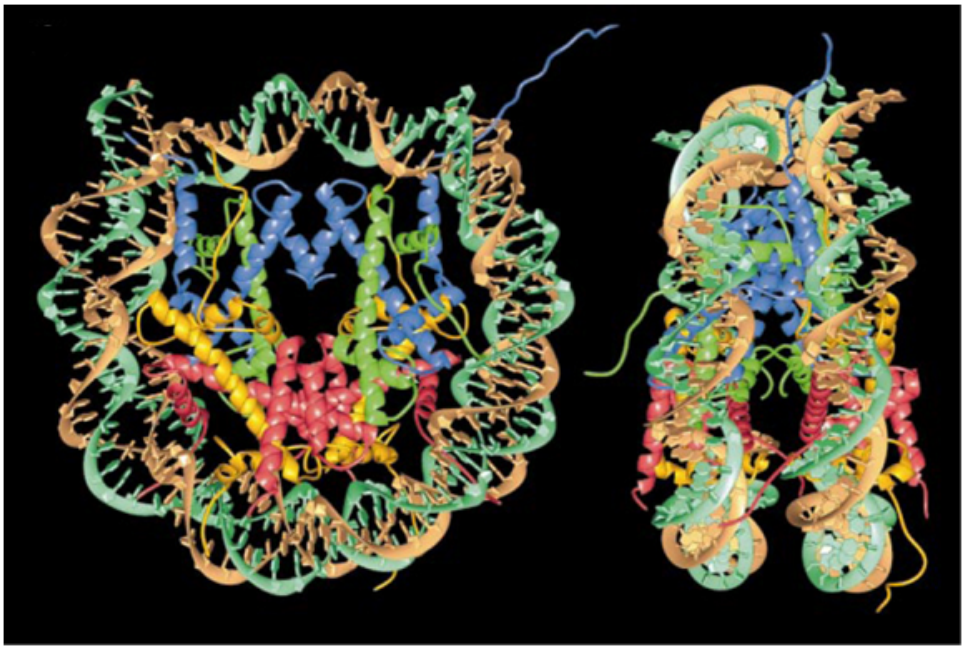


Figure 1-1 Crystal structure of the nucleosome core particle. The structure shows 146-bp DNA (brown and turquoise) wrapped around a histone core octamer with eight histone proteins (blue: H3; green: H4; yellow: H2A; red: H2B) (Cited from Luger et al. 1997).

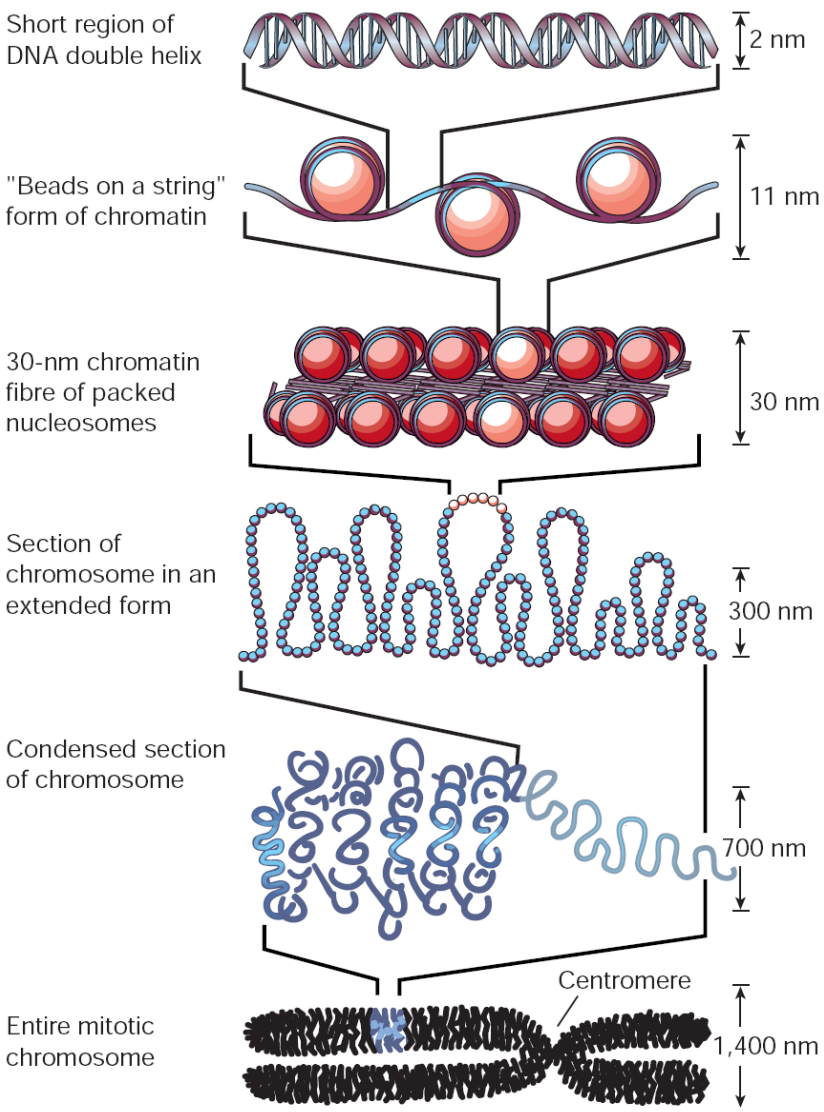


Figure 1-2 The compaction of DNA. DNA double helix wraps around histone octamers to form the lowest chromatin structure unit nucleosomes. Nucleosomes are packed into 30 nm chromatin fibers, which form higher level chromatin structures (Felsenfeld and Groudine, 2003).

accessibility to the transcriptional machinery, while genes located in heterochromatin are repressed (or silenced) because of the closed chromatin confirmation that is less accessible to the transcriptional machinery (Strathern *et al.*, 1982; Terleth *et al.*, 1989; Gottschling, 1992; Loo and Rine, 1994). Chromatin structure is regulated by histone proteins as well as non-histone proteins. My dissertation research focuses mainly on the regulation of gene expression and mitotic recombination in silent chromatin domains by histones and histone-modifying enzymes.

1.1. NUCLEOSOME, HISTONES AND HISTONE MODIFICATIONS

1.1.1. The Nucleosome Structure

The structure of a nucleosome particle has been solved by X-ray crystallography by several groups (Luger *et al.*, 1997; Harp *et al.*, 2000; White *et al.*, 2001; Davey *et al.*, 2002; Richmond and Davey, 2003). As shown in Figure 1-1, each nucleosome particle consists of 146 bp of DNA wrapped around a histone octamer in 1.65 turns of a left-handed superhelix. The central histone octamer can be divided into four dimers defined by two sets of H3-H4 and H2A-H2B histone pairs. The majority of histone octamer is protected by the superhelical DNA gyre, yet there are N-terminal or C-terminal residues of each core histone protruding out of the nucleosome disk. These N- or C-terminal residues are called “histone tails”, which are believed to be relatively unstructured because they are not amenable to X-ray analysis (Luger *et al.*, 1997).

1.1.2. Post-Translational Modifications of Histones

Histone proteins are subjected to a variety of post-translational modifications including acetylation and methylation of lysines (K) and arginines (R), phosphorylation

of serines (S) and threonines (T), ubiquitylation and sumoylation of lysines, as well as ribosylation (Turner, 2005; Millar and Grunstein, 2006). These modifications to histone proteins play essential roles in many aspects of chromatin function. A summary of the amino acid residues of the histones H2A, H2B, H3 and H4 in yeast that are known to be modified is shown in Figure 1-3.

1.1.2.1. Histone Acetylation/Deacetylation

Histone acetylation/deacetylation is best characterized of all known histone modifications. The acetylation state of histones affects many cellular activities, such as nucleosome assembly, chromatin folding, gene transcription and repression, and heterochromatin formation (Shahbazian and Grunstein, 2007). For example, newly synthesized histones are acetylated transiently and deacetylated after deposition onto DNA. Acetyl groups on histones may provide recognition sites for the chaperone proteins (Smith and Stillman, 1991; Ma *et al.*, 1998; Ai and Parthun, 2004; Poveda *et al.*, 2004). These acetyl groups must be removed after the histones are incorporated into chromatin, to allow the compaction of the chromatin into higher-order structures. It has been suggested that the acetylation of lysine residues on histones assembled in nucleosomes can neutralize the positive charge of lysine residues, thereby disrupting electrostatic interactions between histones and DNA and allowing a more opened chromatin confirmation (Mathis *et al.*, 1978; Simpson, 1978; Vidali *et al.*, 1978; Imai *et al.*, 1986; Annunziato *et al.*, 1988; Tse *et al.*, 1998), which is a prerequisite for activation of transcription. Hence, hyperacetylated histones are correlated with active transcription,

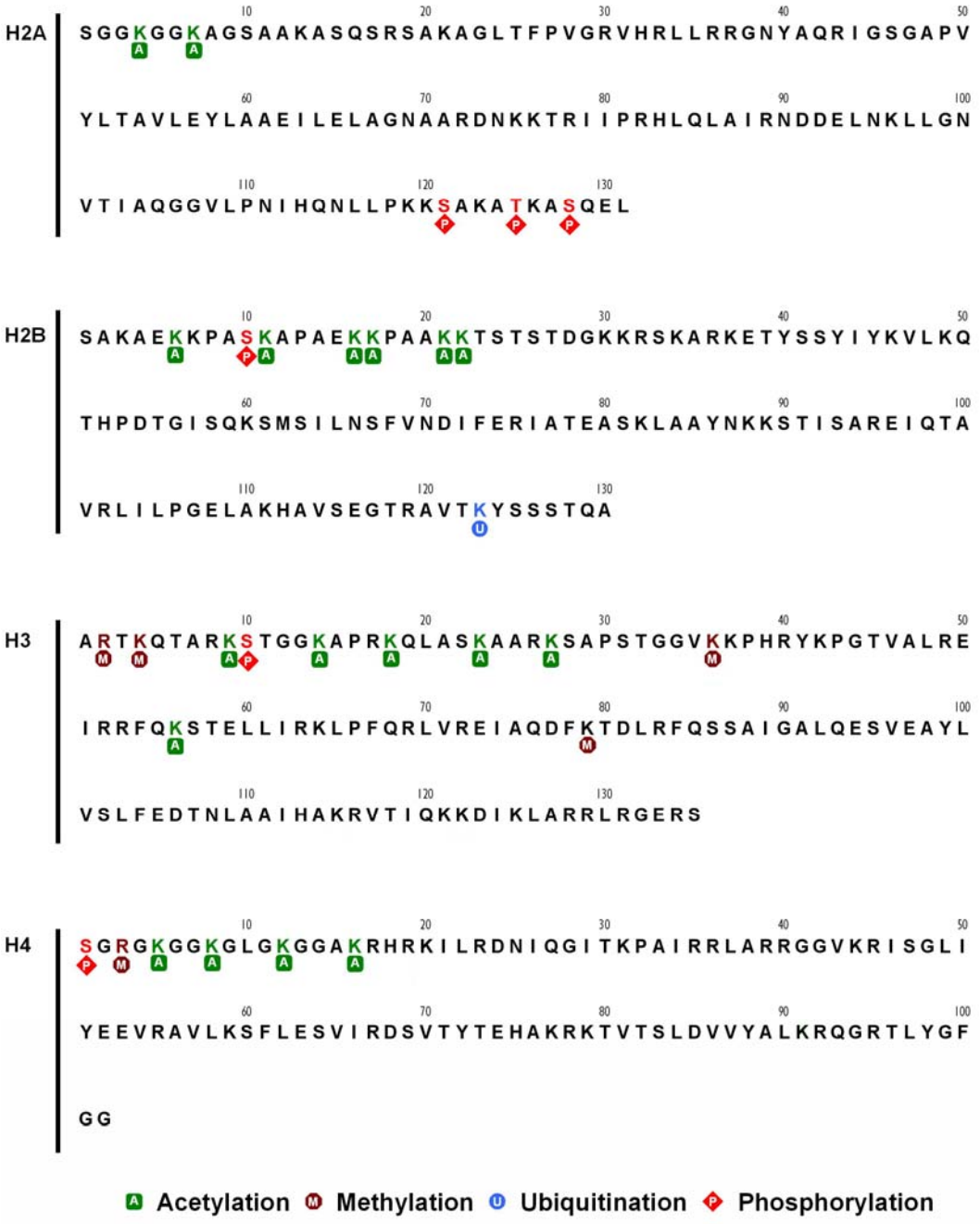


Figure 1-3 Known histone modifications in yeast (Downs *et al*., 2000; Roth *et al*., 2001; *Suka et al.*, 2001; Ahn *et al*., 2005; Foster and Downs, 2005; Hyland *et al.*, 2005; Ye *et al.*, 2005; Krishnamoorthy *et al.*, 2006; Parra *et al.*, 2006; Krebs, 2007).

while hypoacetylated histones are associated with chromatin in a more closed confirmation, equivalent to a transcriptionally repressed state.

The enzymes responsible for acetylation of histones are called histone acetyltransferases (HATs). HATs modify the histones by transferring an acetyl group from acetyl coenzyme A (acetyl-CoA) to the ε-amino group of a lysine residue. This type of modification is reversible. The acetyl group can be removed by enzymes called histone deacetylases (HDACs) (Marmorstein and Roth, 2001; Roth *et al.*, 2001). One group of HDACs that is associated with silent chromatin is the Sir2 family of histone deacetylases, which is conserved from bacteria to humans (Blander and Guarente, 2004). The name of Sir2 stands for Silent information regulator 2, reflecting the role of the founding member of the Sir2 family in regulating silent chromatin in *S. cerevisiae*. Known targets of Sir2 include K9, K14 and K56 of histone H3, and K16 of histone H4 (Imai *et al.*, 2000; Xu *et al.*, 2007). What makes this HDAC family unique from other HDACs is that the Sir2 family members require the metabolic cofactor NAD^+ for their deacetylase activity (Imai et al. 2000; Landry et al. 2000a; Landry et al. 2000b). For each acetyl lysine that is deacetylated by Sir2, one NAD^+ molecule is cleaved to produce nicotinamide and *O*-acetyl-ADP-ribose (AAR) (for review, see Blander and Guarente, 2004). AAR was found to have a direct role in regulating the assembly of the Sir2/Sir3/Sir4 silencing complex in *S. cerevisiae* (Liou *et al.*, 2005). In *S. cerevisiae*, Sir2 regulates transcriptional silencing, mitotic recombination, DNA replication and aging, which will be discussed later.

1.1.2.2. Histone Methylation

Methylation is another type of histone modification that has been extensively studied. This modification occurs on lysine or arginine residues. Lysine residues can be mono-, di- or trimethylated on the ε-nitrogen by enzymes called histone methyltransferases (HMTs) that use *S*-adenosylmethionine as the donor of the methyl group. Arginine residues can be mono- or dimethylated by enzymes known as protein arginine methyltransferases (PRMTs) (Shilatifard, 2006). In budding yeast, the lysine residues on histone H3 that are known to be methylated are K4, K36 and K79. The enzymes that catalyze methylation of these lysine residues are Set1, Set2 and Dot1, respectively (for review, see Klose and Zhang, 2007). In *S. pombe* and higher eukaryotic cells, K9 on histone H3 is a target for methylation, which is required for heterochromatin formation (for review, see Martin and Zhang, 2005; Horn and Peterson, 2006). However, K9 of histone H3 is not methylated in budding yeast. Histone methylation had been thought to be irreversible until histone demethylases were identified recently (for review, see Klose and Zhang, 2007).

In budding yeast, high levels of methylated histones are found in the coding regions of genes (Bernstein *et al.*, 2002; Santos-Rosa *et al.*, 2002). In fact, the enzymes responsible for methylation of H3K4 and H3K36 have been shown to physically associate with RNA polymerase II (Pol II) during transcription elongation, resulting in hypermethylation of the histones in the coding regions of genes (Krogan *et al.*, 2003b; Li *et al.*, 2003; Ng *et al.*, 2003c; Xiao *et al.*, 2003). Paradoxically, methylation of H3K4

and H3K79 is also important for maintaining silent chromatin. A discussion of the roles of methylated histone H3 in gene silencing is provided below.

1.1.2.3. Histone Phosphorylation and Ubiquitylation

Histones are also subjected to other modifications, such as phosphorylation and ubiquitylation. Phosphorylation of histones is known to occur on serine residues. For example, histone H3 can be phosphorylated on serine 10. Serine 10 phosphorylated H3 is crucial for chromosome condensation and cell cycle progression as well as transcriptional activation (Nowak and Corces, 2004). However, it is not clear if phosphorylation of H3S10 is involved in transcriptional silencing.

Ubiquitylation can occur on lysine residues of histones. In budding yeast, the K123 residue of histone H2B has been found to be ubiquitylated in chromatin (Jentsch *et al.*, 1987; Robzyk *et al.*, 2000). Ubiquitylation of H2B on K123 is required for di- and trimethylation of H3 on K4 and K79, indicating a possible interaction between different histone modifications (for review, see Weake and Workman, 2008).

1.2. THREE SILENT DOMAINS IN *Saccharomyces cerevisiae*

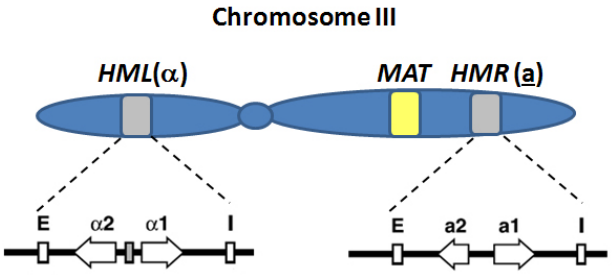
In *S. cerevisiae*, genes located in heterochromatin domains are silenced at the transcriptional level due to silent chromatin structure. This phenomenon is known as gene silencing, a form of transcriptional repression that acts in a regional manner rather than a promoter-specific manner. Three silent loci have been discovered in *S. cerevisia*e: the telomeres, the silent mating-type (*HM*) loci and the ribosomal DNA locus (rDNA). Among these silent loci, silencing at the *HM* loci and the telomeres has been studied extensively.

1.2.1. Silencing at the Silent Mating Type Loci

Gene silencing was first discovered at the silent mating-type loci (Klar *et al.*, 1981; Nasmyth *et al.*, 1981). In budding yeast, haploid cells have two mating types, a or α. Haploid budding yeast cells respond to the mating pheromone produced by haploid cells of the opposite mating type to commence mating and the formation of a diploid cell. The mating type of a cell is determined by a cassette of genetic information present at the mating type locus, *MAT* (*MAT*a or *MAT*α), on chromosome III. Cells carry extra copies of the a and α genetic information at the silent mating type loci, *HMR* (a) and *HML* (α) (Figure 1-4A). In wild-type cells, the genetic information at the two silent mating type loci is silenced, so that the mating type of budding yeast cells is determined by the genetic information present at the *MAT* locus (Moazed, 2001).

The silencing of *HML* and *HMR* is mediated by *cis*-acting DNA elements known as silencers. Each *HM* locus contains two silencer elements, *E* and *I*. The chromatin between the two elements acquires a repressive structure that prevents expression of Pol II transcribed genes in that region (for reviews, see Rusche *et al.*, 2003; Fox and McConnell, 2005). The establishment of silent chromatin starts at the *E* elements, which contain binding sites for several proteins including members of the origin replication complex (ORC), Rap1, and Abf1. These proteins then recruit Silent information regulator (Sir) proteins (Sir1-4) to the *HMR-E* region in an ordered manner (Rusche *et al.*, 2002). Sir2, an NAD^+-dependent histone deacetylase, is brought to the *HM* loci through interaction with Sir4 (Moazed *et al.*, 1997; Ghidelli *et al.*, 2001), and deacetylates histone H3 and H4 in nearby nucleosomes (Hoppe *et al.*, 2002). It has been

A



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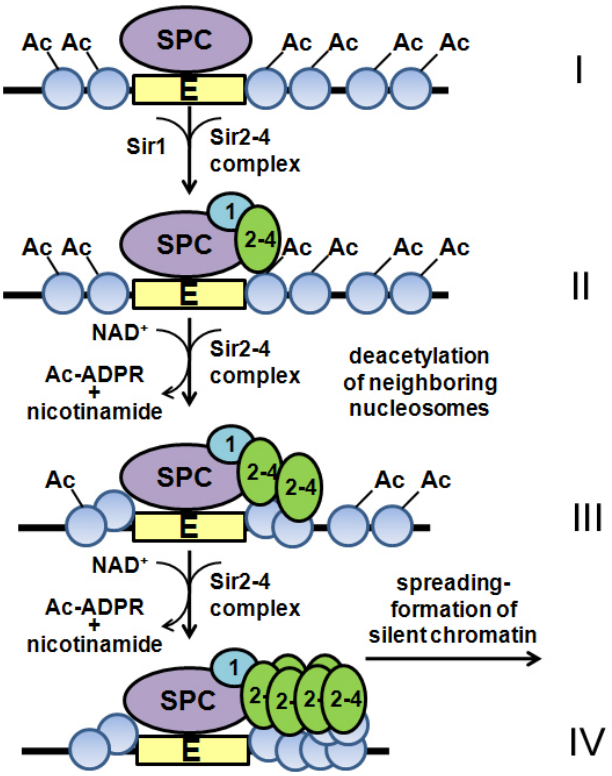

Figure 1-4 The *HM* silent mating type loci. (A) Relative locations of the silent *HML* and *HMR* loci, and the active *MAT* locus on yeast chromosome III. The *HML* and *HMR* loci contain the *E* and *I* silencers and the open reading frames of the α1, α2, and a1, a2 mating type genes. (B) A model illustrating silent chromatin assembly at *HMR* (adapted from Fox and McConnell, 2005). (I) The E element binds silencer protein complex (SPC), which contains ORC, Rap1 and Abf1. (II) The SPC recruits Sir1 and the Sir2-4 complex. Sir2 removes acetyl groups from the neighboring nucleosomes through its NAD^+-dependent HDAC activity. (III) The hypoacetylated nucleosomes recruit additional Sir2-4 complex. (IV) Repetition of this process allows the spreading of the silent chromatin.

shown that Sir3 and Sir4 can interact with the N-terminal tails of histone H3 and H4, preferentially deacetylated forms of H3 and H4 (Hecht *et al.*, 1995; Carmen *et al.*, 2002). The deacetylation of histone H3 and H4 by Sir2 creates more binding sites for Sir3 and Sir4 (Rusche *et al.*, 2002), which allows the spreading of the silent chromatin. A model for silent chromatin spreading in *HMR-E* is shown in Figure 1-4B. The direct interaction between Sir3 and Sir4 also helps the spreading of the silent chromatin (Rudner *et al.*, 2005).

It was believed that the repression of Pol II transcription in the *HM* loci was due to the repressive chromatin structure that is less accessible to the Pol II transcriptional machinery. However, a recent study proposed a model that challenges this idea. In this study, Gao and colleagues found that the promoters of the silenced *HMRa1* and *HML*α*1/*α*2* are permissive to the binding of the pre-initiation complex (PIC) of Pol II transcriptional machinery, while the association of elongation factors and the capping enzymes with these regions was restricted in a Sir2-dependent manner (Sekinger and Gross, 2001; Gao and Gross, 2008).

1.2.2. Telomere Silencing

Telomeres located at the ends of the chromosomes represent another group of sequences in the *S. cerevisiae* genome where silent chromatin is located. A typical *S. cerevisiae* telomere consists of short tandem DNA repeats that are about 300 bp in length and two classes of sub-telomeric repeats, the X and Y' elements (Wright *et al.*, 1992). Over 50 proteins have been implicated in telomere silencing. Among these proteins, Sir2, Sir3, Sir4 (Sir2-3-4), Rap1 and Ku70/80 heterodimer are absolutely

required (Aparicio *et al.*, 1991; Kyrion *et al.*, 1992; Boulton and Jackson, 1998; Laroche *et al.*, 1998). There are several Rap1 binding sites at the telomere, and the initiation of the silent chromatin starts with recruitment of Rap1 to these binding sites (Buchman *et al.*, 1988). The Sir2/Sir4 complex is then recruited to the telomere through the interaction between Sir4 and Rap1 (Moretti *et al.*, 1994). The Ku70/Ku80 heterodimer also interacts with telomeric DNA and assists Rap1 in recruiting the Sir2/Sir4 complex (Mishra and Shore, 1999). Like silent chromatin at *HM* loci, Sir2 deacetylates histone tails and Sir3 is recruited via interactions through Rap1, Sir4 and the hypoacetylated histone tails. The spreading of silent chromatin at the telomeres is similar to the spreading that occurs at the *HM* loci (Moazed, 2001).

1.2.3. Ribosomal DNA Silencing

In a study to determine whether the chromosomal position of a Ty1 retrotransposon affects its expression, Bryk *et al*. (1997) found that when a Ty1 element was integrated into the ribosomal DNA locus (rDNA), it was silenced at the transcriptional level. At the same time, Smith *et al.* (1997) showed that other Pol II transcribed genes are silenced when inserted into the rDNA. This phenomenon is known as rDNA silencing. In addition, silencing at the rDNA includes repression of mitotic recombination (Szstak and Wu, 1980; Zamb and Petes, 1982) and silencing of replication origins (Walmsley *et al*., 1984; Saffer and Miller, 1986). The mechanism of silencing at the rDNA is different from that at the other silent loci. In our lab we use the rDNA as a model system to study gene silencing and I will discuss silencing at the rDNA locus below.

1.3. MODEL SYSTEM: RIBOSOMAL DNA SILENCING

1.3.1. Structure of Ribosomal DNA Locus

The rDNA is a highly repeated locus, containing about 150-200 copies of the tandem rDNA repeats that make up about two-thirds of chromosome XII (Warner, 1989). The rDNA repeats form a unique structure called the nucleolus (Dammann *et al.*, 1995). Each rDNA repeat is about 9.1 kb in length and contains two nontranscribed spacers (NTS1 and NTS2), a 35S ribosomal RNA gene (rRNA) and a 5S rRNA gene (Figure 1-5). The 35S rRNA gene is transcribed by RNA polymerase I (Pol I), and the Pol I transcript is processed into the 18S, 5.8S and 25S rRNAs. The 5S rRNA gene is transcribed by RNA polymerase III (Pol III) (Venema and Tollervey, 1999). These rRNAs serve as structural and catalytic components of ribosomes. The NTS region contains several *cis*-acting regulatory sequences including: a promoter, enhancer and terminator that direct Pol I transcription of the 35S rRNA gene (Paule and White, 2000); a replication fork barrier (RFB) in NTS1 that prevents bidirectional replication of the rDNA (Brewer *et al.*, 1992; Kobayashi *et al.*, 1992); and an origin of DNA replication (ARS) in NTS2 (Figure 1-5) (Brewer and Fangman, 1991). Despite the fact the rRNA genes are actively transcribed by Pol I and Pol III, with the ribosomal RNA making up more than 60% of total RNA (Warner, 1989), Pol II transcribed genes are repressed when inserted into the rDNA. This indicates that the chromatin at the rDNA locus forms a specialized structure that allows Pol I and Pol III transcription but represses Pol II transcription.

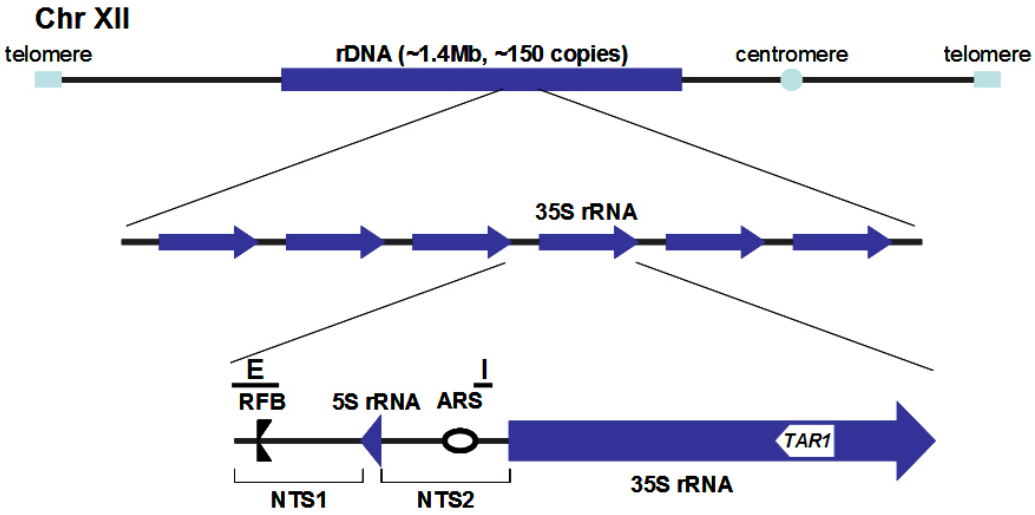

Figure 1-5 Structure of *S. cerevisiae* rDNA locus. The rDNA locus covers about 60% of chromosome XII in *Saccharomyces cerevisiae*. The 35S and 5S rRNA genes, the autonomous replication sequence (ARS), the replication fork barrier (RFB), the non-transcribed spacers 1 and 2 (NTS1 and NTS2), and the *TAR1* gene located within the coding region of 35S rRNA gene are indicated. Black lines above the RFB and ARS, the E and I elements that are *cis*-acting sequences essential to *HOT1*-stimulated recombination. The 35S rRNA gene is not drawn to scale. Arrows indicate the direction of gene transcription.

1.3.2. Factors Involved in Transcriptional Silencing in Ribosomal DNA Locus

1.3.2.1. The RENT Complex

Since the discovery of rDNA silencing in 1997, a growing number of factors have been identified that participate in rDNA silencing. Unlike silencing at the *HM* loci and telomeres, silencing at the rDNA does not require the Sir2-Sir3-Sir4 complex. Instead, it requires a complex called RENT (regulator of nucleolar silencing and telophase exit), which contains Net1, Sir2 and Cdc14. The RENT complex was identified by Straight *et al*. (1999) in an attempt to identify proteins that associate with Sir2. Deletion of either Net1 or Sir2 results in disruption of rDNA silencing (Bryk *et al.*, 1997; Fritze *et al.*, 1997; Smith and Boeke, 1997; Straight *et al.*, 1999).

As the core component of the RENT complex, Net1 associates with the rDNA, serving as an anchor to recruit the other two subunits of the RENT complex, Sir2 and Cdc14 (Straight *et al.*, 1999; Huang and Moazed, 2003). It has been shown that the RENT complex binds the rDNA at two distinct regions, NTS1 and the Pol I promoter region in NTS2, including the 5' end of 35S rRNA coding region (Huang and Moazed, 2003). The association of Net1 with the NTS1 region requires the RFB-binding protein Fob1, which is required for the replication fork blocking and recombination hotspot activities (See Chapter I, 4.3). In the absence of Fob1, the RENT complex is not recruited to NTS1 and the silencing of Pol II transcription is abolished in NTS1 (Huang and Moazed, 2003). The recruitment of the RENT complex to the Pol I promoter region may be mediated through Pol I since both Net1 and Sir2 were found to physically

interact with Pol I (Shou *et al.*, 2001; Huang and Moazed, 2003). This is also consistent with the finding that Pol I transcription directs the spreading of silent chromatin (Buck *et al.*, 2002; Cioci *et al.*, 2003). Sir2 deacetylates histones at the rDNA (Bryk *et al.*, 2002; Huang and Moazed, 2003). Deletion of Sir2 results in a significant increase in the levels of K9-acetylated H3 and K14-acetylated H3 in NTS1 and NTS2, but has minor effects, if any, on the levels of acetylated H4 (K5, K8, K12, and K16) (Bryk *et al.*, 2002; Buck *et al.*, 2002). Sir2 also regulates physical chromatin structure of the rDNA. In cells lacking Sir2, the number of micrococcal nuclease sensitive sites in the rDNA is increased, consistent with a more accessible chromatin structure (Fritze *et al.*, 1997; Cioci *et al.*, 2002; Li *et al.*, 2006a). The function of Cdc14 in rDNA silencing has not been studied.

1.3.2.2. The Compass Complex

Another multiprotein complex that is important for rDNA silencing is the COMPASS complex (Complex Proteins Associated with Set1), whose members include Set1, Bre2, Sdc1, Shg1, Spp1, Swd1, Swd2 and Swd3 (Roguev *et al.*, 2001; Krogan *et al.*, 2002; Nagy *et al.*, 2002). The COMPASS complex is required for mono-, di- and trimethylation of K4 of histone H3 in S. *cerevisiae* (Briggs *et al.*, 2001; Roguev *et al.*, 2001; Krogan *et al.*, 2002; Nagy *et al.*, 2002; Santos-Rosa *et al.*, 2002). The catalytic and core component of this complex is Set1, a histone methyltransferase that contains a conserved SET domain (Roguev *et al.*, 2001; Krogan *et al.*, 2002; Nagy *et al.*, 2002). Most advances in our understanding of Set1 are related to its function at euchromatin in association with Pol II. It has been shown that COMPASS is recruited to the promoter of actively transcribed genes by the Paf1 complex (Krogan *et al.*, 2003a; Ng *et al.*, 2003c).

High levels of K4-trimethylated histone H3 are associated with the genes that have been recently transcribed by Pol II (Bernstein *et al.*, 2002; Santos-Rosa *et al.*, 2002; Boa *et al.*, 2003). However, a Set1-dependent low level of K4-methylated histone H3 is also required for gene silencing at the three silent loci in S. *cerevisiae* (Nislow *et al.*, 1997; Briggs *et al.*, 2001; Bryk *et al.*, 2002; Nagy *et al.*, 2002; Krogan *et al.*, 2003a). While deletion of the *SET1* gene does not alter the association of Sir2 or the acetylation state of histone H3 within rDNA, an increase of K4-methylated histone H3 is observed at this locus in *sir2*Δ cells (Bryk *et al.*, 2002). The result was unexpected because high levels of K4-methylated H3 are not predicted to be associated with a genomic region that is free of Pol II-transcribed genes. This observation indicates that Sir2 is involved in repressing Set1-mediated methylation of histone H3 on K4 at the rDNA, and perhaps the localization of Pol II to the rDNA. These are two issues that will be addressed in this dissertation.

1.3.2.3. Chromatin Remodeling Complex

Chromatin remodelers are complexes that can change the chromatin structure through their enzymatic activity that alters histone-DNA interactions. It is not surprising that chromatin remodelers are involved in regulating transcriptional silencing at the rDNA. In fact, two chromatin remodelers, Swi/Snf complex and Isw1 complex, were found to be required for rDNA silencing (Dror and Winston, 2004; Mueller and Bryk, 2007). Swi/Snf is an ATP-dependent multi-subunit complex that can activate as well as repress transcription (Sudarsanam and Winston, 2000). Snf2 is the catalytic member of this complex. Deletion of the *SNF2* gene results in loss of silencing at the rDNA and

telomeres. The repressive activity of Swi/Snf at the rDNA has been shown to be independent of Sir2 and Set1. It is proposed that Swi/Snf may regulate the rDNA chromatin structure at a higher-order level, but this has not been investigated and it is not known if the effect of Swi/Snf is direct or indirect (Dror and Winston, 2004). The Imitation Switch (ISWI) class of chromatin remodelers is another type of ATP-dependent chromatin remodeling complex known to be involved in the ordering and spacing of nucleosomes after DNA replication as well as transcriptional activation and repression (for review see Tsukiyama, 2002; Saha *et al.*, 2006). In *S. cerevisiae*, the ISWI class of chromatin remodeler has two members, Isw1 and Isw2. The Isw1 complex is required for gene silencing at the *HMR* locus, but not at the telomeres (Cuperus and Shore, 2002). A recent study from our lab showed that Isw1is also required for transcriptional silencing at the rDNA locus (Mueller and Bryk, 2007). Deletion of *ISW1* results in loss of Pol II gene silencing and changes in the chromatin structure in the NTS1 region of the rDNA. Since Isw1 physically associates with the rDNA, it is likely that Isw1 functions directly on rDNA silencing (Mueller and Bryk, 2007). In another study from our lab, we also showed that, Isw2 is required for silencing at the rDNA but not at the telomeres (Mueller *et al.*, 2007). Unlike Isw1, deletion of *ISW2* does not affect chromatin structure at the rDNA (Mueller *et al.*, 2007). This is likely to reflect different mechanisms between Isw1 and Isw2 in regulating silent chromatin.

1.3.2.4. Histones and Histone Modifying Enzymes

Histones, the basic components of the chromatin structure, play direct roles in regulating gene silencing. The link between histones and their modifications with

silencing at the telomeres and the *HM* loci has been studied extensively. For instance, it has been shown that the N-terminal tails of H3 and H4 are required for silencing at telomeres and the *HM* loci (Johnson *et al.*, 1990; Megee *et al.*, 1990; Park and Szostak, 1990; Thompson *et al.*, 1994; Hecht *et al.*, 1995; Martin *et al.*, 2004). All of the tested lysine residues on the tails of histone H3 (K9, 14, 23, 27) and H4 (K5, 8, 12, 16) are hypoacetylated within the telomeres and *HM* loci (Braunstein *et al.*, 1993; Braunstein *et al.*, 1996; Suka *et al.*, 2001; Martin *et al.*, 2004). These findings favor a model that the Sir proteins preferentially bind the hypoacetylated histone tails, which promotes the spreading of the silent chromatin. Modifications of histone proteins at sites other than the tails also regulate the silent chromatin. Several other studies have identified residues located at a specific nucleosome surface of histone H3 that are required for all three forms of silencing in budding yeast (Park *et al.*, 2002; van Leeuwen *et al.*, 2002; Ng *et al.*, 2003c). This region is involved in interaction between nucleosomes and the silencing proteins (Park *et al.*, 2002). Hyland et al. (2005) and Xu et al. (2005) demonstrated that amino acid substitutions within the nucleosome core domain of histone H3 or H4 can influence transcriptional silencing as well.

Studies looking for factors required for rDNA silencing also identified histones and histone-modifying enzymes as key players at this heterochromatic domain. Global analysis indicates that the levels of acetylated histone H3 (K9, K14), acetylated H4 (K5, K8, K12, K16) and the methylated histone H3 (K4) are significantly lower at all the three silent loci, including the rDNA locus, than the global average level (Bernstein *et al.*, 2002). Deletion of *SET1*, the histone methyltransferase of H3K4, and replacement of K4

of histone H3 with arginine cause loss of silencing at the rDNA (Briggs *et al.*, 2001; Bryk *et al.*, 2002). It is not clear whether the effect of H3K4 methylation on rDNA silencing is direct or indirect. One possibility is that deletion of *SET1* leads to genome wide loss of methylation on K4 of histone H3, which may result in mislocalization of Sir2 proteins. This model is supported by the finding that Sir3 spreads away from telomeres in cells lacking Set1 (Santos-Rosa *et al.*, 2004). However, this does not hold true for the rDNA. First of all, Sir3 is not required for rDNA silencing. Secondly, deletion of *SET1* does not affect the association of Sir2 or Net1 with the rDNA (Bryk *et al.*, 2002). Perhaps a low level of K4-methylated H3 is directly required for forming a specialized chromatin structure, which is refractory for Pol II transcription at the rDNA. Interestingly, methylation of another lysine residue of histone H3 (K79) by Dot1 is also required for silencing at all three silent loci, although the effect is relatively modest at the rDNA locus (Singer *et al.*, 1998; van Leeuwen *et al.*, 2002). It was proposed that one of Dot1's roles in maintaining the silent loci is to prevent the Sir proteins from binding indiscriminately along the chromosomes via methylating most of the total histone H3 in the cells (van Leeuwen *et al.*, 2002). Both H3K4 methylation and H3K79 methylation indicate that although histone methylation is correlated to active Pol II transcription, it also participates in regulating silent chromatin directly or indirectly.

It is well known that highly acetylated histones are associated with active Pol II transcription, while deacetylated histones are found in silent chromatin. However, there are some exceptions, too. The MYST family HAT Esa1was found to play a key role in transcriptional silencing at telomeres and the rDNA (Clarke *et al.*, 2006). As a HAT,

Esa1 primarily acetylates histone H4, and to a less extent H3 and H2A (Smith *et al.*, 1998a; Clarke *et al.*, 1999). It is surprising that the catalytically inactive temperature-sensitive mutants of Esa1 cause telomere and rDNA silencing defects at the non-permissive temperature. Further analysis indicated that Esa1 physically binds to the rDNA and mutations of Esa1 result in hypoacetylation of histone H3 and H4 at this locus. More interestingly, overexpression of Esa1 can bypass the requirement of Sir2 in rDNA silencing (Clarke *et al.*, 2006). These data suggest that the opposing chromatin modifying activities of Sir2 and Esa1 contribute to repressing Pol II transcription at the rDNA locus. In addition, another MYST family HAT, Sas10, was shown to be involved in transcriptional silencing. Overexpression of Sas10 disrupted silencing at all the three silent loci (Kamakaka and Rine, 1998), although the mechanism behind this phenotype remains unknown.

In addition to histones H3 and H4, the histones H2A and H2B are also important in regulating rDNA silencing. Reducing the copy number of genes encoding histone H2A and H2B can cause silencing defects at the rDNA locus (Bryk *et al.*, 1997), while mutations in Hir3, a subunit of the HIR complex that controls the level of histone expression, cause an increase in rDNA silencing (Smith *et al.*, 1999). This may be due to the higher levels of H2A and H2B when Hir3 is mutated (Spector *et al.*, 1997). These findings imply that histone stoichiometry is critical for gene silencing. In addition, it is known that histone H2B can be ubiquitylated at the K123 residue. This modification is a prerequisite for methylation of histone H3 on K4 and K79 (Briggs *et al.*, 2002; Dover *et al.*, 2002; Ng *et al.*, 2002b; Sun and Allis, 2002; Zhang, 2003; Shahbazian *et al.*, 2005).

Deletion of *UBC2*/*RAD6*, the histone H2B ubiquitin-conjugating enzyme, results in loss of gene silencing at the rDNA in *S. cerevisiae* (Bryk *et al.*, 1997). On the other hand, *UBP10*, an ubiquitin protease whose targets include histone H2B, was shown to associate with the rDNA locus (Emre *et al.*, 2005). In cells lacking Ubp10, the level of Sir2 at the NTS region was reduced, resulting in hyperacetylation of H4K16 and hypermethylation of H3K4 and H3K79 at this region (Emre *et al.*, 2005; Calzari *et al.*, 2006). These data together suggest that ubiquitylation of histone H2B on K123 may be required for rDNA silencing. However, whether H2B K123 ubiquitylation is involved in rDNA silencing is not shown yet.

1.3.2.5. Other Factors

Most of the factors mentioned above are either components of chromatin or enzymes that modify chromatin. There are many other factors that are required for rDNA silencing. In a screen to identify factors other than Sir2 that affect rDNA silencing, Smith and colleagues identified several genes that are usually involved in DNA replication and modulation of chromatin structure, such as DNA polymerase α and ε, replication factor C (*CDC44*), Cac1 (subunit of nucleosome assembly complex during replication) (Smith *et al.*, 1999). Mutations in the genes encoding these proteins result in loss of rDNA silencing, indicating a possible interaction between DNA replication and rDNA silencing. Alternatively, because some DNA replication factors also function in DNA repair, the finding suggests a link between rDNA silencing and DNA repair.

DNA topoisomerase I (Top1) is an enzyme that modifies the superhelicity of DNA that has been found to be involved in several cellular processes, such as DNA

replication, transcription, recombination and chromatin remodeling. Studies have demonstrated that Top1 is essential for rDNA silencing (Bryk *et al.*, 1997; Smith *et al.*, 1999). In *top1Δ* cells, the level of acetylated histones at the rDNA locus is increased, and the rDNA chromatin becomes more accessible to MNase (Cioci *et al.*, 2002), consistent with previous finding that Top1 is required to maintain the normal structure of the rDNA locus (Christman *et al.*, 1993). Thus, the rDNA-silencing defect in cells lacking Top1 may be due to the altered chromatin structure that favors the binding of Pol II transcription machinery.

1.3.3. Repression of Replication at the Ribosomal DNA Locus

As shown in Figure 1-5, each rDNA repeat contains two *cis*-acting elements that regulate rDNA replication, the autonomously replicating sequence (ARS) in NTS2 (Skryabin *et al.*, 1984) and the replication fork barrier (RFB) in NTS1 (Brewer and Fangman, 1988; Linskens and Huberman, 1988). In S phase of the cell cycle, replication in the rDNA initiates bidirectionally from the ARS. Replication forks moving in the direction that is opposite of that of Pol I transcription are arrested at the RFB in NTS1. Forks moving in the same direction as Pol I transcription are not blocked and replicate several rDNA repeats. This unidirectional mode of replication is a unique feature of rDNA replication. The RFB is a polar block that blocks the replication forks approaching from its right side, without affecting the forks coming from its left side (Fangman and Brewer, 1991). The blocking activity of RFB requires a nucleolar protein called Fob1 (Kobayashi and Horiuchi, 1996); (Gadal *et al.*, 2002). Fob1 associates with the RFB sequence in an unusual "wrapping structure" that is dependent on its zinc finger

motif (Kobayashi, 2003). One of the roles of the RFB is thought to be in preventing the collision between replication and the Pol I transcription machineries (Brewer *et al.*, 1992; Kobayashi *et al.*, 1992). The RFB and Fob1 also play a role in rDNA recombination (See Chapter I, 4.4).

Although the sequence of each rDNA repeat is identical, not all ARSs in the rDNA locus are active. Actually, only twenty percent of the rDNA ARSs fire in any single S phase, while the rest of the rDNA ARSs remain inactive or silenced (Saffer and Miller, 1986; Brewer and Fangman, 1988). Just like the transcriptional silencing at the rDNA, the repression of rDNA ARS firing also requires the histone deacetylase Sir2. Using BrdU incorporation and DNA combing to visualize replication origins on single rDNA molecules, Pasero and colleagues showed that in cells lacking Sir2, the number of active ARSs increased about 2-fold compared to wild-type cells (Pasero *et al.*, 2002). This indicates that Sir2 represses the use of rDNA ARSs in wild-type cells. Interestingly, Sir2 also has a repressive role in regulating DNA replication at several ARSs outside of the rDNA locus (Palacios DeBeer and Fox, 1999; Pappas *et al.*, 2004; Crampton *et al.*, 2008). Deletion of *SIR2* rescues the growth of some pre-replication complex (pre-RC) mutants, presumably through rescuing loading of the MCM complex at these ARSs (Pappas *et al.*, 2004; Crampton *et al.*, 2008). Although Sir2 binding is not detectable at these ARSs, mutation of the H4K16 which is one of Sir2's targets to glutamine to mimic the acetylated state of histone H4 on K16 could also rescue the growth defect of the pre-RC mutants (Crampton *et al.*, 2008). This suggests that the inhibitory effect of Sir2 on these ARSs requires its histone deacetylase activity.

The mechanism of how Sir2 represses the rDNA replication is not clear. It is known that Sir2 physically associates with the rDNA (Gotta *et al.*, 1997), and deletion of Sir2 results in hyperacetylation of the rDNA chromatin as well as altered chromatin structure in the nontranscribed spacer region, making the region more accessible to MNase digestion (Fritze *et al.*, 1997; Cioci *et al.*, 2002; Li *et al.*, 2006a). Therefore it is possible that Sir2 acts as a structural component of the rDNA chromatin, which is repressive for ARS firing. Alternatively, since Sir2 is a histone deacetylase, it might exert its inhibitory effect via its deacetylation activity or via a crosstalk between other histone modifying enzymes such as Set1.

1.3.4. Repression of Mitotic Recombination at the Ribosomal DNA Locus

By definition, recombination is a process by which a strand of DNA is broken and then joined to a different DNA molecule. It plays important roles in many cellular processes, such as DNA repair, chromosome segregation, rescuing stalled replication forks and it is also one of the driving forces of evolution (Haber, 1999; McGlynn and Lloyd, 2002; San Filippo *et al.*, 2008). On the other hand, improper recombination can cause genome instability (Hartwell and Kastan, 1994). Thus, tight control of genetic recombination is a critical issue for all living organisms. There are four general types of recombination, including homologous recombination, site-specific recombination, DNA transposition and non-homologous recombination. In budding yeast, homologous recombination is very active (Christman *et al.*, 1988) and occurs preferentially within repetitive sequences. The rDNA is a highly repetitive locus and is considered to be one of the most fragile regions in the *S. cerevisiae* genome, because the density of repeated

sequences makes the rDNA a hot spot for homologous recombination. The consequence of this could be fatal, leading to deletion or expansion of the rDNA repeats, and resulting in unwanted genome instability and cell death. Fortunately, *S. cerevisiae* cells have developed mechanisms to protect the rDNA locus from excessive and inappropriate recombination events that help the cell maintain a stable number of the rDNA repeats.

1.3.4.1. Mitotic Recombination at the rDNA Locus

Mitotic recombination at the rDNA locus has been studied since the 1980s' (Szostak and Wu, 1980). The most commonly used method for studying mitotic recombination at the rDNA is achieved by introducing a marker gene into one of the rDNA repeats, and then assaying the rate of loss of the marker gene per generation. With this method, the rate of mitotic recombination at the rDNA was estimated to be $5x10^{-4}$ per cell division in wild type cells (Szostak and Wu, 1980). There are several *cis*-acting elements and *trans*-acting factors involved in stimulating rDNA recombination.

Recombination at the rDNA requires a sequence called *HOT1* (Keil and Roeder, 1984; Lin and Keil, 1991). It was originally identified as a region encompassing the entire NTS region. When placed elsewhere in the genome, the *HOT1* sequence can stimulate mitotic recombination both between and within chromosomes (Keil and Roeder, 1984). Subsequently, the *HOT1* sequence was narrowed down to two essential *cis*-acting elements, the E and I elements (Figure 1-5) (these two elements are different from the "*E*" and "*I*" elements in *HM* loci). The E element overlaps with the RFB and the Pol I transcription enhancer in NTS1 and the I element corresponds to the Pol I promoter region in NTS2 (Elion and Warner, 1984; Voelkel-Meiman *et al.*, 1987). It was

shown that Pol I transcription is required for *HOT1* mediated recombination activity (Voelkel-Meiman *et al.*, 1987; Huang and Keil, 1995). One possible cause of this may be the unwinding of the DNA duplex and changes in the DNA supercoiling or in the chromatin structure during transcription, exposing the DNA to the damaging agents which provide source of mitotic recombination (Aguilera *et al.*, 2000). Besides Pol I transcription, Fob1 is also critical for the rDNA recombination (Kobayashi and Horiuchi, 1996; Johzuka and Horiuchi, 2002). Since Fob1 was shown to be involved in blocking the replication fork through RFB, it was proposed that the replication forks arrested at the RFB were prone to double strand breaks (DSB) that initiate recombination (Kobayashi and Horiuchi, 1996; Kobayashi *et al.*, 1998; Defossez *et al.*, 1999; Rothstein and Gangloff, 1999; Weitao *et al.*, 2003b; Burkhalter and Sogo, 2004; Kobayashi *et al.*, 2004).

1.3.4.2. Factors Involved in Regulating rDNA Recombination

Many factors have been shown to repress rDNA recombination. The most well characterized protein is Sir2, which is also required for transcriptional silencing and repression of rDNA replication. It was shown that deletion of *SIR2* results in hyperrecombination at the rDNA locus, with a 10-fold increase in the rate of marker loss (Gottlieb and Esposito, 1989). Kobayashi *et al*. showed that deletion of *SIR2* reduces the association of the cohesin subunit Mcd1 with rDNA, which would allow unequal paring of sister chromatids. When sister chromatids are unequally aligned, recombination events between replicated chromatids causes contraction and/or expansion of the rDNA locus (Kobayashi *et al.*, 2004).

Other factors, including DNA topoisomerases Top1, Top2 and Top3 (Christman *et al.*, 1988; Gangloff *et al.*, 1994; Bryk *et al.*, 1997), Hpr1 (Aguilera and Klein, 1990; Merker and Klein, 2002) and Sgs1 (a RecQ helicase required for genome stability) (Bryk *et al.*, 2001; Weitao *et al.*, 2003a) have been implicated in repressing recombination at the rDNA. Since these factors are not the focus of this dissertation and I will not discuss them in detail here.

1.3.4.3. Maintenance of the rDNA Copy Number and Genome Stability

As a result of recombination, the copy number of the rDNA repeats can change, leading to expansion or contraction of the rDNA array. Expansion of the rDNA array is an outcome of repairing the DSB generated at the RFB via gene conversion, which introduces additional repeats into the rDNA array (Gangloff *et al.*, 1996). While unequal recombination events could cause deletion of rDNA repeats, resulting in a shortened rDNA array or expansion of rDNA repeat number and leading to a lengthened array. However, each organism is known to maintain a specific copy number of the rDNA repeats (Long and Dawid, 1980), indicating that the copy number of the rDNA repeats is tightly regulated.

Several lines of evidence suggest the factors involved in chromatin architecture play important roles in maintaining the rDNA copy number (Kobayashi *et al.*, 2004; Kobayashi and Ganley, 2005; Huang *et al.*, 2006; Johzuka *et al.*, 2006; Johzuka and Horiuchi, 2007). One of these factors is cohesin, the complex that holds sister chromatids together during S and G2 phase in the cell cycle. Cohesin associates with the

rDNA in the NTS in a Sir2-dependent manner (Laloraya *et al.*, 2000; Kobayashi *et al.*, 2004). Huang and colleagues have demonstrated that cohesin complex is clamped to the rDNA by Fob1, the RENT complex (including Sir2, Net1 and Cdc14), and the Lrs4/Csm1 complex. These proteins act together to repress the unequal sister-chromatid recombination (Huang *et al.*, 2006). Kobayashi and Ganley (2005) showed that in cells lacking Sir2, a region in NTS1 became transcriptionally active. Interestingly, they also found that the level of cohesin in NTS1 was reduced compared to wild-type cells. By integrating the bidirectional *GAL1/10* promoter in NTS1 and growing the cells in galactose-containing media, they showed that cohesin levels were reduced as well, just like in *sir2Δ* cells. These data suggest that Pol II transcription could induce dissociation of cohesin, increasing the chances for improper alignment of the two sister chromatids and unequal sister-chromatid recombination, which causes changes in the rDNA copy number (Kobayashi and Ganley, 2005). Hence, Sir2 may contribute to maintain proper alignment of the two sister chromatids and repress unequal sister-chromatid exchange at the rDNA locus through repressing Pol II transcription at the NTS1 region.

It is worth mentioning that another outcome of the unequal sister-chromatid recombination at the rDNA is the generation of extrachromosomal rDNA circles (ERCs). Accumulation of ERCs has been related to genome instability and premature aging of yeast cells (Sinclair and Guarente, 1997). Because Sir2 has a role in repressing rDNA recombination, it is not surprising that deletion of Sir2 causes accumulation of ERCs and aging phenotypes (Kaeberlein *et al.*, 1999).

1.4. DISSERTATION OVERVIEW

Chapter II of this dissertation describes my investigation of the role of the NAD^+-dependent histone deacetylase Sir2 in regulating the level of K4-methylated histone H3 and the function of Pol II at the ribosomal DNA locus in *S. cerevisiae*. My work showed that in cells lacking Sir2, a high level of K4-methylated histone H3 is associated with the rDNA, primarily at the NTS2 region. Consistent with the notion that high levels of K4 methylated histone H3 correlates with active Pol II transcription, I discovered novel endogenous Pol II transcription units in NTS2 whose transcription is repressed in a Sir2-dependent manner.

Chapter III focuses on my work to understand the function of transcription by Pol II in NTS2. I found that overexpression of the NTS2 transcription units from a plasmid does not disrupt rDNA silencing. In contrast, overexpression of the NTS2 transcripts at their endogenous location by introducing a *GAL7* promoter into one of the rDNA repeats causes loss of transcriptional silencing as well as an increase in mitotic recombination leading to loss of a marker gene located at the same repeat. The result suggests that repressing Pol II transcription in the NTS2 region supports transcriptional silencing and repression of mitotic recombination at the rDNA. It is also consistent with the idea proposed by Ganley and Kobayashi (2005) that Sir2 represses mitotic recombination at the rDNA by inhibiting an endogenous transcription unit at NTS1.

Chapter IV describes a study that I did in collaboration with other scientists in our lab to examine the role of histone H1 in regulating silent chromatin at the rDNA. Our results indicate that histone H1 does not regulate Pol II gene silencing at the rDNA.

However, it is required to repress mitotic recombination at the rDNA. Our recombination assays showed that histone H1 and Sir2 repress different pathways of recombination at the rDNA. We followed up our recombination experiments with physical and aging studies and found that, unlike cells lacking Sir2, cells lacking histone H1 do not accumulate ERCs and they do not exhibit a premature aging phenotype. These results indicate that cells lacking histone H1 form different recombination intermediates than those formed in cells lacking Sir2. These studies are now being pursued by others in the lab to identify the specific recombination pathways that histone H1 represses at the rDNA.

Finally, the Appendix includes three parts. The first part presents the work showing that Isw2, a chromatin remodeling protein, is required for rDNA silencing. The second part shows some of the work I performed to investigate the relationship between mitochondria DNA (mtDNA) and cell division. In this part, I showed that increasing the amount mtDNA in cells by overexpressing *ABF2* promotes dissociation of Sir2 from the rDNA locus. The last part shows that the histone variant H2A.Z does not regulate rDNA silencing.

1.5. SIGNIFICANCE

Studying how modified histones and chromatin structure regulate gene silencing will contribute to the general understanding of gene regulation in eukaryotes. The mechanisms that regulate gene expression and gene silencing in *S. cerevisiae* are conserved in higher eukaryotes. As our understanding of higher eukaryotes, e.g. human,

has benefited by the study of simple organisms, my findings may provide valuable insight into the human situation as well.

CHAPTER II

SIR2 REPRESSES ENDOGENOUS POLYMERASE II TRANSCRIPTION UNITS IN THE RIBOSOMAL DNA NONTRANSCRIBED SPACER*

Silencing at the rDNA, *HM* loci, and telomeres in *Saccharomyces cerevisiae* requires histone-modifying enzymes to create chromatin domains that are refractory to recombination and RNA polymerase II transcription machineries. To explore how the silencing factor Sir2 regulates the composition and function of chromatin at the rDNA, the association of histones and RNA polymerase II with the rDNA was measured by chromatin immunoprecipitation. We found that Sir2 regulates not only the levels of K4-methylated histone H3 at the rDNA but also the levels of total histone H3 and RNA polymerase II. Furthermore, our results demonstrate that the ability of Sir2 to limit methylated histones at the rDNA requires its deacetylase activity. In *sir2Δ* cells, high levels of K4-trimethylated H3 at the rDNA nontranscribed spacer are associated with the expression of transcription units in the nontranscribed spacer by RNA polymerase II and with previously undetected alterations in chromatin structure. Together, these data suggest a model where the deacetylase activity of Sir2 prevents euchromatinization of the rDNA and silences naturally occurring intergenic transcription units whose expression has been associated with disruption of cohesion complexes and repeat amplification at the rDNA.

2.1. INTRODUCTION

Modified histones at silent genomic domains contribute to a chromatin environment that is refractory to gene expression and genetic recombination (reviewed in Strahl and Allis, 2000; Turner, 2000; Jenuwein and Allis, 2001). In *Saccharomyces cerevisiae*, chromatin at the homothallic mating-type loci *HML* and *HMR*, telomeres, and the ribosomal DNA locus (rDNA) silences genetic recombination and expression of native and ectopic genes transcribed by RNA polymerase (Pol) II. Silencing at the *HM* loci and telomeres has been studied extensively, whereas the mechanisms of Pol II silencing at the rDNA are not well characterized (reviewed in Moazed, 2001; Rusche *et al.*, 2003). Increasing our understanding of the factors and mechanisms that regulate silencing at the rDNA will provide insight into the pathways that regulate gene expression and genome stability.

In *S. cerevisiae*, the rDNA contains ~150-200 tandem copies of a 9.1-kilobase (kb) repeat, with each repeat containing a Pol I-transcribed 35S rRNA gene and a nontranscribed spacer (NTS) that is subdivided into NTS1 and NTS2 by the Pol III-transcribed 5S rRNA gene (reviewed in Warner, 1999; Figure 2-1). Despite high levels of transcription by Pol I and Pol III in the rDNA locus, Pol II-transcribed genes integrated into the rDNA are silenced (referred to as rDNA silencing) (Bryk *et al.*, 1997; Fritze *et al.*, 1997; Smith and Boeke, 1997). Additionally, silent chromatin at the rDNA is essential for repression of genetic recombination (Gottlieb and Esposito, 1989; Davis *et al.*, 2000; Kobayashi *et al.*, 2004) and extension of replicative life span (reviewed in Guarente, 2000).

Chromatin-associated proteins and modified histones regulate silencing of Pol II transcription at the rDNA (Bryk *et al.*, 1997; Fritze *et al.*, 1997; Smith and Boeke, 1997; Smith *et al.*, 1999; Straight *et al.*, 1999; Hoppe *et al.*, 2002; Park *et al.*, 2002; Dror and Winston, 2004; Kobayashi *et al.*, 2004; Kuzuhara and Horikoshi, 2004; Machin *et al.*, 2004; Ye *et al.*, 2005). Net1 and the NAD-dependent histone deacetylase Sir2 are members of the RENT complex, which functions in silencing at the rDNA (Shou *et al.*, 1999; Straight *et al.*, 1999). The RENT complex is the functional equivalent of the Sir2-3-4 complex necessary for silencing at the *HM* loci and telomeres. In addition to being required for the association of Sir2 with the rDNA, Net1 interacts with Pol I and regulates the structure of the nucleolus (Straight *et al.*, 1999; Shou *et al.*, 2001). The histones H3 and H4 are substrates for the deacetylase activity of Sir2. At the *HM* loci and telomeres, hypoacetylated H3 and H4 promote the interaction of Sir3 and Sir4 with nucleosomes, thereby facilitating the formation and spread of silent chromatin (Braunstein *et al.*, 1993; Hecht *et al.*, 1995; Braunstein *et al.*, 1996; Wu and Grunstein, 2000; Carmen *et al.*, 2002; Liou *et al.*, 2005). Hypoacetylated histones are present at the rDNA (Bryk *et al.*, 2002; Buck *et al.*, 2002; Sandmeier *et al.*, 2002; Huang and Moazed, 2003), although how they contribute to rDNA silencing remains unclear.

Cells lacking K4-methylated histone H3 exhibit defects in transcriptional silencing at the rDNA and telomeres (Nislow *et al.*, 1997; Briggs *et al.*, 2001; Bryk *et al.*, 2002; Nagy *et al.*, 2002; Mueller *et al.*, 2006). Set1 is the catalytic subunit of the COMPASS complex that is required for mono-, di-, and trimethylation of histone H3 on K4. In *set1Δ* cells, the lack of K4-methylated histone H3 is associated with the aberrant

spreading of Sir proteins from telomeres into adjacent sequences. Reduced concentration of Sir complexes at telomeres in cells lacking Set1 is hypothesized to cause the loss-of-telomeric-silencing defect in *set1Δ* cells (Ng *et al.*, 2002a; Meneghini *et al.*, 2003; Ng *et al.*, 2003a; Martin *et al.*, 2004; Katan-Khaykovich and Struhl, 2005). In contrast, at the rDNA, the levels of the silencing factors Sir2 and Net1 are equivalent in *set1Δ* and wild-type cells (Bryk *et al.*, 2002). Moreover, in *set1Δ* cells, the levels of acetylated histone H3 and H4 remain low, indicating that Sir2 maintains its deacetylase function at the rDNA in the absence of K4-methylated H3. Thus, the mechanism by which silencing is lost at the rDNA in *set1Δ* cells is not equivalent to that at telomeres and has yet to be discovered.

In wild-type cells, silent chromatin at the rDNA contains low levels of acetylated H3 and H4 and low levels of K4-methylated histone H3. In cells lacking Set1 where rDNA silencing is impaired, histones at the rDNA remain hypoacetylated but are unmethylated. In contrast, in cells lacking Sir2, the levels of acetylated histone H3 and H4 are increased at several positions in the rDNA repeat and the level of K4-dimethylated H3 is increased at the NTS (Bryk *et al.*, 2002). Together, these observations indicate that rDNA silencing is controlled by a specific combination of hypoacetylated and hypomethylated histones and that perturbations that alter the profile of modified histones disrupt rDNA silencing. To gain insight into the relationship between Sir2 and K4-methylated histone H3, we used chromatin immunoprecipitation (ChIP) and real-time PCR analysis to generate high-density profiles of K4-methylated histone H3 and Pol II across the rDNA repeat in wild-type and *sir2Δ* cells. Here, we

show that novel changes occur at the rDNA in *sir2Δ* cells that reveal the central role of deacetylase activity of Sir2 in preventing aberrant Pol II transcription and protecting the structure and function of the unique chromatin domain present at the rDNA in *S. cerevisiae*. Furthermore, our findings provide mechanistic insight into the recent observation that high levels of transcription in the rDNA NTS are associated with amplification of rDNA repeats (Kobayashi and Ganley, 2005).

2.2. MATERIALS AND METHODS

2.2.1. Yeast strains, plasmids, and media

Standard media recipes were used (Rose, 1990). YPADT is YPD medium supplemented with adenine sulfate (40 mg/l) and l-tryptophan (20 mg/l). Yeast strains MBY1198 (wild type), MBY1217 (*set1Δ*), and MBY1238 (*sir2Δ*) have been described previously (Briggs *et al.*, 2001; Bryk *et al.*, 2002). The XhoI-NotI fragment containing *SIR2* or *sir2H364Y* from plasmids pAR455 (*SIR2 HIS3* CEN) or pAR456 (*sir2H364Y HIS3* CEN) (Rudner *et al.*, 2005) were subcloned into pRS415 (*LEU2 CEN*; Stratagene) by using standard techniques. The resulting plasmids, MBB406 (p*SIR2 LEU2 CEN*) and MBB407 (p*sir2H364Y LEU2 CEN*), were verified by DNA sequencing. p*SIR2* and p*sir2H364Y* were transformed into MBY1238 (Bryk *et al.*, 2002). p*SIR2* was also transformed into MBY1249 (*MATα his3Δ200 ade2Δ::hisG leu2Δ0 trp1Δ63 ura3Δ0 met15Δ0* Ty1*his3AI*-236 Ty1*ade2AI*-515 *set1Δ1::TRP1 sir2Δ::KANMX4*). The integrity of the p*SIR2* and *psir2H364Y* plasmids was verified in silencing assays and by measuring the level of K9, K14-acetylated histone H3 at the rDNA by ChIP (our

unpublished data). ChIP experiments (Figure 2-6) using an anti-Sir2 antibody showed that the level of Sir2 protein at the rDNA in *sir2Δ* cells containing p*sir2H364Y* was 0.5- to 1.6-fold of wild type, consistent with previously published reports (Tanny *et al.*, 1999; Hoppe *et al.*, 2002). Constructs for NTS-specific RNA probes were made by cloning PCR-generated NTS1 or NTS2 fragments into pSP70 (Promega, Madison, WI) to make MBB419 (pSP70-NTS1) or MBB413 (pSP70-NTS2). In vitro transcription reactions with SP6 or T7 RNA polymerase and linearized plasmid in the presence of [γ-^{32}P]CTP were performed to generate strand-specific RNA probes. Yeast strains with wild-type (wt) or temperature-sensitive (ts) Pol II used in temperature-shift experiments were made by genetic crosses: MBY1987 [*MATα his3Δ200 leu2Δ0 ura3Δ0 lys2-128δ rpb1Δ187::HIS3* (pRP114 *RPB1 LEU2 CEN*)]; MBY1988 [*his3Δ200 leu2Δ0 ura3-52 lys2-128 δ rpb1Δ187::HIS3 sir2Δ::KANMX4*) (pRP114 *RPB1 LEU2 CEN*)]; MBY1989 [*MATα his3Δ200 leu2Δ0 ura3Δ0 rpb1Δ187::HIS3* (pRP1-1 *rpb1-1 URA3 CEN*)]; and MBY1992 [*his3Δ200 leu2Δ0 lys2-128δ met15Δ0 trp1Δ63 ura3-52 sir2Δ::KANMX4 rpb1Δ187::HIS3* (pRP1-1 *rpb1-1 URA3 CEN*)].

2.2.2. Oligonucleotides for ChIP

For analysis of histone H3 at *HMR* and *TEL-VIR* by real-time PCR, oligonucleotides were designed to amplify ~250- to 300-base pair products (sequences available upon request). The primers used to analyze the rDNA (Huang and Moazed, 2003), *RPS16A*, and the intergenic region on chr *VIII* (Ng *et al.*, 2003c) have been described previously. For analysis of the promoter and 5' end of the rDNA-Ty1 element

by quantitative radioactive PCR, the primers annealed to the Ty1A region and downstream of the 5S rRNA gene (Bryk *et al.*, 2002).

2.2.3. Chromatin immunoprecipitation

Cells were grown and lysates prepared as described previously (Strahl-Bolsinger *et al.*, 1997; Bryk *et al.*, 2002) with the following modifications. Strains containing p*SIR2* or p*sir2H364Y* were grown in synthetic complete media lacking leucine with 2% glucose. Chromatin solutions were sonicated 12 times for 20 s each at 4°C by using a Branson Sonifier 250 at power setting 1.5 and 100% duty cycle to shear the chromatin to an average length of <500 base pairs. For each ChIP, 200 μl of sheared chromatin was incubated in a volume of 500 μl with specific antibodies for ~12 h at 4°C. The antisera used were anti-histone H3, 5 μl (ab1791; Abcam, Cambridge, MA); anti-K4monoMeH3, 6 μl (ab8895; Abcam); anti-K4diMeH3, 15 μl (07-030; Upstate Biotechnology, Lake Placid, NY); anti-K4triMeH3, 5 μl (ab8580; Abcam); anti-Sir2, 1 μl (gift from Danesh Moazed, Department of Cell Biology, Harvard Medical School, Boston, MA); and anti-RNA Pol II carboxy-terminal domain (CTD) (4H8), 10 μl (ab5408; Abcam). The specificity of the antisera for K4-methylated H3 was verified by peptide blots by using unmodified and K4-methylated H3 peptides (our unpublished data).

2.2.4. Analysis of ChIPs

Quantitative radioactive PCRs were performed as described previously (Bryk *et al.*, 2002) to determine the level of K4-di- and -trimethylated H3 at the rDNA-Ty1 element in wild-type (MBY1198), *sir2Δ* (MBY1238), and *set1Δ* (MBY1217) cells.

ChIP experiments shown in Figures 2-1 to 2-4 and Figure 2-6 to 2-8 were analyzed by quantitative real-time PCR (qPCR). Duplicate reactions containing 1 μl of input DNA (1:100) or immunoprecipitated DNA (1:10) were amplified in 20 μl containing 1.25 μM of each oligonucleotide and 1× SYBR Green Dynamo Hot Start PCR mix (Finnzymes, Espoo, Finland) by using an iCycler Iq real-time PCR machine (Bio-Rad, Hercules, CA). The PCR parameters were 1 cycle of 95°C, 15 min; 40 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s; 1 cycle at 95°C, 1 min; 1 cycle at 55°C, 1 min; and 80 cycles starting from 55°C with an increasing gradient of 0.5°C for 10 s each cycle to acquire a melting curve for each primer pair. The threshold cycle value was detected at the 72°C elongation step. Percentage of immunoprecipitation (%IP) was calculated by dividing the signal from IP DNA by the signal from input DNA. The %IP values from wild-type and *sir2Δ* cells analyzed by ChIP for K4-monomethylated H3, K4-dimethylated H3, and K4-trimethylated H3 were corrected for background by subtracting the %IP value obtained for *set1Δ* cells. For the 33 pairs of rDNA oligonucleotides, the range of average %IP values obtained from *set1Δ* cells were 0.031-0.128 for K4-monomethylated H3, 0.086-0.384 for K4-dimethylated H3, and 0.012-0.207 for K4-trimethylated H3. Graphs showing %IP data obtained for K4-methylated forms of histone H3 at the rDNA and two control loci before normalization to total histone H3 levels are shown in Figure 2-3. The %IP values of K4-di- and -trimethylated H3 from cells containing p*SIR2* or p*sir2H364Y* were corrected for background by subtracting the %IP values obtained from *set1Δ sir2Δ* cells (MBY1249) containing p*SIR2*. The %IP values from wild-type and

sir2Δ cells analyzed by ChIP for RNA Pol II and total histone H3 were corrected for background by subtracting the %IP value from a no-antibody control.

2.2.5. Northern analysis

Total RNA was isolated from yeast cells as described previously (Bryk *et al.*, 1997). Northern analyses were performed as described previously (Swanson *et al.*, 1991). Strand-specific ^{32}P-labeled RNA probes were used to detect NTS transcripts and *PYK1* RNA. An *ACT1* (+564 to +1200) probe and 35S rRNA probe were made by PCR amplification of yeast genomic DNA and then purified from an agarose gel and labeled with [α-^{32}P]dATP by random priming (Ausubel and K., 1988). Growth conditions for the Pol II shut-off experiments (Figure 2-10) were as described previously (Herrick *et al.*, 1990). Briefly, for each strain (MBY1987, MBY1988, MBY1989, and MBY1992), a 50-ml culture in YPADT was grown at 24°C to 1-2$\times$10^7 cells/ml before being split into two 25-ml cultures. Twenty-five milliliters of fresh YPADT at 24°C was added to one culture, and growth was continued at 24°C for 30 min. To the other culture, 25 ml of YPADT at 48°C was added, and the culture was incubated at 36°C for 30 min. For time-course experiments, the volumes of the initial culture and fresh YPADT added were increased so that RNA was isolated from 50 ml of culture at each time point (0, 15, 30, and 60 min). Northern blots were quantified on a GE Healthcare (Little Chalfont, Buckinghamshire, United Kingdom) Storm 860 PhosphorImager by using ImageQuant software.

2.2.6. Primer extension

Total RNA (30-50 μg) from MBY1198, MBY1217, and MBY1238 was annealed to 1 pmol of ^{32}P-labeled oligonucleotide (OM454; 5'-GTTGGTTTTGGTTTCGGTTG-3') at 58°C for 20 min. Extension reactions were performed using the Primer Extension System-AMV Reverse Transcriptase kit (Promega) according to the manufacturer's protocol. Sequencing reactions with oligonucleotide OM454 and double-stranded DNA template were performed using Sequenase Version 2.0 (USB, Cleveland, OH). Primer extension products and sequencing ladders were separated on 8 M urea, 8% polyacrylamide gels in 1× Tris borate-EDTA. Dried gels were visualized using a GE Healthcare Storm 860 PhosphorImager with ImageQuant software and autoradiography.

2.2.7. Analysis of Chromatin Structure with Micrococcal Nuclease

Yeast cells MBY1198, MBY1217, and MBY1238 were grown in 100 ml of YPADT medium to ~1.2×10^7/ml and harvested by centrifugation. Preparation of spheroplasts and digestion with micrococcal nuclease (MNase; Worthington, Biochemicals, Freehold, NJ) was performed as described previously with slight modifications (Kent and Mellor, 1995). Spheroplasts (~1.7×10^8) were incubated with MNase (0, 2.15, 4.3, 6.45, or 8.6 U/ml) for 4 min at 37°C and then the DNA was purified. Purified DNA that had not been treated with MNase was digested with 0.86 U of MNase for 2 min at 37°C to control for MNase sequence preferences. All DNAs were digested to completion with EcoRI or PvuII, separated on 1.5% agarose gels, transferred to nylon membranes, and analyzed by indirect end labeling. Blots of DNA digested with EcoRI were hybridized with a ^{32}P-labeled probe that anneals to +2229 to +2496 of the

rDNA locus (beginning of NTS1 is +1), and blots with DNA digested with PvuII were hybridized with a ^{32}P-labeled probe that anneals to +1015 to +1268 of the rDNA. Positions of MNase cleavages and nucleosomes were calculated using restriction fragments as a reference.

2.3. RESULTS

2.3.1. Sir2 Excludes K4-Methylated Histone H3 from Silent Chromatin

Cells lacking Sir2 have high levels of acetylated histones at the *HM* loci and telomeres and the rDNA (Braunstein *et al.*, 1993; Braunstein *et al.*, 1996; Bryk *et al.*, 2002; Buck *et al.*, 2002; Sandmeier *et al.*, 2002; Huang and Moazed, 2003). We observed that K4-dimethylated histone H3 was also increased at the rDNA NTS in *sir2Δ* cells (Bryk *et al.*, 2002), suggesting that Sir2 regulates the levels of K4-methylated histone H3 at the rDNA. Specific antisera against three forms of K4-methylated H3, K4-mono-, di-, and -trimethylated histone H3 have been used in numerous studies investigating the association of K4-methylated H3 with genes that are actively transcribed by RNA Pol II. These studies, in *S. cerevisiae*, have shown that K4-dimethylated H3 is enriched at euchromatin and that K4-trimethylated H3 is present at high levels at genes transcribed by Pol II (Bernstein *et al.*, 2002; Santos-Rosa *et al.*, 2002; Krogan *et al.*, 2003a; Ng *et al.*, 2003b; Ng *et al.*, 2003c; Santos-Rosa *et al.*, 2004). Therefore, high levels of K4-methylated H3 at the rDNA NTS were unexpected due to the lack of Pol II-transcribed genes at the rDNA.

We examined the effect of Sir2 on the association of each of the three forms of K4-methylated H3 across the rDNA repeat. ChIPs using antisera against histone H3 as well as K4-mono-, di-, or -trimethylated histone H3 were analyzed by qPCR by using 33 primer pairs that amplify 260- to 280-base pair regions at intervals of ~300 base pairs across the 9.1-kb rDNA repeat and two sets of primers that amplify control loci. Samples from cells lacking Set1 that are devoid of K4-mono-, di-, and -trimethylated histone H3 were included to provide a measurement of background that was subtracted from the signal obtained from wild-type and *sir2Δ* cells (see *Materials and Methods*). To determine whether Sir2 affected the level of total histone H3 at the rDNA, ChIP experiments were performed with antisera that recognize the C-terminal tail of histone H3. The interaction of the anti-histone H3 antisera with histone H3 is independent of modifications present at the N terminus of H3. The results of these experiments revealed that the level of total histone H3 was significantly lower at several positions in the rDNA repeat in *sir2Δ* cells compared with wild-type cells (Figure 2-2). To control for differences in the amount of total histone H3 at the rDNA, in Figure 2-1, we have normalized data examining the levels of K4-methylated histones at the rDNA to the level of total histone H3 in wild-type and *sir2Δ* cells.

The results of ChIP experiments using antisera specific for K4-mono, di-, and -trimethylated H3 showed significant changes in the profile of K4-methylated histone H3 at the rDNA in *sir2Δ* cells compared with wild-type cells (Figure 2-1 and Figure 2-3). We observed that K4-methylated histone H3 was excluded from the rDNA repeat in a Sir2-dependent manner. The profile of each form of K4-methylated H3 across the rDNA

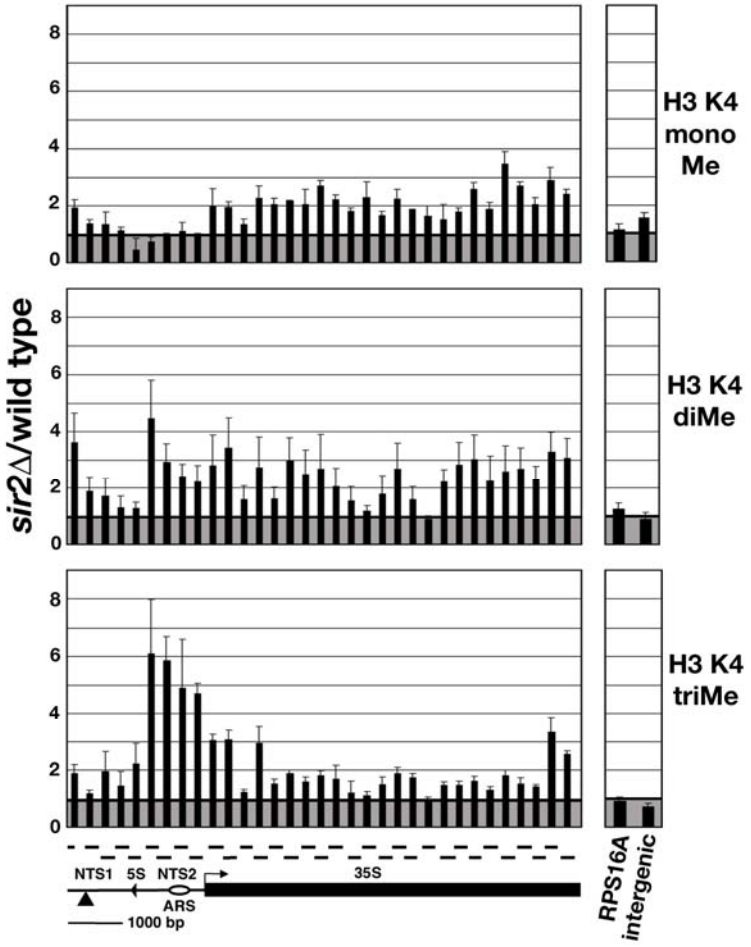

Figure 2-1. Altered distribution of K4-methylated histone H3 at the rDNA in *sir2Δ* cells relative to wild-type cells. Graphical representations of the average ratio of %IP of K4-monomethylated (top; ± SE; n = 2 or 3), K4-dimethylated (middle; ± SE; n = 3), and K4-trimethylated (bottom; ± SE; n = 4) histone H3 in *sir2Δ* cells (MBY1238) relative to wild-type cells (MBY1198) at the rDNA, the *RPS16A* gene, and an intergenic region on chr *VIII*. Data presented were normalized to total histone H3 to correct for reduced levels of H3 in *sir2Δ* cells at some regions of the rDNA repeat (see text and Figure 2-2). The structure of a 9.1-kb rDNA repeat unit is shown below the bottom panel, indicating the location of the Pol I-transcribed 35S rRNA (35S) gene and the NTS that is divided into NTS1 and NTS2 by the Pol III-transcribed 5S rRNA gene (5S). ARS, replication origin; bent line with arrow, transcription start site of the 35S rRNA gene; ▲, location of a silenced Ty1*his3AI* element present in one repeat, referred to as rDNA-Ty1 in text. Horizontal lines above the rDNA indicate PCR products generated during the analysis of ChIP experiments by qPCR.

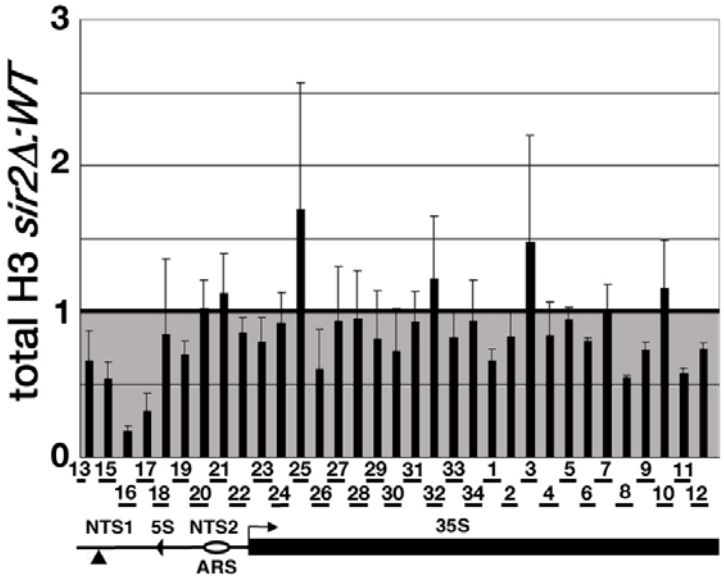

Figure 2-2. Cells lacking Sir2 have significantly lower levels of histone H3 at several positions in the rDNA repeat. Graphical representation of the average ratio of %IP of histone H3 in *sir2Δ* cells (MBY1238) relative to wild type cells (MBY1198) at the rDNA. The structure of an rDNA repeat unit is shown below the bottom panel with the Pol I transcribed 35S ribosomal RNA (35S) gene and a non-transcribed spacer divided into NTS1 and NTS2 by the Pol III-transcribed 5S rRNA gene (5S). ARS, replication origin; bent line with arrow, transcription start site of the 35S rRNA gene; ▲, location of a silenced Ty1*his3AI* element present in one repeat. Horizontal numbered lines above the rDNA indicate PCR products generated during the analysis of ChIP experiments by quantitative real-time PCR. The average ratio of % IP ± range from *sir2Δ* cells to wild-type cells from two independent experiments is plotted.

repeat in *sir2Δ* cells was distinct. In cells lacking Sir2, the level of K4-monomethylated histone H3 was increased over the 35S rRNA gene and the NTS1 portion of the NTS (Figure 2-1, top; and Figure 2-3, top). The level of K4-dimethylated H3 was higher across most of the rDNA repeat, including regions in NTS1, NTS2, and the 35S rRNA gene (Figure 2-1, middle; and Figure 2-3, middle). Strikingly, the level of K4-trimethylated histone H3 was increased primarily at the NTS2 region of the rDNA with levels that were approximately five- to six fold higher in *sir2Δ* cells than wild-type cells (Figure 2-1, bottom and Figure 2-3, bottom). Sir2 also affected the level of K4-trimethylated H3 at the beginning and end of the Pol I-transcribed 35S rRNA gene. The levels of K4-methylated H3 were similar in wild-type and *sir2Δ* cells at genomic loci that were analyzed as controls, *RPS16A*, a highly expressed Pol II-transcribed gene, and an intergenic region on chr *VIII* that is devoid of known Pol II-transcribed open reading frames (ORFs), indicating that Sir2 does not regulate K4-methylated H3 at these regions (Figure 2-1, right and Figure 2-3, right). In summary, our data indicate that in cells lacking Sir2, the rDNA is exposed to the Set1-containing complex COMPASS that is required for the methylation of histone H3 on K4. Moreover, because the K4-trimethylated form of histone H3 has been found exclusively at genes that are actively transcribed by Pol II, our results suggest that Pol II may also be present at the rDNA NTS.

To understand how Sir2 affected the distribution of K4-methylated histone H3 at other silent loci, we performed ChIP experiments using wild-type, *sir2Δ*, and *set1Δ* cells to obtain profiles of K4-methylated H3 at the *HMR* locus and near the telomere on the

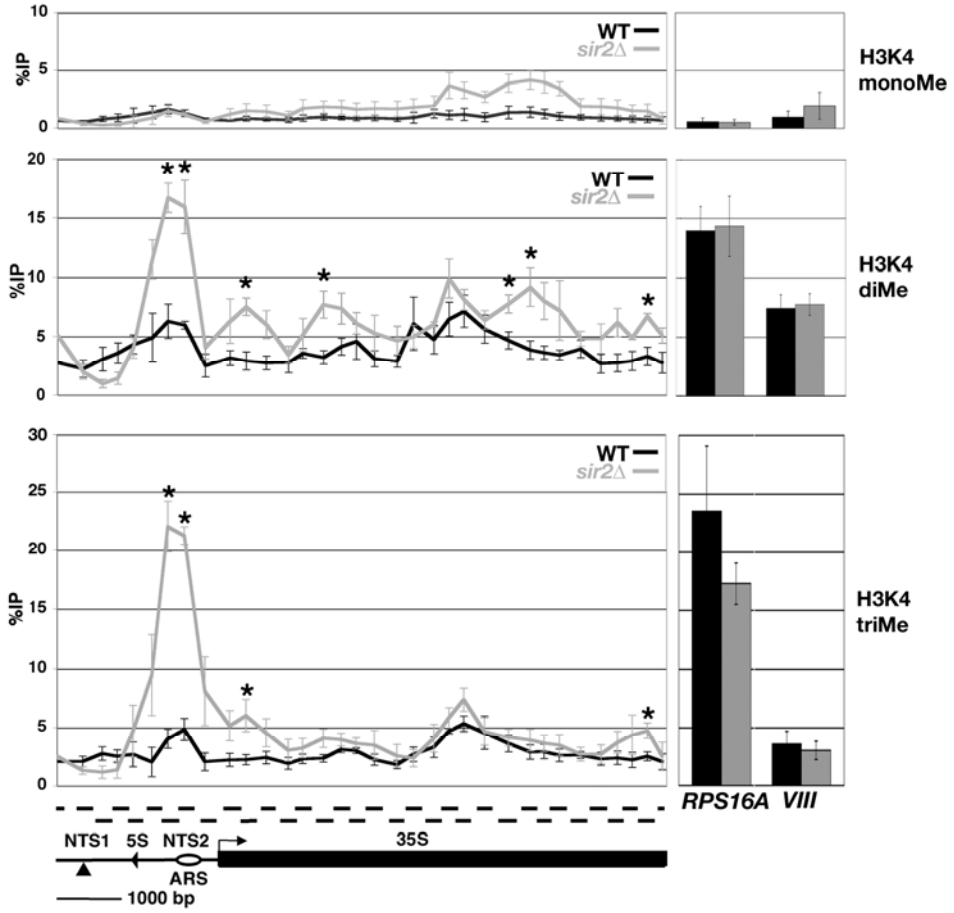

Figure 2-3. The levels of K4 di- and trimethylated histone H3 in the rDNA repeat are increased significantly in *sir2Δ* cells. Graphical representations of % IP of K4-monomethylated (top; ± range; n = 2 or 3), K4-dimethylated (middle; ± SE; n=3), and K4-trimethylated (bottom ; ± SE; n = 4) histone H3 with the rDNA, the *RPS16A* gene, and an intergenic region of chr *VIII* (*VIII*) in wild-type (black line and bars) and *sir2Δ* cells (gray lines and bars). The plotted values have not been normalized to total histone H3 levels, which are reduced at the rDNA repeat in cells lacking Sir2 (See Figure 2-2). Asterisks, regions of the rDNA with significantly higher levels of K4-di- (middle panel) or trimethylated (bottom panel) H3 in *sir2Δ* cells compared to wild-type cells, as determined by Student's t-test ($P < 0.05$). Levels of K4-monomethylated H3 in wild type and *sir2Δ* cells are not significantly different. The structure of a 9.1 kb rDNA unit is shown below the bottom panel (labels as in legend to Figure 2-1).

right arm of chr *VI* (*TEL-VIR*). Experiments measuring histone H3 at *HMR* and *TEL-VIR* revealed that the average ratio of histone H3 in *sir2Δ* to wild-type cells was between 0.9 and 1.2, indicating that the level of histone H3 was similar in wild-type and *sir2Δ* cells (our unpublished data). In contrast to the rDNA where we observed distinct profiles of K4-methylated H3, the levels of K4-di- and -trimethylated H3 were increased similarly across ~4 kb of the *HMR* locus and up to 2.8 kb from the right end of chr *VI* (Figure 2-4, A and B). At *HMR*, the highest levels of K4 di- and -trimethylated histone H3 were present at a region containing the Pol II-transcribed *a1* and *a2* genes (Figure 2-4A, primer pair X/Ya). The region of *HMR* where we detect changes in the association of K4-methylated histone H3 falls within the boundaries of the silent domain that were mapped in previous studies (Donze *et al.*, 1999; Donze and Kamakaka, 2001). At *TEL-VIR*, the highest levels of K4 di and-trimethylated histone H3 were present 1.0 -1.5 kb from the end of the chromosome. This region of chr *VI* contains the promoter and 5' end of the Pol II-transcribed gene *YFR057W*, whose expression has been shown to be increased in cells lacking Sir2 or Ubp10 (Wyrick *et al.*, 1999; Emre *et al.*, 2005). In contrast, we did not observe an effect of Sir2 on the levels of K4-methylated H3 at *YFR055W* a Pol II-transcribed gene located 4.7 kb from the end of chr *VI*. In summary, in cells lacking Sir2, the rDNA, *HMR*, and *TEL-VIR* are exposed to factors that are required for the methylation of histone H3 on K4.

2.3.2. K4-Methylated H3 at the Promoter of the rDNA-Ty1 Element Is Regulated by Sir2

Several of the strains used in this study contain a single copy of a genetically

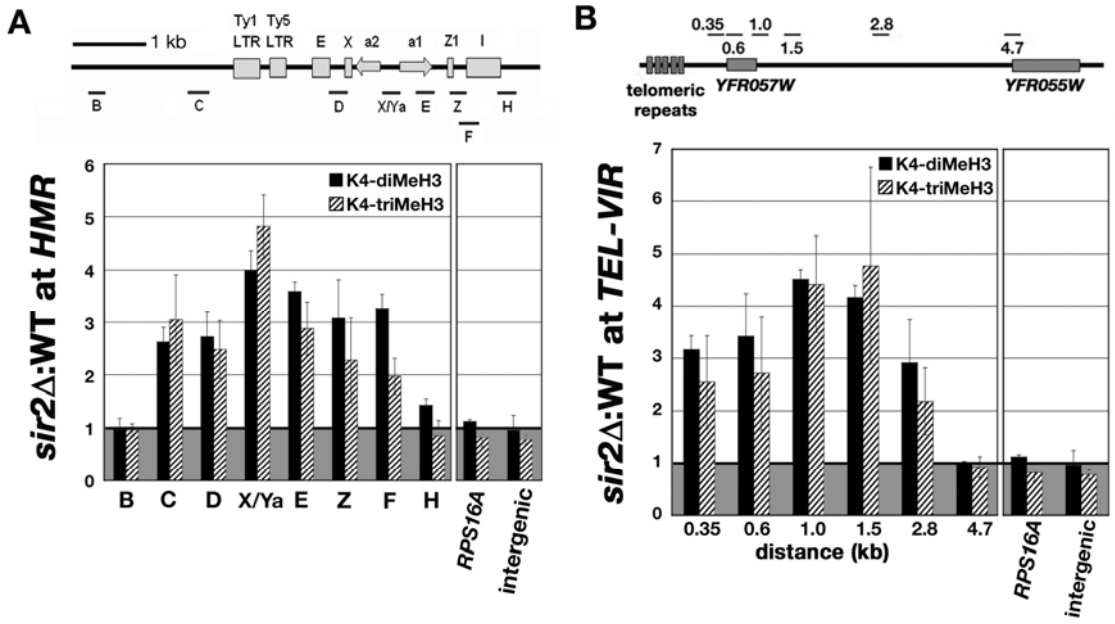

Figure 2-4. The levels of K4 di- and trimethylated histone H3 were increased at *HMR* and *TEL-VIR* in *sir2Δ* cells. (A) Diagram of *HMR* located on chr *III* with the positions of silencers (E and I), *cis*-sequences (X and Z1), Ty long terminal repeats (LTR), and the genes *HMRa1* and *HMRa2*. Short lines labeled B, C, D, X/Ya, E, Z, F, and H indicate the location of the PCR products used in ChIP analysis. The graph shows the average ratio of %IP of K4-dimethylated H3 (black bars) and K4-trimethylated H3 (hatched bars) at *HMR*, the *RPS16A* gene, and an intergenic region on chr *VIII* in *sir2Δ* cells relative to wild-type cells. (B) Diagram of *TEL-VIR* (right telomere on chr *VI*) with the positions of ORFs (*YFR057W* and *YFR055W*) and telomeric repeats indicated. Short lines labeled 0.35, 0.6, 1.0, 1.5, 2.8, and 4.7 indicate regions analyzed by qPCR. The numbers indicate the distance (in kb) from the right end of the PCR product to the right end of chr *VI*. Graph shows the average ratio of %IP of K4-di and -trimethylated H3 at *TEL-VIR* in *sir2Δ* cells relative to wild-type cells. For both panels, the average ratio of % IP ± SE from *sir2Δ* cells to wild-type cells from three independent experiments is plotted.

marked Ty1 element located in NTS1 in one of the repeats of the rDNA array (see Figure 2-1; Bryk *et al.*, 1997). In previous work, we showed that expression of the rDNA-Ty1 element is increased in *sir2Δ* cells and that the promoter of the rDNA-Ty1 element contains low levels of K4-dimethylated H3 in wild-type cells (Bryk *et al.*, 1997; Bryk *et al.*, 2002). It is unlikely that the rDNA-Ty1 element is responsible for the increased levels of K4-methylated H3 observed in *sir2Δ* cells because it is present in only one of the ~150-200 rDNA repeats. However, we wanted to determine whether Sir2 affected the levels of K4-methylated H3 at the promoter and 5' end of the Pol II-transcribed rDNA-Ty1 element, which would be consistent with its increased expression in *sir2Δ* cells. Using ChIP and radioactive PCR, we compared the levels of K4-di- and -trimethylated H3 at the rDNA-Ty1 element by using primers that amplify a 540-base pair fragment containing the promoter and 5' end of the rDNA-Ty1 element (Figure 2-5). We found that the levels of K4-dimethylated H3 and total histone H3 present at the rDNA-Ty1 element were not significantly different in *sir2Δ* and wild-type cells (Figure 2-5; our unpublished data). In contrast, the level of K4-trimethylated H3 at the rDNA-Ty1 element was 1.8-fold higher in *sir2Δ* cells than wild-type cells, consistent with increased expression of the rDNA-Ty1 element in *sir2Δ* cells (Bryk *et al.*, 1997).

2.3.3. Deacetylase Activity of Sir2 Excludes K4-Di and -Trimethylated H3 from the rDNA

One model to account for increased levels of K4 methylated histone H3 at the rDNA is that the physical presence of Sir2 prevents the association of factors required for the methylation of histone H3. In this steric model, in the absence of Sir2, rDNA

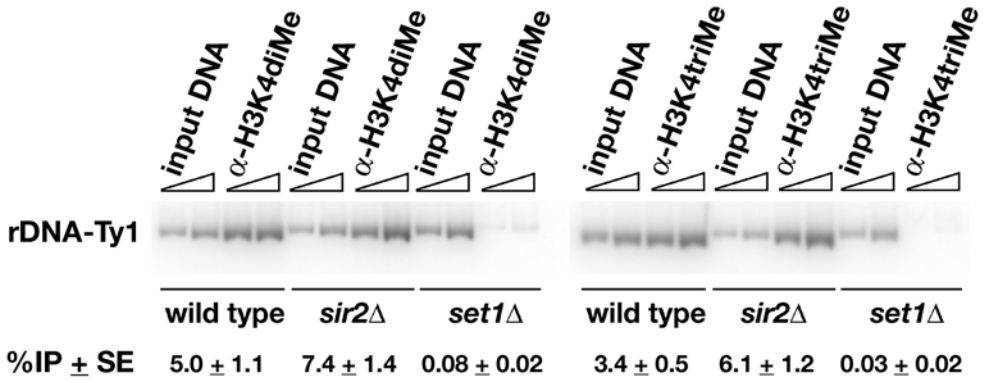

Figure 2-5. *sir2Δ* cells have higher levels of K4-trimethylated H3 at the rDNA-Ty1 element. ChIP experiments were analyzed by quantitative radioactive PCR to measure K4-di- and -trimethylated H3 at the promoter region of the silent Ty1*his3AI* element in the rDNA in wild-type and *sir2Δ* cells. Immunoprecipitations using *set1Δ* cells were performed to provide a measure of background for the ChIP experiments. The average %IP ± SE of K4-dimethylated H3 at the rDNA Ty1 promoter region from three independent experiments in wild-type cells was 5.0 ± 1.1, in *sir2Δ* cells is 7.4 ± 1.4, and in *set1Δ* cells is 0.08 ± 0.02. The average %IP ± SE of K4-trimethylated H3 at the rDNA-Ty1 promoter region from four independent experiments in wild-type cells was 3.4 ± 0.5, in *sir2Δ* cells is 6.1 ± 1.2, and in *set1Δ* cells is 0.03 ± 0.02. Slanted triangles indicate a twofold increase in the amount of template DNA used in the PCR reactions.

chromatin would be more accessible, allowing higher levels of K4-methylated histone H3. An alternative model posits that the deacetylase activity of Sir2 contributes to a hypoacetylated chromatin domain at the rDNA that hinders the methylation of histone H3.

To distinguish between these two modes of regulation by Sir2, we used a catalytically inactive allele of *SIR2*, *sir2H364Y*, which encodes a mutant Sir2 protein that retains the ability to bind to the rDNA but lacks histone deacetylase activity (Tanny *et al.*, 1999; Hoppe *et al.*, 2002; also see Figure 2-6). ChIP experiments were performed using antisera against K4-di- and -trimethylated H3 and cross-linked chromatin from *sir2Δ* cells containing a plasmid with either a wild-type copy of *SIR2* (p*SIR2*) or the mutant allele of *SIR2* (p*sir2H364Y*). Signal obtained from *set1Δ sir2Δ* double mutant cells containing the p*SIR2* plasmid was subtracted as background in the analysis of these ChIP experiments (see *Materials and Methods*). The data revealed that the levels of K4-di- and -trimethylated H3 were higher at the rDNA in cells expressing *sir2H364Y* compared with cells with p*SIR2* (Figure 2-7). Our results indicate that the deacetylase activity of Sir2 is required to maintain low levels of K4-methylated H3 at the rDNA. In addition, whereas the mutant Sir2 protein did not affect the levels of methylated H3 at *RPS16A* or the intergenic region, the levels of K4-di- and -trimethylated histone H3 were increased significantly at a region 0.6 kb from the right end of chr *VI* (Figure 2-7, right). Together, our results indicate that the deacetylase activity of Sir2 is required to maintain low levels of K4-di- and -trimethylated H3 at the rDNA and *TEL-VIR*.

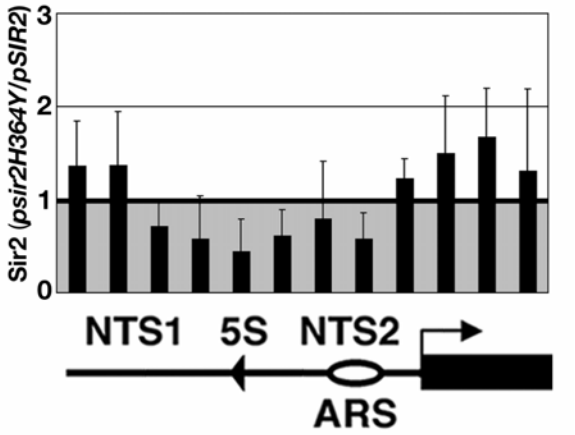

Figure 2-6. ChIP with anti-Sir2 antisera show that the levels of Sir2 protein are similar at the rDNA in cells containing p*SIR2* and p*sir2H364Y*. Graph showing the average ratio of %IP of Sir2 (± SE; n = 3) across a region of the rDNA repeat in p*sir2H364Y*/p*SIR2* cells. %IP values from *sir2Δ* cells containing an empty vector were subtracted from the %IP values to correct for background. The regions of the rDNA analyzed include the 2.5 kb NTS and 1.4 kb of the 35S rRNA gene. Labels for representation of rDNA repeat as in legend to Figure 2-1.

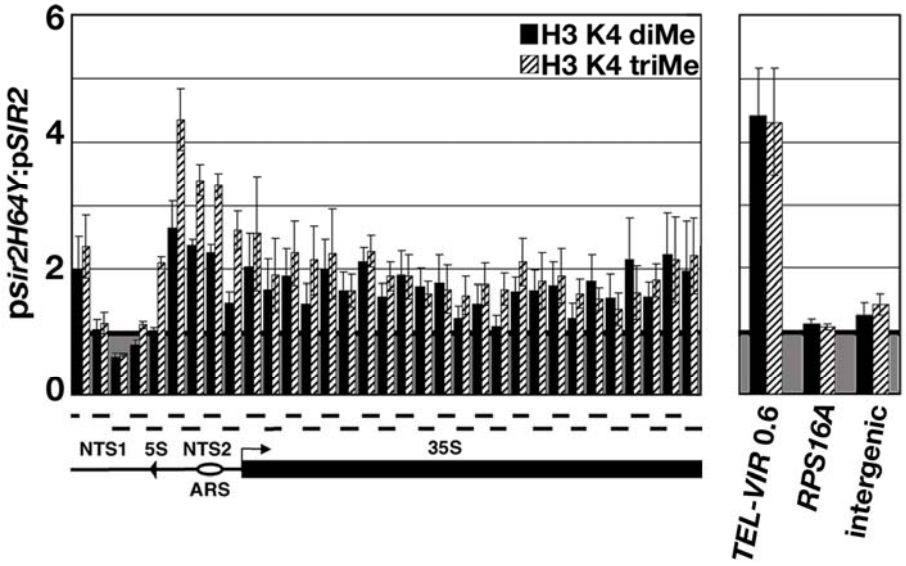

Figure 2-7. Deacetylase activity of Sir2 is required to exclude K4-di- and -trimethylated H3 from the rDNA. Graph showing the average ratio of %IP of K4-dimethylated H3 (H3 K4 diMe, black bars) and K4-trimethylated H3 (H3 K4 triMe, hatched bars) across rDNA repeat (left) and control loci (right) in p*sir2H364Y*/p*SIR2* cells. The labels for the representation of rDNA repeat are defined in legend to Figure 2-1. Average ratio of %IP ± SE for three independent experiments is shown.

2.3.4. Sir2 Excludes RNA Pol II from Silent Chromatin

In *sir2Δ* cells, the average %IP value of K4-trimethylated H3 of 21-22% measured at NTS2 was similar to the value of 23% measured at the Pol II-transcribed *RPS16A* gene (Figure 2-3), suggesting that Pol II might be present at higher levels at the rDNA in *sir2Δ* cells. This idea is supported by several lines of evidence. First, in *S. cerevisiae*, K4-trimethylated histone H3 has been shown to be associated with genes transcribed by RNA Pol II (Bernstein *et al.*, 2002; Santos-Rosa *et al.*, 2002; Santos-Rosa *et al.*, 2004). In addition, at *HMR* and *TEL-VIR* in *sir2Δ* cells, we observed the highest levels of K4-trimethylated H3 at regions that contain one or more Pol II-transcribed genes (Figure 2-4), whose expression is increased in *sir2Δ* cells (Wyrick *et al.*, 1999; Rusche *et al.*, 2003; Emre *et al.*, 2005). To determine whether the higher level of K4-trimethylated H3 observed over the rDNA NTS region in *sir2Δ* cells was associated with increased levels of Pol II, we performed ChIP experiments by using antisera that recognize the CTD of Pol II. We found that the association of Pol II with the NTS1 and NTS2 regions of the rDNA, the 5' end of the rDNA-Ty1 element *HMR*, and *TEL-VIR* was higher in *sir2Δ* cells than in wild-type cells (Figure 2-8). In contrast, Pol II levels at *RPS16A* and the intergenic region were equivalent in *sir2Δ* and wild-type cells (Figure 2-8A, right). Previous work has shown that Pol II was increased at *HMR* and *TEL-VIR* in silencing-defective cells (Chen and Widom, 2005). Our data reveal that Pol II was also increased at the rDNA in *sir2Δ* cells. Considering our observations of increased levels of K4-trimethylated H3 and Pol II at the rDNA, we wanted to determine whether Pol II transcription was occurring in the NTS in *sir2Δ* cells.

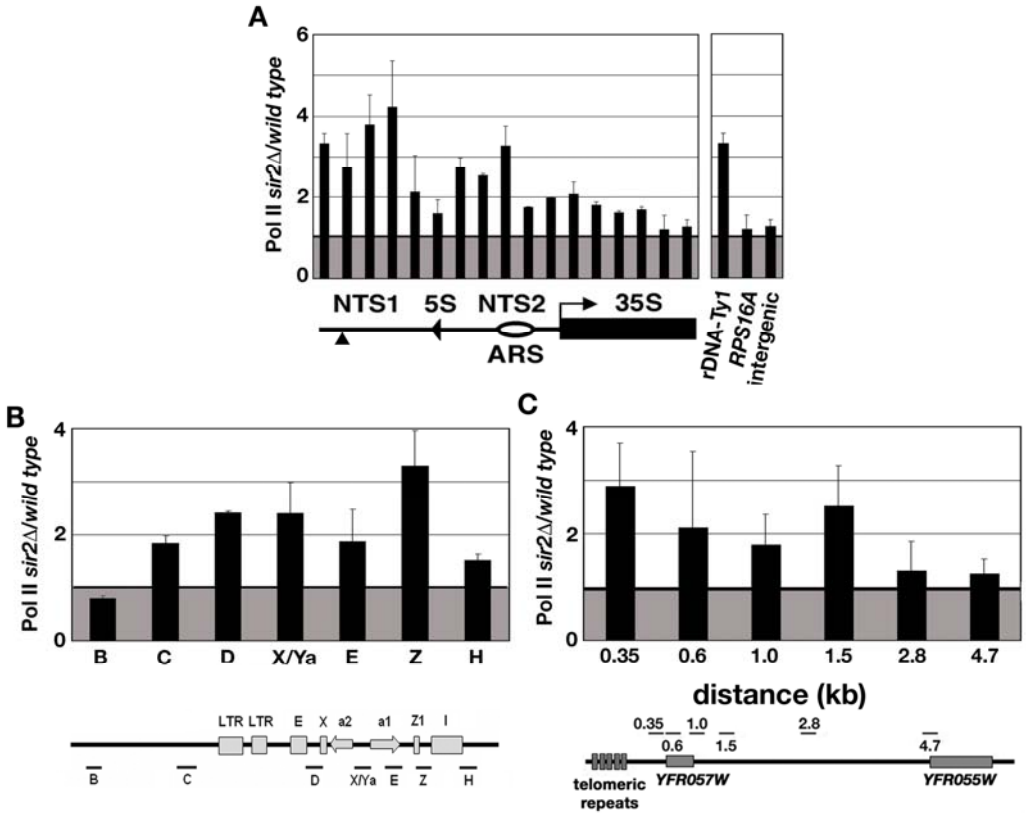

Figure 2-8. Association of Pol II with silent loci is increased in *sir2Δ* cells. Graphs show the average ratio of %IP of Pol II at the (A) rDNA (left), the promoter of the rDNA-Ty1 element and control loci (right); (B) *HMR*; and (C) *TEL-VIR* in *sir2Δ* cells relative to wild-type cells. The representation of the rDNA in A indicates the portion of the rDNA repeat analyzed. Labels for the representations of the rDNA, *HMR*, and *TEL-VIR* are in the legends to Figures 2-1 and 2-4. The average ratio (± range) of Pol II measured in *sir2Δ*:wild-type cells from two independent experiments is plotted.

2.3.5. Sir2 Regulates Expression of Endogenous Transcription Units in the rDNA NTS

We detected high levels of Pol II at NTS1 and NTS2 consistent with Pol II transcription in the NTS region. A recent report has shown that bidirectional transcripts can be detected from NTS1 in *sir2Δ* cells (Kobayashi and Ganley, 2005). The observation of high levels of K4-trimethylated H3 in NTS2 suggested to us that Pol II transcription might be occurring in NTS2 as well. Northern analyses were performed using strand-specific probes to detect transcripts with the same polarity as the NTS1 top, NTS1 bottom, NTS2 top, or NTS2 bottom strand to determine whether transcription was occurring in the rDNA NTS region in *sir2Δ* cells or *set1Δ* cells, which both exhibit defects in rDNA silencing. To control for equivalent loading of RNA, we used a *PYK1*-specific RNA probe. NTS-specific transcripts, whose levels were increased significantly in *sir2Δ* and *set1Δ* cells, were identified using Northern blot experiments (Figure 2-9). Hybridization with a probe that detects transcripts with the same polarity as the top strand of NTS1 revealed transcripts in RNA from *sir2Δ* and *set1Δ* cells, similar to those previously identified by (Ganley *et al.*, 2005) (Figure 2-9B, wild type, lane 1; *sir2Δ*, lane 2; and *set1Δ*, lane 3). Likewise, a transcript of ~1.7 kb with the same polarity as the bottom strand of NTS1 was detected in RNA from *sir2Δ* (lane 5) and *set1Δ* cells (lane 6). On longer exposure of this blot, a faint 1.5-kb transcript could also be detected in RNA from *sir2Δ* and *set1Δ* cells. In lanes 7-9, by using a probe with the same polarity as the top strand of NTS2, we detected a faint smear in RNA from *sir2Δ* and *set1Δ* cells that

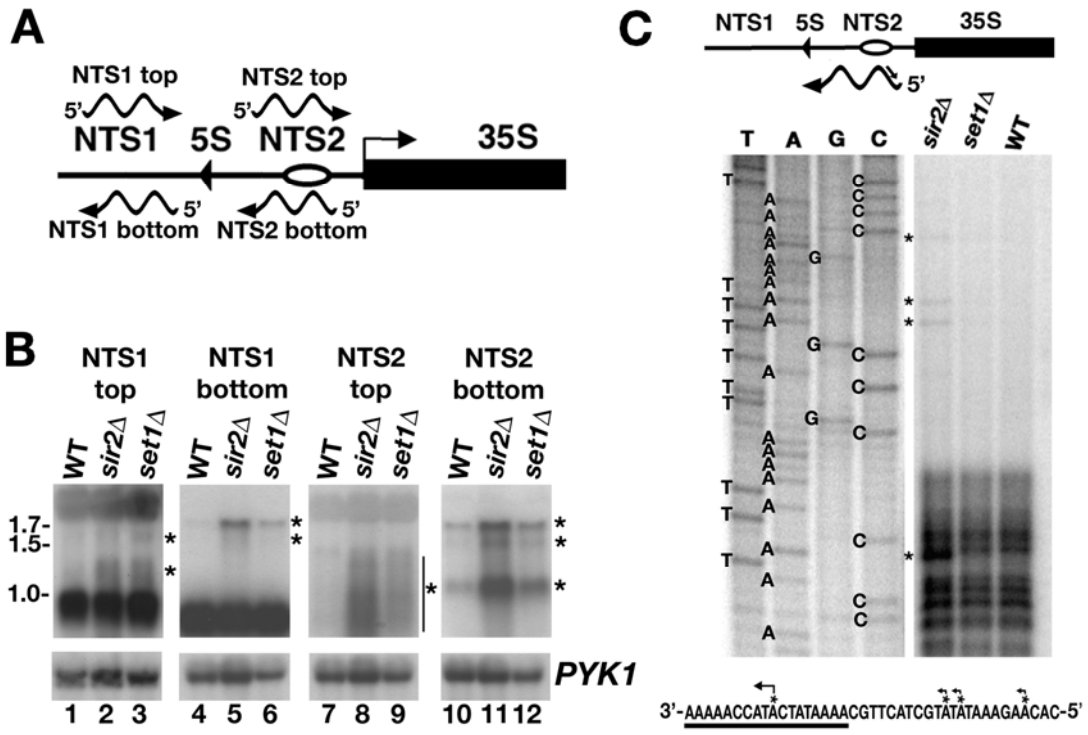

Figure 2-9. Endogenous transcription units in the rDNA NTS are expressed in rDNA silencing-defective mutants. (A) Schematic of rDNA NTS showing the polarity of transcripts detected by probes used in Figure 2-9B. Other labels, as in Figure 2-1. (B) Representative Northern blots of total RNA from wild-type, *sir2Δ*, and *set1Δ* cells hybridized to strandspecific probes that are complementary to transcripts with the same polarity as the NTS1 top (lanes 1-3), NTS1 bottom (lanes 4-6), NTS2 top (lanes 7-9), and NTS2 bottom (lanes 10-12) strands. For each blot, *PYK1* RNA levels were used to normalize for the amount of RNA analyzed. Numbers on left, transcript length in kb. Asterisks or line with asterisks, position of NTS-specific transcripts. (C) Primer extension analysis to map the 5' ends of the NTS2 bottom-strand transcripts. Schematic at top represents an NTS2 transcript and the oligonucleotide (OM454, line with arrowhead) that was used to map the 5' ends (not to scale). DNA sequence ladder and primer extension reactions revealed one major 5' end within the consensus TSS (underlined in DNA sequence representation of the bottom strand of NTS2 at base of C) and three minor start sites upstream of the TSS. Asterisks and/or bent arrows indicate 5' ends.

was likely to represent transcripts that ranged in length from ~0.8 to 1.5 kb. In lanes 10-12, by using a probe to detect RNA with the same polarity as the bottom strand of NTS2, we identified three transcripts of ~1.7, 1.5, and 1.0 kb in RNA from *sir2Δ* and *set1Δ* cells. Although these 1.7-, 1.5-, and 1.0-kb transcripts could be detected at low levels in RNA from wild-type cells (lane 10), they were enriched significantly in RNA from *sir2Δ* (lane 11) and *set1Δ* cells (lane 12). Thus, these data show that, in addition to transcripts from NTS1 that have been characterized previously, transcripts can be detected from NTS2.

The sequence of the rDNA NTS2 region was scanned by eye for the presence of Pol II-specific regulatory sequences. We identified a consensus Pol II transcription start site (TSS) sequence, 5'-A-$(A_{rich})_5$-N-Py-*A*-(A/T)-N-N-$(A_{rich})_6$-3', where *A* is the TSS (Zhang and Dietrich, 2005), between the rDNA autonomously replicating sequence (ARS) in NTS2 and beginning of the 35S rRNA gene (Figure 2-9C, bottom). This sequence was recognized previously as a conserved sequence element (rCNS6) in the rDNA NTS of several yeasts related to *S. cerevisiae* (Ganley *et al.*, 2005). No sequences with an exact match to a consensus TATA box sequence, 5'-TATA(A/T)A(A/T)-3', were found upstream of the TSS sequence in NTS2. After narrowing down the endpoints of the transcripts by using Northern analysis with oligonucleotide probes that spanned the rDNA NTS (our unpublished data), we performed primer-extension reactions to map the 5' ends of the transcripts with the same polarity as the bottom strand of NTS2 (hereafter referred to as NTS2 transcripts). Total RNA from wild-type, *sir2Δ*, and *set1Δ* cells was subject to reverse transcription with a primer that was extended toward the

predicted 5' end of the NTS2 transcripts (see schematic at top of Figure 2-9C). In RNA from *sir2Δ* cells, the major 5' end of the NTS2 transcripts was detected within the TSS consensus sequence at the position corresponding to the *A* (Figure 2-9C), indicating that the major transcription start site for the NTS2 transcripts was at the same base that was identified for >200 Pol II-transcribed genes in *S. cerevisiae* (Zhang and Dietrich, 2005). Minor 5' ends were detected upstream of the major TSS. Although longer exposures of the primer-extension gels revealed the presence of these bands in the *set1Δ* lane, they were barely or not at all detectable in the wild-type lane. Based on our Northern and 5'-end mapping data, we conclude that the NTS2 transcripts initiate ~300 base pairs upstream of the 35S rRNA gene and extend across NTS2 and the 5S rRNA gene into NTS1. Consistent with this conclusion, we were able to detect the 1.7- and 1.5-kb transcripts in RNA from *sir2Δ* cells using a probe to the 5S rRNA gene (our unpublished data).

To determine whether the NTS2 transcripts were made by Pol II, we used wild-type and *sir2Δ* strains lacking the genomic *RPB1* gene that encodes the largest subunit of Pol II with either the wt *RPB1* gene or a ts mutant allele, *rpb1-1* provided from a plasmid (see *Materials and Methods*). Strains with *rpb1-1* make functional Pol II at 24°C; however, upon shift of these cells to 36°C, Pol II becomes nonfunctional. RNA from cultures grown continuously at 24°C and from cultures shifted from 24 to 36°C for 30 min was hybridized with probes specific for the NTS2 transcripts, Pol II-transcribed *ACT1* RNA, and Pol I-transcribed rRNA (Figure 2-10A). In wild-type and *sir2Δ* cells with *RPB1* (wt RNA Pol II), *ACT1* and rRNA transcript levels were maintained in cells

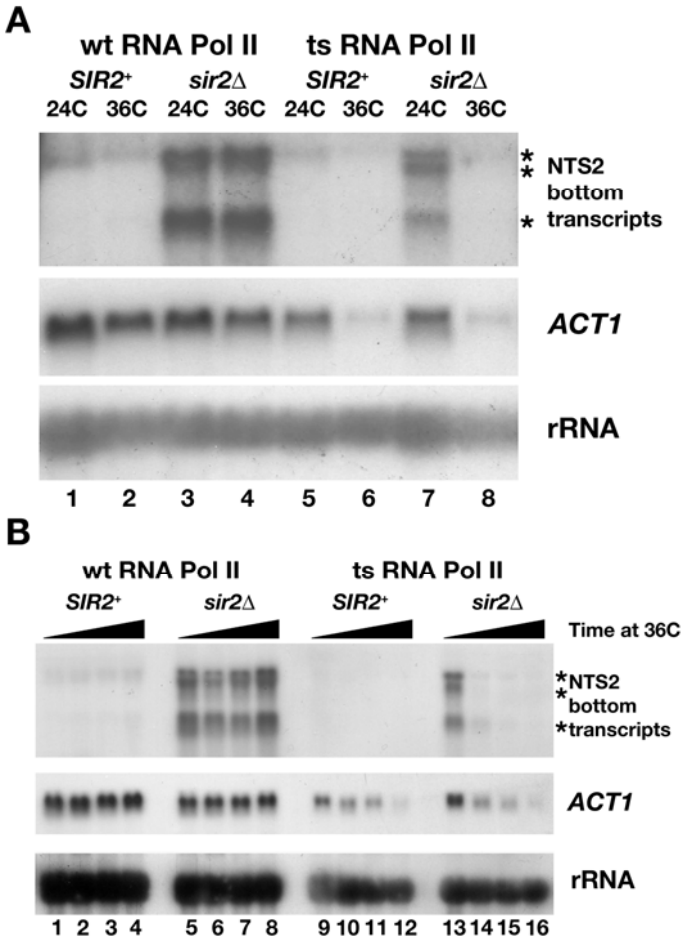

Figure 2-10. NTS2 transcripts are made by RNA polymerase II. (A) Cultures of *SIR2*$^+$ and *sir2Δ* cells containing a wild-type allele of *RPB1* (wt RNA Pol II) or a ts allele *rpb1-1* (ts RNA Pol II) were grown at 24°C and either maintained at 24°C or shifted to 36°C for 30 min (see *Materials and Methods*). Northern analysis of total RNA shows that NTS2 and *ACT1* transcript levels are stable at 24 and 36°C in cells with wt RNA Pol II (lanes 1-4, top and middle) but are depleted after 30 min at 36°C in cells with ts RNA Pol II (lanes 5-8). The levels of Pol I transcribed rRNA are stable at 24 and 36°C (lanes 1-8, bottom). (B) Time-course analysis reveals that NTS2 transcripts are depleted 15 min after inactivation of Pol II. Cultures of cells grown at 24°C were shifted to 36°C and aliquots were removed at 0, 15, 30, and 60 min. Total RNA was analyzed as described in A. Between 15 and 30 min after the shift to 36°C, the NTS2 transcripts were depleted significantly in cells containing the ts RNA Pol II but continue to be made in cells with the wt RNA Pol II (compare lanes 13-16 to 5-8, top). By 60 min, the level of *ACT1* RNA was reduced significantly in cells with ts RNA Pol II, whereas rRNA levels remain stable (compare lanes 1-8 to 9-16, middle and bottom). Asterisks indicate positions of NTS2 transcripts.

shifted to 36°C (Figure 2-10A, lanes 1-4, middle and bottom), as were the NTS2 transcripts in total RNA from *sir2Δ* cells (Figure 2-10A, lanes 3 and 4, top). However, in cells with *rpb1-1* (ts RNA Pol II, Figure 2-10A, lanes 5-8), *ACT1* RNA was present in cultures grown at 24°C but disappeared after 30 min at 36°C (compares lanes 5 and 6 or 7 and 8). Likewise, the NTS2 transcripts present in the *sir2Δ* cells were gone after 30 min at 36°C (compare lanes 7 and 8). The level of transcript from the Pol I-transcribed 35S rRNA gene was stable at 36°C. These data suggest that the NTS2 transcripts were made by Pol II and not by Pol I. In addition, it is unlikely that the NTS2 transcripts were made by Pol III, as numerous poly$T_{>5}$ stretches, which act as terminators for Pol III (Allison and Hall, 1985; Braglia *et al.*, 2005), are present in the rDNA NTS.

The stability of *ACT1* and NTS2 transcripts in *RPB1* and *rpb1-1* cells was measured over time at 36°C. Cultures grown at 24°C were shifted to 36°C, and aliquots of culture were removed for isolation of total RNA at 0, 15, 30 and 60 min after the shift to 36°C. A representative Northern blot in Figure 2-10B shows that although the levels of NTS2 transcripts were maintained in *sir2Δ* cells with wt RNA Pol II (lanes 5-8), the NTS2 transcripts were depleted in the *rpb1-1* cells 15-30 min after the shift to 36°C (lanes 13-16). Likewise, *ACT1* transcript levels were decreased over the time course at 36°C in *rpb1-1* cells. Together, these data indicate that the NTS2 transcripts were made by Pol II. In further support of this conclusion, we found that the NTS2 and *ACT1* transcripts were enriched in RNA immunoprecipitations using antisera against the trimethylguanosine cap present at the 5' end of Pol II-transcribed RNAs (our unpublished data).

2.3.6. Nucleosome Positioning at NTS2 Is Altered in Cells with rDNA-silencing Defects

Previous studies revealed that cells lacking Sir2 have altered chromatin structure in the rDNA (Fritze *et al.*, 1997; Cioci *et al.*, 2002); however, these studies did not focus on NTS2 where we predict changes in nucleosome positioning might occur because of increased transcription of the NTS2 by Pol II. To determine whether nucleosome positioning in the NTS2 region of the rDNA was altered in cells lacking Sir2 or Set1, we performed indirect end-labeling analysis of MNase digested chromatin. The rDNA repeats in *S. cerevisiae* exist in two configurations that are correlated with their accessibility to a cross-linking reagent psoralen and Pol I transcription activity (Dammann *et al.*, 1993). Open rDNA repeats are accessible to psoralen and transcribed by Pol I, whereas closed repeats are inaccessible to psoralen and not transcribed. It is important to consider that similar to the ChIP analyses, MNase experiments at the rDNA evaluate populations of cells, with each cell containing 150-200 rDNA repeats that are in different conformations with variable MNase accessibilities. It is possible that there are stronger effects on individual repeat, however, the alterations we observed represent the average accessibility of all the rDNA repeats. Thus, the ability to see even modest alterations in MNase cleavage over 150-200 repeats is significant.

Five positioned nucleosomes have been mapped in the NTS2 region of the rDNA (Figure 2-11, open circles labeled 1-5; Vogelauer *et al.*, 1998). We detected alterations in MNase accessibility at several positions in the NTS2 region in *sir2Δ* cells and *set1Δ*

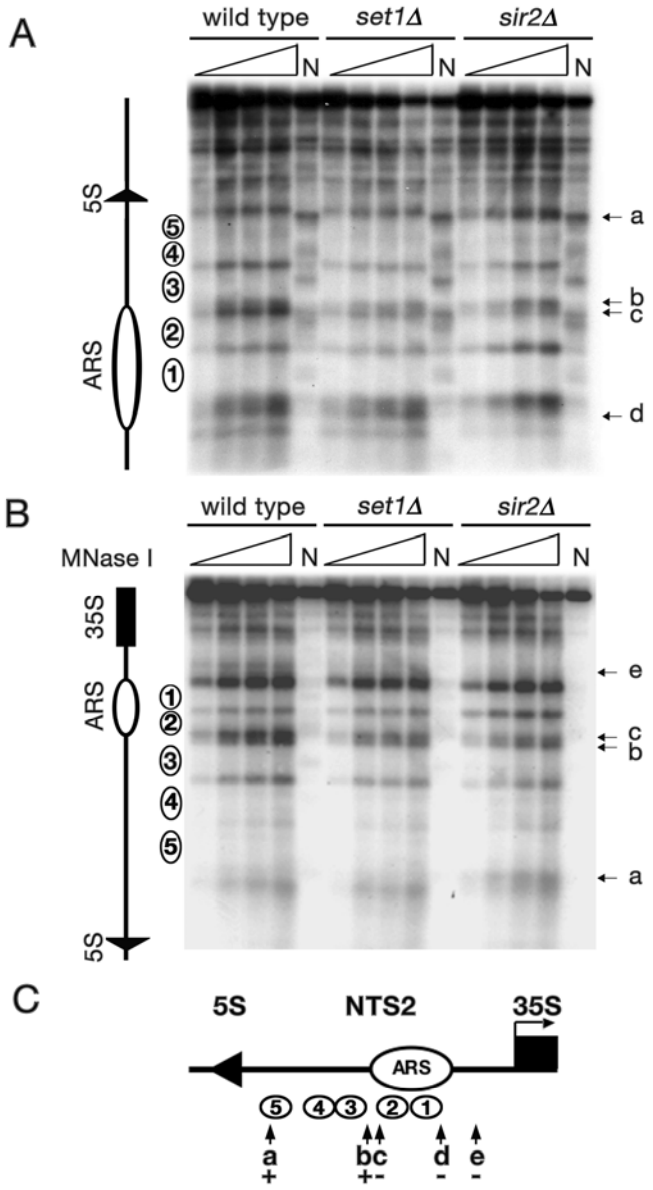

Figure 2-11. MNase accessibility of chromatin in the NTS2 region of the rDNA is altered in *set1Δ* and *sir2Δ* cells. DNA purified after MNase treatment of spheroplasts from wild-type, *set1Δ*, or *sir2Δ* cells was digested with EcoRI (A) or PvuII (B). Schematic of NTS2 with five positioned nucleosomes (numbered ovals) identified here and in a previous study (Vogelauer *et al*., 1998) is shown on the left of each panel and in C. Triangles, increasing amounts of MNase; N, naked DNA; arrows with lowercase letters, altered MNase accessibility (see text); other labels as in Figure 2-1. (C) The NTS2 region of the rDNA. Numbered circles, positioned nucleosomes; + and -, extent of MNase digestion in *sir2Δ* cells relative to wild-type cells.

cells (Figure 2-11, A-C). Between the ARS and the transcription start site of the 35S rRNA gene, in the region containing the TSS for the NTS2 transcripts, an MNase cleavage site was clearly missing in chromatin from *sir2Δ* cells and *set1Δ* cells that was present in wild-type chromatin (Figure 2-11, B and C, arrow marked e). This change may reflect protection by Pol II or its associated factors in *sir2Δ* and *set1Δ* cells. Other changes in MNase accessibility were detected in *sir2Δ* cells only, including a new cleavage site in NTS2 upstream of the Pol III-transcribed 5S gene (Figure 2-11, A-C, arrow marked a) and a missing cleavage site near the ARS (Figure 2-11, A and C, arrow marked d). In addition, we detected subtle changes in two MNase cleavage sites between nucleosomes 2 and 3 (Figure 2-11, A-C, arrows marked b and c). Our results indicate that rDNA chromatin structure was altered near the TSS sequence in the NTS2 region of the rDNA repeat in *sir2Δ* cells and *set1Δ* cells. These changes in chromatin structure are consistent with the observation that *sir2Δ* and *set1Δ* cells, which have profoundly different types of modified histones at the rDNA, both have defects in silencing of Pol II-transcribed genes at the rDNA.

2.4. DISCUSSION

Modified histones play a central role in regulating gene expression and gene silencing in eukaryotes. We have uncovered a functional relationship between Sir2, Pol II, and K4-methylated histone H3 at the rDNA in *S. cerevisiae*. Cells lacking Sir2 not only have high levels of acetylated histones but also high levels of K4-methylated H3 at the rDNA. Our data suggest that the change, from silent chromatin containing K4-

hypomethylated H3 to active chromatin containing high levels of K4-methylated H3, reflects increased transcription by Pol II at the rDNA NTS in cells lacking Sir2.

2.4.1. K4-Methylated Histone H3 and Pol II Are Excluded from Silent Chromatin by Sir2

We have analyzed K4-methylated H3 at silent loci in wild-type and *sir2Δ* cells by ChIP. Our results show that Sir2 excludes K4 mono-, di- and -trimethylated histone H3 from silent chromatin domains, including *HMR*, *TEL-VIR*, and the rDNA (Figures 2-1, 2-4 and 2-5). Using a catalytically inactive form of Sir2, we have determined that the deacetylase activity of Sir2 is required to exclude K4-di- and -trimethylated H3 from the rDNA (Figure 2-7). Consistent with the primary role of Sir2 at silent chromatin, no significant difference in the levels of K4-methylated H3 was detected at the euchromatic and non-rDNA intergenic regions in *sir2Δ* cells.

In *S. cerevisiae*, high levels of K4-trimethylated histone H3 are associated with actively transcribed genes in euchromatic domains of the genome (Bernstein *et al.*, 2002; Santos-Rosa *et al.*, 2002). These observations are supported by work showing that the Pol II elongation complex Paf1C recruits the histone H3 K4-methylation complex OMPASS to genes being transcribed by Pol II (Krogan *et al.*, 2003a; Ng *et al.*, 2003b; Ng *et al.*, 2003c). The results of experiments in Figures 2-8, 2-9 and 2-10 show that the level and activity of Pol II are increased at the rDNA NTS in *sir2Δ* cells. Interestingly, in cells lacking Paf1, the levels of K4-methylated H3 at the rDNA NTS are reduced, suggesting that even the low levels of K4-methylated H3 observed at the rDNA are dependent on transcription (Mueller *et al.*, 2006). Finally, our MNase data show that the

structure of chromatin in NTS2 is altered in *sir2Δ* cells. Based on these results, we propose that the deacetylase activity of Sir2 prevents the conversion of silent chromatin at the rDNA, normally inaccessible to Pol II, to active chromatin with the potential to be transcribed. This conclusion is supported by results showing that the level of K4-dimethylated H3, a mark of chromatin with the potential to be transcribed by Pol II, was increased over the rDNA repeat in cells lacking Sir2 and that the highest level of K4-trimethylated H3 was in the NTS, where Pol II transcription was occurring.

The observation of increased K4-methylated H3 and increased transcription at the rDNA in *S. cerevisiae* cells lacking Sir2 is reminiscent of recent reports regarding the role of modified histones in regulating Pol I transcription and rDNA chromatin structure in higher eukaryotes (reviewed in Grummt and Pikaard, 2003; McStay, 2006). In several *Arabidopsis* species, disruption of the histone deacetylase (HDAC) activities of HDT1, a plant-specific HDAC, or HDA6, a member of the RPD3 family of HDACs, caused normally silenced rDNA repeats to acquire characteristics of active rDNA repeats, including high levels of Pol I and K4-methylated H3, and low levels of K9-methylated H3 and DNA methylation (Lawrence *et al.*, 2004; Probst *et al.*, 2004; Earley *et al.*, 2006). Unlike the *Arabidopsis* system, in *S. cerevisiae*, transcription by Pol I is not increased in cells that lack the histone deacetylase *RPD3* (Oakes *et al.*, 1999; Oakes *et al.*, 2006b). Likewise, no changes in Pol I transcription have been observed in cells lacking Sir2 (Oakes *et al.*, 1999; Oakes *et al.*, 2006b). Despite the differences, a common thread between the yeast and plant systems is that loss of specific HDACs results in rDNA that is more like euchromatin, and, subsequently, has altered function.

It is important to note that each rDNA repeat in *S. cerevisiae* contains an ARS; however, only a small fraction (~20%) of these replication origins fire during S phase to replicate the rDNA locus (Linskens and Huberman, 1988). Molecular imaging experiments have shown that a large proportion of rDNA origins (50%) are activated in cells lacking Sir2 (Pasero *et al.*, 2002). The high level of K4-trimethylated H3 at the rDNA may be associated with increased firing of the rDNA ARS elements in *sir2Δ* cells. Experiments to address the role of K4-methylated H3 and NTS transcription in origin firing at the rDNA are currently underway.

2.4.2. Additional Factors May Limit K4-Methylated H3 at the rDNA

In cells lacking Sir2, increased levels of K4-trimethylated histone H3 were observed across an ~4-kb region of *HMR* and up to 2.8 kb from *TEL-VIR*, whereas the increase at the rDNA was limited primarily to NTS2 (Figures 2-1 and 2-4). The region of *HMR* where we detect changes in the association of K4-methylated histone H3 falls within the boundaries of the silent domain mapped in previous studies (Donze *et al.*, 1999; Donze and Kamakaka, 2001). From these observations, we conclude that COMPASS has greater access to *HMR* and *TEL-VIR* than to the rDNA in *sir2Δ* cells. Given that Pol II transcription units have been identified in the 35S rRNA gene and NTS1 (see below), it is possible that Sir2-independent mechanisms exist that limit the association of Pol II and K4-methylated H3 with these other regions. One possibility is that transcription by Pol I excludes factors required for methylation of H3. This can be tested by measuring the levels of K4-methylated H3 at the rDNA in cells that lack Pol I and make rRNA from a plasmid-borne 35S rRNA gene under the control of a Pol II

promoter, similar to the cells used to demonstrate the requirement for Pol I transcription in rDNA silencing (Buck *et al.*, 2002; Cioci *et al.*, 2003).

In addition, a *trans*-histone regulatory pathway has been identified where the ubiquitylation of histone H2B by Rad6 is required for the di- and trimethylation of histone H3 on K4 and K79 (Briggs *et al.*, 2002; Sun and Allis, 2002; Shahbazian *et al.*, 2005). In cells lacking Ubp10, an enzyme required for the removal of ubiquitin from H2B at silent domains in *S. cerevisiae*, the level of K4-trimethylated H3 was increased at the 5S and 35S rRNA gene (Emre *et al.*, 2005). We measured low levels of K4-trimethylated H3 at these regions in *sir2Δ* cells, suggesting that Ubp10 limits K4-methylated H3 even in the absence of Sir2.

Silencing at the rDNA, *HM* loci, and telomeres requires the deacetylase activity of Sir2. The known protein targets of Sir2 are lysine residues in histones H3 and H4, including K9 and K14 of H3 and K16 of H4 (reviewed in Rusche *et al.*, 2003). Recent work has demonstrated that *O*-acetyl-ADP-ribose (AAR), a product of the Sir2 deacetylation reaction, promotes a conformational change in the Sir2-3-4 complex that may contribute to the formation of silent chromatin (Liou *et al.*, 2005). Although we have determined that Sir2 restricts the access of Pol II and the K4-methylation machinery to silent chromatin, our in vivo studies do not separate the contributions of hypoacetylated histone tails and AAR. In vitro studies using purified factors and chromatin templates should distinguish between the functions of these products of Sir2.

Cells lacking Set1 exhibit defects in silencing at the rDNA and telomeres (Nislow *et al.*, 1997; Briggs *et al.*, 2001; Bryk *et al.*, 2002; Nagy *et al.*, 2002; Mueller *et*

al., 2006). Unlike what has been observed at telomeres (Ng *et al.*, 2002a; Meneghini *et al.*, 2003; Ng *et al.*, 2003a; Martin *et al.*, 2004; Katan-Khaykovich and Struhl, 2005), silencing factors remain at the rDNA in *set1Δ* cells (Bryk *et al.*, 2002), and thus the mechanism behind loss of silencing at the rDNA in *set1Δ* cells is not consistent with the model proposed to explain loss of silencing at telomeres. One model that we are currently testing is that release of Sir proteins from telomeres in *set1Δ* cells interferes with rDNA silencing. However, at this time, we are unable to determine whether the changes in chromatin accessibility or the loss of rDNA silencing seen in *set1Δ* cells are direct or indirect.

2.4.3. Other Pol II Transcription Units in the rDNA

In addition to the NTS2 transcripts that we have identified, the rDNA contains other Pol II transcription units. A naturally occurring Pol II-transcribed gene *TAR1* has been identified on the antisense strand of the 35S rRNA gene ~3-4 kb away from NTS2 (Coelho *et al.*, 2002). Although its expression was shown to be responsive to Sir2, we did not observe high levels of K4-trimethylated H3 over the *TAR1* ORF. Previous studies have characterized mutants that lack subunits of Pol I and survive by transcribing the 35S rRNA genes with Pol (Conrad-Webb and Butow, 1995; Vu *et al.*, 1999). However, in RNA from *sir2Δ* cells, Pol II-derived 35S rRNA transcripts have not been detected (Oakes *et al.*, 1999; Oakes *et al.*, 2006b). Moreover, we did not detect high levels of K4-trimethylated H3 or Pol II over the 35S rRNA gene, which would be expected if the 35S rRNA gene was being transcribed by Pol II.

Recent work has shown that in *sir2Δ* cells, bidirectional transcripts from NTS1 displace cohesin complexes from the rDNA leading to high levels of unequal sister chromatid exchange and rDNA repeat amplification (Kobayashi and Ganley, 2005). In addition to the transcripts from NTS1, we have identified transcripts from NTS2. Although transcription and the level of Pol II is increased clearly over both NTS1 and NTS2 in *sir2Δ* cells, we were surprised that the level of K4-trimethylated H3 over NTS1 was increased only ~1.5- to 2.0-fold. Despite our efforts to correct for total histone H3, we suspect reduced levels of total H3 at NTS1 (Figure 2-2) may have contributed to the lower level of K4-trimethylated H3 measured at NTS1 in ChIP experiments (Figure 2-1 and Figure 2-3). Nonetheless, the NTS transcription units display histone marks found on euchromatic genes, suggesting that not only Pol II but also Pol II-associated factors required for the trimethylation of H3 on K4 have access to the rDNA in *sir2Δ* cells.

Three possible ORFs of 71, 65, and 60 amino acids were identified in the sequence of the 1.7-kb NTS2 transcript, but no significant matches to known eukaryotic proteins from the nonredundant protein database were identified using BLASTp searches. Likewise, significant conservation of *S. cerevisiae* NTS DNA sequences was noted in several yeast species related to *S. cerevisiae* by BLASTn, but not in nonyeast organisms. It is possible that the NTS RNAs are noncoding RNAs. Intergenic transcription has been associated with regulatory pathways involving chromatin and gene expression in several organisms (reviewed in Bernstein and Allis, 2005). Interestingly, intergenic transcripts from the rDNA spacer that are made by Pol I have recently been shown to regulate the heterochromatin structure of rDNA repeats in mouse cells (Mayer *et al.*, 2006).

Experiments are currently underway to analyze the Pol II-transcribed NTS transcripts from *S. cerevisiae* in detail and to determine whether transcription through NTS2 alters rDNA recombination, silencing of Pol II marker genes, and/or DNA replication from the origin present in each rDNA repeat.

CHAPTER III

FUNCTIONAL ANALYSIS OF THE ENDOGENOUS POLYMERASE II TRANSCRIPTION UNITS IN THE RIBOSOMAL DNA NONTRANSCRIBED SPACER

3.1. INTRODUCTION

Silent chromatin in the eukaryotic genome refers to regions of chromosomes where some cellular processes, such as gene transcription, genetic recombination and DNA replication, are repressed. In the budding yeast *Saccharomyces cerevisiae*, silent chromatin includes the *HM* loci, telomeres and the ribosomal DNA (rDNA) locus. The rDNA locus is located on chromosome XII and contains ~150-200 tandem copies of the RNA polymerase I (Pol I)-transcribed 35S ribosomal RNA (rRNA) gene, each separated by a non-transcribed spacer (NTS) that is subdivided into NTS1 and NTS2 by the RNA polymerase III (Pol III)-transcribed 5S rRNA gene (reviewed in Warner, 1999; Figure 1-5). Although a subset of the rDNA repeats are actively transcribed by Pol I and Pol III (Dammann *et al.*, 1993), Pol II-transcribed genes integrated into the rDNA are silenced (referred to as rDNA silencing) (Bryk *et al.*, 1997; Fritze *et al.*, 1997; Smith and Boeke, 1997). In addition, genetic recombination is repressed at the rDNA locus, which contributes to the stability of the rDNA locus (Gottlieb and Esposito, 1989; Davis *et al.*, 2000; Kobayashi *et al.*, 2004).

The Silent information regulator 2 (Sir2) protein regulates silent chromatin at the rDNA locus. Sir2 is an NAD^+-dependent histone deacetylase, whose targets include histone H3 and H4. Together with Net1 and Cdc14, Sir2 forms a complex called RENT,

which is required for rDNA silencing (Huang and Moazed, 2003). It has been shown that Sir2 is recruited to the rDNA by Net1 and deacetylates acetyl-lysine residues in the N-terminal tail of histone H3 (Huang and Moazed, 2003). In cells lacking Sir2, Pol II-transcribed genes inserted at the rDNA array are no longer silenced, and the accessibility of rDNA chromatin to micrococcal nuclease is altered (Bryk *et al.*, 1997; Fritze *et al.*, 1997; Smith and Boeke, 1997; Li *et al.*, 2006a). The rate of mitotic recombination at the rDNA is also increased significantly in *sir2Δ* cells (Gottlieb and Esposito, 1989).

In our previous study to determine how Sir2 regulates the composition of silent chromatin at the rDNA locus, we identified several endogenous transcription units in both top strand and bottom strand of the rDNA NTS region (Li *et al.*, 2006a). These include NTS1 transcription units initiated from a promoter region called E-pro (Kobayashi and Ganley, 2005). In addition, novel transcripts of multiple sizes were identified with the same polarity as the top strand of NTS2 and three distinct transcripts of 1.0, 1.5 and 1.7 kb were identified with the same polarity as the bottom strand of NTS2 (Li *et al.*, 2006a). The silencing of each of these NTS transcription units is dependent on Sir2 (Kobayashi and Ganley, 2005; Li *et al.*, 2006a).

In this study I focus on characterizing the function of the transcripts with the same polarity as the bottom strand of NTS2, referred to as the NTS2 bottom strand transcripts. Three potential open reading frames (ORFs) were identified in the1.7 kb NTS2 bottom strand transcript, with the longest ORF encoding a protein of 71 amino acids (aa). The hypothetic proteins encoded by these ORFs do not match any known proteins, suggesting that these RNAs may be non-coding RNAs (Li *et al.*, 2006a).

Despite this, we can't rule out the possibility that the NTS2 bottom strand RNAs encode novel proteins.

Intergenic transcripts have been considered to be products of spurious transcription without biological function. However, recent evidence suggests that intergenic transcripts actively participate in fundamental processes in cells. In fact, most regions of the genome in eukaryotes are known to be transcribed into non-coding RNAs (Bertone *et al.*, 2004; David *et al.*, 2006; Yazgan and Krebs, 2007).

The functions of the non-coding RNAs transcribed from intergenic regions are diverse. The most well characterized non-coding RNAs are small RNAs, including microRNAs (miRNAs) and short interfering RNAs (siRNAs). They are known to control mRNA stability as well as chromatin structure (Mathieu and Bender, 2004; Bernstein and Allis, 2005). In addition, there are large non-coding RNAs derived from introns or intergenic regions. The function of these large non-coding RNAs is not known specifically but it has been hypothesized that they are involved in various aspects of gene regulation (for review, see Yazgan and Krebs, 2007). For instance, the Xist (X inactive specific transcript) RNAs are transcribed from one of the two copies of the X chromosomes in female mammals. The Xist RNAs coat the X chromosome from where they were transcribed, resulting in silencing of this chromosome. This is known as X-inactivation and the process acts to compensate for the extra dosage of X-linked genes in female cells (reviewed in Chow *et al.*, 2005; Ng *et al.*, 2007).

While non-coding RNAs have been demonstrated to be a functional component in some regulatory mechanisms, there is also evidence that the act of transcription itself

can modulate transcriptional regulation and chromatin activities. One example is the regulation of the *SER3* gene in *S. cerevisiae*. *SER3* encodes a protein called 3-phosphoglycerate dehydrogenase, which catalyzes the biosynthesis of serine and glycine. Martens *et al.* (2004, 2005) showed that under repressive conditions a regulatory sequence upstream of the *SER3* gene is expressed at high levels. Transcription of this upstream regulatory element represses transcription of the downstream *SER3* gene. An example of intergenic transcription regulating chromosomal processes was uncovered at the rDNA is the intergenic transcription of the rDNA NTS1 region. Kobayashi *et al*. (2005) suggested that bidirectional transcription of NTS1 that occurs in *sir2Δ* cells displaces cohesin complexes from the rDNA, thereby promoting mispairing of the replicated rDNA loci and deleterious unequal sister chromatin exchanges. These types of events can result in deletion or amplification of rDNA repeats. Similar events in repeat-containing loci in human chromosomes are associated with X-linked color blindness (Vorallrath *et al.* 1988).

Based on the fact that the non-coding RNAs and the act of transcription regulate gene expression and chromatin function, we have investigated the effects of transcription of the rDNA NTS2 region to determine if there is a discernable function in regulating the silent chromatin at the rDNA locus. To address this question, we overexpressed the NTS2 bottom strand RNAs either from a plasmid ("*in trans*") or from the endogenous locus ("*in cis*"), and assayed the effects of overexpression on Pol II gene silencing and mitotic recombination at the rDNA locus. The result showed that overexpression of the NTS2 bottom strand RNAs *in cis* disrupts Pol II gene silencing and increases the rate of

mitotic recombination at the rDNA locus. In contrast, no silencing defects were observed when the transcripts were overexpressed *in trans*, which suggests that the act of transcription but not the RNA itself disrupts silent chromatin at the rDNA locus. These data provide insights into the mechanism of how Sir2 represses rDNA recombination and maintains rDNA stability.

In addition, we tested the coding potential of the NTS2 bottom strand transcripts. Interestingly, our result showed that the NTS2 bottom strand transcripts could be translated *in vitro*. A polysome fractionation experiment also showed that these transcripts associate with polysomes *in vivo*. These data suggest that the NTS2 bottom strand transcripts may encode proteins or they may be involved in regulating protein synthesis.

3.2. MATERIALS AND METHODS

3.2.1. Yeast strains, plasmids, and media

Standard media recipes were used (Rose, 1990). YPADT is YPD medium supplemented with adenine sulfate (40 mg/L) and L-tryptophan (0.4 mM). All media contain 2% glucose if not specified otherwise. In experiments requiring the transcription of the NTS2 bottom strand transcripts under the control of a *GAL* promoter, the media contained 2% galactose. Yeast strains used in this study are listed in Table 3-1.

Plasmid MBB426 was constructed by amplifying the NTS fragment (-2481 to -241 relative to the transcriptional start site of 35S rRNA precursor) using primers containing BamH I (OM714) and XhoI (OM692) restriction sites. The PCR product was digested with BamHI and XhoI, and ligated into the corresponding sites on MBB345

Table 3-1 Strains and plasmids

Strain	Genotype
MBY30	*MATα*, *ura3-167*, *his3Δ200*, GAL$^+$, Ty1*his3AI-236*
MBY1447	*MATα*, *his3Δ200*, ura3-167, GAL$^+$, Ty1*HIS3*-242
MBY1487	*MATα*, *his3Δ200*, *leu2:hisG*, Ty1*HIS3-236*
MBY1211	*MAT*a, *ura3Δ0*, *leu2Δ0*, *his3Δ200*, *ade2Δ::hisG*, *trp1Δ63*, *met15Δ0,* Ty1*ade2AI-515*
MBY1198	*MAT*α, *ade2*Δ*::his*G, *his3*Δ*200*, *leu2*Δ*0*, *met15*Δ*0*, *trp*1Δ*63, ura3*Δ*0*, Ty1*his3AI*-236
MBY1238	MBY1198, *sir2*Δ*::KANMX4*
MBY1290	*MATα*, *ura3-167*, *his3Δ200*, GAL$^+$, Ty1*his3AI-236*, *set1Δ::kanMX4#1*
MBY2067	MBY1447, MBB345 [pRS426-GAL1p, *URA3*, *CEN*]
MBY2068	MBY1447, MBB426 [pRS426-GAL1p-NTS, *URA3*, *CEN*]
MBY2069	MBY1487, MBB345 [pRS426-GAL1p, *URA3*, *CEN*]
MBY2070	MBY1487, MBB426 [pRS426-GAL1p-NTS, *URA3*, *CEN*]
MBY2206	MBY30, MBB345 [pRS426-GAL1p, *URA3*, *CEN*]
MBY2207	MBY30, MBB426 [pRS426-GAL1p-NTS, *URA3*, *CEN*], isolate #1
MBY2208	MBY30, MBB426 [pRS426-GAL1p-NTS, *URA3*, *CEN*], isolate #2
MBY2352	MBY1290, MBB345 [pRS426-GAL1p, *URA3*, *CEN*]
MBY2367	MBY1211, one of the rDNA repeats carries a *HIS3* gene in NTS1 (PvuII; same orientation as 35S rRNA gene) and *GAL7* promoter in NTS2 (SmaI; orientation as 35S rRNA gene) #2
MBY2368	MBY1211, one of the rDNA repeats carries a *HIS3* gene in NTS1 (PvuII; same orientation as 35S rRNA gene) and *GAL7* promoter in NTS2 (SmaI; orientation as 35S rRNA gene) #3
MBY2371	MBY1211, one of the rDNA repeats carries a *HIS3* gene in NTS1 (Pvu II; same orientation as 35S rRNA gene) and *GAL7* promoter in NTS2 (SmaI; orientation as 5S rRNA gene) #1
MBY2372	MBY1211, one of the rDNA repeats carries a *HIS3* gene in NTS1 (PvuII; same orientation as 35S rRNA gene) and *GAL7* promoter in NTS2 (SmaI; orientation as 5S rRNA gene) #9
MBY2373	MBY1211, one of the rDNA repeats carries a *HIS3* gene in NTS1 (PvuII; same orientation as 35S rRNA gene) #2
MBY2374	MBY1211, one of the rDNA repeats carries a *HIS3* gene in NTS1 (PvuII; same orientation as 35S rRNA gene) #5
MBY2377	MBY2373, *sir2*Δ*::KANMX4*, #11
MBY2378	MBY2374, *sir2*Δ*::KANMX4*, #14
Plasmids	**Description**
MBB345	pRS426 *GAL1*, *URA3,* 2μ, CEN
MBB426	pRS426 *GAL1*-NTS, *URA3,* 2μ, CEN
MBB413	pSP70-NTS2
MBB511	pSP70-NTS, *HIS3* at NTS1, *GAL7p* at NTS2 (towards 35S)
MBB514	pSP70-NTS, *HIS3* at NTS1, *GAL7p* at NTS2 (towards 5S)
MBB515	pSP70-NTS, *HIS3* at NTS1, no *GAL7p*

(pRS426-*GAL1*promoter, 2μ). In MBB426, the NTS2 bottom strand transcripts are transcribed under the control of a *GAL1* promoter. The plasmid MBB345 was transformed into MBY30 to make MBY2206, and the plasmid MBB426 was transformed into MBY30 to make MBY2207 and MBY2208 (two identical isolates from the same transformation). All of these strains carry a Ty1*his3AI* element integrated in the rDNA array, which was used to test if overexpression of the NTS2 bottom strand transcripts from a plasmid affects rDNA silencing. Alternatively, the two plasmids were transformed into MBY1487, which carries a Ty1*HIS3* element at the rDNA locus, to make MBY2069 and MBY2070, respectively. These strains were used to test if overexpression of the NTS2 bottom strand transcripts from a plasmid affects mitotic recombination at the rDNA.

To make the plasmids for integrating a *GAL7* promoter as well as the *HIS3* gene into the rDNA array, the sequence containing the entire rDNA NTS with part of the 35S rRNA gene (-2481 to +519 relative to the transcriptional start site of 35S rRNA precursor) was amplified using primers containing XhoI sites (OM692 and OM717). The PCR product was digested with XhoI and cloned into the corresponding site of plasmid pSP70 to make MBB425. The *GAL7* promoter sequence was amplified with primers containing SmaI sites (OM723 and OM724) and cloned into the corresponding site on MBB425. The orientation of the *GAL7* promoter in the resulting plasmids was determined by PCR. A construct with the *GAL7* promoter in the same orientation as the 35S rRNA gene was assigned as MBB430, while the one with the *GAL7* promoter in the opposite orientation was assigned as MBB431. The *HIS3* sequence (-248 to +689) was

amplified using primers containing PvuII sites (OM986 and OM987) from genomic DNA, and cloned into the corresponding site in NTS1 of MBB430 and MBB431 to make MBB511 and MBB514, respectively. MBB515 was constructed by inserting a *HIS3* fragment into the PvuII site in NTS1 region of MBB425, and it does not contain the *GAL7* promoter. The orientation of the *HIS3* gene in MBB511, MBB514 and MBB515 is the same as 35S rRNA gene as determined by PCR.

Yeast strains MBY2367 and MBY2368 are two isolates obtained by integrating the XhoI fragment from plasmid MBB511 into the rDNA array of the parental strain MBY1211. MBY2371 and MBY2372 are two isolates obtained by integrating the XhoI fragment from plasmid MBB514 into the rDNA array of MBY1211. Finally, the strains MBY2373 and MBY2374 are two isolates obtained by integrating XhoI fragment from plasmid MBB515 into MBY1211. The *SIR2* gene in MBY2373 and MBY2374 was deleted with *KANMX4* to make MBY2377 and MBY2378, respectively (Brachmann *et al*., 1998). These strains carry a single copy of the XhoI fragment at the rDNA array, as identified by restriction digestion with agarose-embedded genomic DNA and CHEF gel analysis (see below).

3.2.2. Contour-clamped homogenous electric fields electrophoresis (CHEF)

Agarose-embedded genomic DNA was prepared as described (Gerring *et al.*, 1991; Bryk *et al.*, 1997). Chromosomal DNA was digested with 8 units of AvrII (which is unique in the *HIS3* gene, but not present in the rDNA array) at 37°C for 16 hours. The DNA was then separated on 1% agarose gel (BioRad, Pulsed Field Certificated) using BioRad CHEF Mapper apparatus in 0.5×TBE buffer. The electrophoresis was performed

under 6 v/cm at 14°C with 60- to 110-sec pulse time for 24 hours. The gel was stained with ethidium bromide, photographed, and treated with UV irradiation for 2 min. After denaturation and neutralization, the DNA was transferred onto a nylon membrane and probed with a ^{32}P-labeled *HIS3* specific DNA probe (from +271 to +615 region of the *HIS3*). The *HIS3* probe was removed by stripping at 70°C for 2 hours in the presence of 1×SSC and 50% formamide (v:v) and the blot was hybridized with an rDNA probe that recognizes the NTS region. If the XhoI-XhoI fragment is integrated only once in the rDNA, AvrII should cut the rDNA array into two pieces and the *HIS3* probe should light up two bands. The same banding pattern should be seen with the rDNA probe. Using this method, I identified strains that have a single integration of the XhoI-XhoI fragment.

3.2.3. Northern analysis

Total RNA was isolated from yeast cells as described previously (Bryk *et al.*, 1997). Northern analyses were performed as described (Swanson *et al.*, 1991). NTS2 bottom strand transcripts, Ty1*his3AI*, total Ty1 and *PYK1* mRNAs were detected using strand specific ^{32}P-labeled riboprobes (Li *et al.*, 2006a; Li *et al.*, 2008). A ^{32}P-labeled double-stranded DNA probe was used to detect the *ACT1* mRNA (Li *et al.*, 2006a). Northern blots were quantified on a Molecular Dynamics Storm 860 phosphorimager using ImageQuant software.

3.2.4. Mitotic recombination analysis

Mitotic stability of the Ty1*HIS3* element in MBY2067, 2068, 2069 and MBY2070, and the *HIS3* in MBY2367, 2368, 2371-2374, 2377 and MBY2378 was measured as described (Bryk *et al.*, 1997) with some modifications. Cells of MBY2067-

2070 were pregrown in 5 ml SC-Ura-His media for one overnight at 30°C. One microliter of each culture was then diluted into 10 ml fresh SC-Ura containing 2% galactose and grown to stationary phase (~ 48 hours) at 30°C. Cells of MBY2367, MBY2368, MBY2371-2374, MBY2377 and MBY2378 were pre-grown in 5 ml SC-His media for one overnight at 30°C, and 1μl of each culture was then diluted into 10 ml fresh YPAT media containing 2% galactose and grown to stationary phase (~ 48 hours) at 30°C. The dilution was repeated 8 times. Cells from the last culture were plated onto YPADT plates, and replicated to SC-His plates to determine the fraction of His^- auxotrophs.

3.2.5. Reverse transcription

Total RNA was isolated from yeast cells as described previously (Bryk *et al.*, 1997). The RNA was treated with RQ1 RNase-free DNase (Promega) to remove genomic DNA contamination followed by phenol extraction. The RNA was then transcribed into first strand cDNA using M-MLV Reverse Transcriptase (Ambion) with Oligo-dT (Ambion) or a strand-specific primer (OM869) that hybridizes to the sense strand of the *HIS3* gene.

3.2.6. Quantitative real-time PCR (qPCR)

One microliter of the diluted (1:5) cDNA was amplified in 20 μl reaction containing 1.25 μM of each oligonucleotide and 1× SYBR Green Dynamo Hot Start PCR mix (Finnzymes, Espoo, Finland) by using an iCycler Iq real-time PCR machine (Bio-Rad, Hercules, CA). The PCR parameters were described in (Li *et al.*, 2006a). The *HIS3* specific primers OM869 and OM879 were used to amplify a 293 bp PCR product,

and the *LYS2* specific primers OM16 and OM17 were used to amplify a 285 bp PCR product. Each primer pair has its own standard curve.

3.2.7. In vitro transcription and in vitro translation

To prepare DNA templates for transcription of the NTS2 bottom strand RNA, the complete NTS2 sequence was amplified with OM679 and OM680 which contain XhoI site at one end and HindIII site at the other end. The PCR fragment was digested with XhoI and HindIII and cloned into the corresponding sites on pSP70 bearing the T7 promoter upstream of HindIII site. The resulting plasmid is MBB413. The T7 promoter driven RNA in this construct is complementary to the NTS2 top strand. (The T7 promoter can drive transcription of NTS2 bottom strand.) To prepare DNA template for *in vitro* transcription, MBB413 was linearized by XhoI digestion. Transcription was carried out in a volume of 20 μl by incubating 0.5 μg of DNA in the presence of 1 mM NTPs, 20 units of RNase inhibitor (Promega), 20 units of T7 RNA polymerase, and accompanying buffer (Roche) for 2 hours at 37°C. One unit of RNase-free DNase (Promega) was then added into the reaction to remove the template. The RNA product was purified by phenol extraction.

All the purified *in vitro* transcribed RNA was used in a 50 μl reaction of Rabbit Reticulocyte Lysate *in vitro* translation system (Promega, Madison, WI) according to the manufacturer's instruction. Five microliter of the in vitro translated product was resolved on 15% SDS-PAGE gels and visualized by autoradiography.

3.2.8. Polysome analysis

The polysome fractions from MBY1198 and MBY1238 were isolated according to (Cigan *et al.*, 1991) with minor modifications. Briefly, cells were grown in 1 L YPADT media to a final density of 3-4×10^6 cells/ml. Cycloheximide was added to the cultures to a final concentration of 50 μg/ml and the cultures were placed on ice water immediately. The cells were harvested by centrifugation, washed with 10 ml of pre-chilled breaking buffer (10 mM Tris-HCl pH7.4, 100 mM NaCl, 30 mM $MgCl_2$, 50 μg of cycloheximide/ml, and 200 μg of heparin/ml), and resuspended in 1 ml of breaking buffer. Glass beads were added to approximately one-fourth of the final volume. The cells were vortexed on ice for 8×15 sec with 15 sec intervals, followed by addition of 1 ml of breaking buffer. The extract was cleared by two sequential centrifugations (5,000 g for 5 min and 12,000 g for 10 min). Twenty-five OD_{260} units of the supernatant was layered on 12 ml linear 7-47% sucrose gradients equilibrated with 50 mM Tris-acetate (pH 7.6), 50 mM NH_4C1, 12 mM $MgCl_2$, and 1 mM dithiothreitol and centrifuged at 4°C in an SW41 rotor (Beckman) at 39,000 rpm for 2 hours. The gradients were fractionated by pipetting 1 ml at a time. One hundred microliters of each fraction were removed and combined with 400 μl of H_2O to measure OD_{206} to generate a polysome profile. Total RNA of each fraction was precipitated and phenol extracted according to (Cigan *et al.*, 1991). Finally, the purified RNA was resuspended in 10 μl of H_2O and half of the RNA was subjected to Northern analysis.

3.3. RESULTS

3.3.1. NTS2 bottom strand transcripts do not regulate Pol II gene silencing and mitotic recombination at the rDNA locus

Non-coding RNAs have been shown to be involved in establishing heterochromatin in the fission yeast *Schizosaccharomyces pombe* (*S. pombe*), plants, flies and mammals (for review, see Pezer and Ugarkovic, 2008). The mechanism of non-coding RNA-mediated chromatin silencing is best studied in the centromeres of *S. pombe*, which consist of highly repeated DNA sequence. In *S. pombe,* a low level of double-stranded RNA is transcribed from the repeated satellite DNA sequence in the pericentromeric or centromeric regions. The RNA is processed by Dicer, and can guide the establishment of heterochromatin formation at the centromere via RNAi pathway (for reviews, see Grewal and Jia, 2007; Pezer and Ugarkovic, 2008). In this case, it is the RNA molecules but not the act of transcription that is important for establishing heterochromatin. Despite the fact that the components of RNAi pathway have not been identified in *S. cerevisiae*, it is possible that the RNAs arise from the rDNA NTS2 bottom strand may hybridize to the NTS2 region, helping to open the chromatin structure and allowing Pol II transcription and genetic recombination to occur.

To determine if the NTS2 bottom strand RNAs regulate Pol II gene silencing at the rDNA locus, a plasmid that contains the NTS2 region under the control of *GAL1* promoter to drive the transcription of the NTS2 RNAs *in trans* was constructed (MBB426; Figure 3-1A). The *GAL1* promoter is an inducible promoter, which is repressed by glucose and activated by galactose (Johnston, 1987). The plasmid was

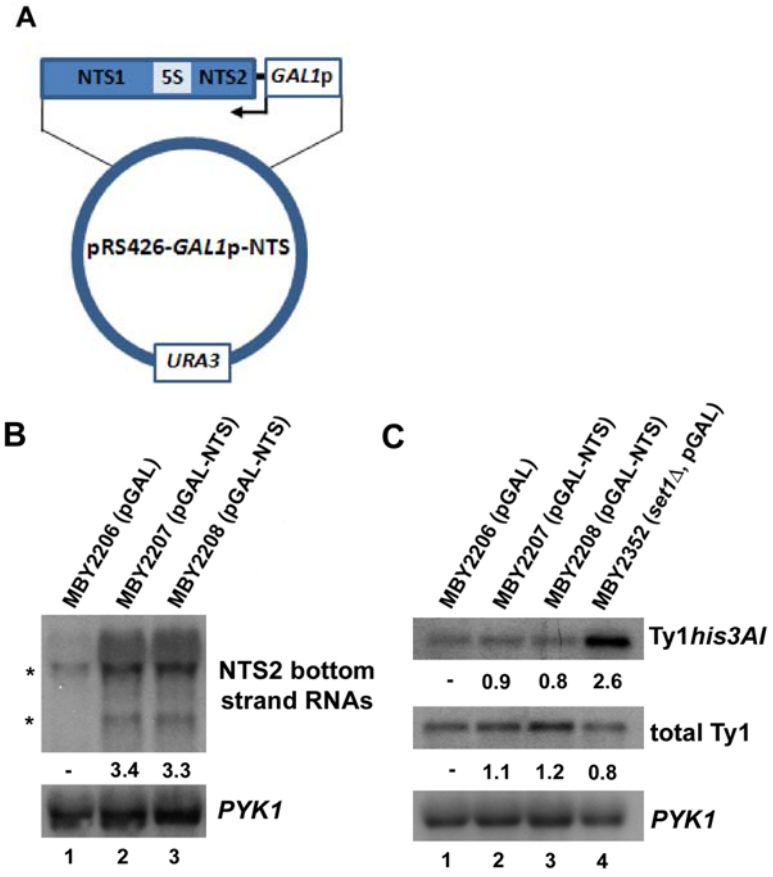

Figure 3-1. Overexpression of the NTS2 bottom strand transcripts *in trans* does not disrupt Pol II gene silencing at the rDNA locus. (A) Plasmid used to drive the transcription of NTS2 bottom strand RNAs. The *GAL1* promoter is placed upstream of the NTS2 bottom strand transcription unit. (B) Northern blot analysis to verify the overexpression of NTS2 bottom stand RNAs from the plasmid shown in A. Total RNA from cells containing an empty vector (pGAL, lane 1) or the overexpression plasmid (pGAL-NTS, lane 2 and 3) were hybridized to a strand-specific probe that recognizes the NTS2 bottom strand RNAs (top panel). *PYK1* RNA levels were measured to check equal loading of RNA in each lane (bottom panel). Asterisks, position of the NTS2 bottom strand transcripts. The average ratio of NTS2 RNA/*PYK1* for the overexpression strains after normalization to the strain containing an empty vector are shown below the top panel. The normalized values of the average ratio ± range were 3.4±0.6 and 3.3± 0.6 for MBY2207 and MBY2208, respectively (n=2). (C) Northern blot analysis of total RNA isolated from strains as shown in B. Strand specific probes were used to measure the steady-state mRNA levels of Ty1*his3AI* (top panel), total Ty1 (middle panel) or *PYK1* (bottom panel). A *set1Δ* strain was used as a silencing-defect control (MBY2352, lane 4). The average ratio of Ty1*his3AI*/*PYK1* and total Ty1/*PYK1* after normalization to the strain containing an empty vector is shown below the top and middle panel. The normalized values of the average ratio ± range for Ty1*his3AI*/*PYK1* were: MBY2207: 0.9 ± 0.1; MBY2208: 0.8 ± 0.2; MBY2352, 2.6 ± 1.1. The normalized values of the average ratio ± range for total Ty1/*PYK1* were: MBY2207: 1.1 ± 0.1; MBY2208: 1.2 ± 0.2; MBY2352, 0.8 ± 0.2.

introduced into a strain that contains a genetically marked Ty1 element, Ty1*his3AI*, located in a single repeat of the rDNA array. The Ty1 element is transcribed by Pol II, and the level of the mRNA from the Ty1*his3AI* has been used as an indicator for Pol II gene silencing at the rDNA (Bryk *et al.*, 1997). Cells containing an empty vector or the overexpression plasmid were grown in media containing galactose to activate the *GAL1* promoter, and total RNA was isolated and subjected to Northern analysis. As shown in Figure 3-1B, the level of NTS2 bottom strand RNA was increased about 3-fold when the transcription was driven by *GAL1* promoter (Figure 3-1B, lane 2 and 3) compared to the level from cells containing an empty vector (Figure 3-1B, lane 1). However, the increased amount of NTS2 RNA did not affect Pol II gene silencing at the rDNA (Figure 3-1C), since the level of Ty1*his3AI* mRNA from cells overexpressing NTS2 RNA (lane 2 and 3, top panel) was similar to the level from cells containing an empty vector (lane 1, top panel). As a positive control, we also measured the Ty1*his3AI* mRNA from cells lacking *SET1*, a gene encoding the histone methyltransferase Set1 that is required for Pol II silencing at the rDNA (Nislow *et al.*, 1997; Briggs *et al.*, 2001; Bryk *et al.*, 2002; Nagy *et al.*, 2002; Krogan *et al.*, 2003a). As expected, deletion of *SET1* disrupted Pol II gene silencing at the rDNA and the level of Ty1*his3AI* mRNA was increased about 2.6-fold (Figure 3-1C, lane 4, top panel). Because most *S. cerevisiae* strains carry about 30 copies of Ty1 elements throughout the genome, we also measured the mRNA level of total Ty1 elements using a probe that recognizes all the Ty1 mRNA. The result indicated that overexpression of the NTS2 bottom strand RNA from a plasmid did not increase the mRNA of total Ty1elements significantly (Figure 3-1C, middle panel). These data

indicate that overexpression of the NTS2 bottom strand RNA does not affect Pol II gene silencing at the rDNA.

It is known that the silent chromatin at the rDNA locus also represses homologous recombination between the rDNA repeats. We next tested if the NTS2 bottom strand RNA plays a role in regulating mitotic recombination at the rDNA locus. To address this question, the empty vector and the overexpression plasmid were transformed in to a strain containing a Ty1*HIS3* element located in a single repeat of the rDNA array. The cells containing the *HIS3* gene are histidine prototrophs and can grow on media lacking histidine. If the cells lose the *HIS3*gene, which is caused by mitotic recombination between flanking rDNA repeats or by recombination between LTRs of Ty1 elements, they become histidine auxotrophs and will not be able to grow on media without histidine. The rate of loss of *HIS3* marker in these strains is used as an indicator for the stability of the rDNA array. We grew the strains that harboring either the empty vector or the overexpression plasmid in SC-Ura media (to keep the selection for the plasmids) with 2% galactose for about 120 generations, and measured the rate of loss of the *HIS3* marker. The result showed that the rate of loss of the *HIS3* marker from the overexpression strain is about 1.1×10^{-4} per generation, and is not significantly different from the strain containing an empty vector (2.5×10^{-4} per generation) (P= 0.096). We also measured the rate of loss of a Ty1*HIS3* element located outside of the rDNA array, and found that overexpressing NTS2 bottom strand RNAs did not affect the rate of loss of this *HIS3* marker either (Table 3-2). These data indicate that the NTS2 bottom strand RNAs do not have a role in regulating mitotic recombination at the rDNA locus.

Table 3-2 Mitotic stability of Ty1*HIS3* elements when NTS2 region is overexpressed *in trans*

Strain (location[a])	**Relevant genotype**	**Loss *HIS3*/generation[b], average (± SE; n)**	**Loss relative to pGAL strains**
MBY2069 (in rDNA)	pGAL	$2.52\ (\pm\ 0.72;\ 3)\times10^{-4}$	-
MBY2070 (in rDNA)	pGAL-NTS	$1.12\ (\pm\ 0.27;\ 4)\times10^{-4}$	0.44[c]
MBY2067 (outside rDNA)	pGAL	$<5.13(\pm\ 0.66;\ 3)\times10^{-6}$	-
MBY2068 (outside rDNA)	pGAL-NTS	$<5.63(\pm\ 0.81;\ 3)\times10^{-6}$	1.1

a. Location of Ty1*HIS3* element: in rDNA, in NTS1 of a single rDNA repeat at position 460482; outside the rDNA, in *YLR460C* at position 1060536.
b. Determined after 120 generations of growth in SC-URA medium containing 2% galactose.
c. The rate of loss of *HIS3* in pGAL-NTS cells is not significantly different from the one in pGAL cells, P=0.096 determined by Student's t-test.

3.3.2. Overexpression of the NTS2 bottom strand transcripts in cis disrupts Pol II gene silencing and mitotic recombination at the rDNA locus

The process of transcription in NTS1 in *sir2Δ* cells has been shown to disrupt silent chromatin at the rDNA (Kobayashi and Ganley, 2005). We want to test if the process of transcription of the NTS2 bottom strand would also have an effect on regulating the silent chromatin at the rDNA locus. The hypothesis is that the act of transcription opens the chromatin at the rDNA and a more open chromatin conformation will disrupt Pol II gene silencing as well as promote mitotic recombination. To test this idea, we overexpressed the NTS2 bottom strand RNA at its endogenous location to mimic the loss of silencing of the NTS2 transcription units in the *sir2Δ* strain.

A series of strains were constructed for these experiments (Figure 3-2A). In the first strain, a galactose-inducible promoter, *GAL7*, was placed upstream of the TSS of the NTS2 bottom strand transcriptional unit of one of the rDNA repeats. The direction of the *GAL7* promoter is towards 5S rRNA gene (Figure 3-2A, top panel). A *HIS3* marker gene is also inserted into the NTS1 region of the same repeat (Figure 3-2A). This arrangement should allow transcription of the NTS2 bottom strand when the cells are grown in galactose-containing media. The second strain is similar to the first one, except that the *GAL7* promoter is in the same direction as the 35S rRNA gene and will not drive transcription of the NTS2 bottom strain RNA (Figure 3-2A, bottom panel). As a control, we also constructed strains with the *HIS3* gene inserted at NTS1 and lacking a *GAL7* promoter at NTS2 in a $SIR2^{+}$ background and *sir2Δ* background.

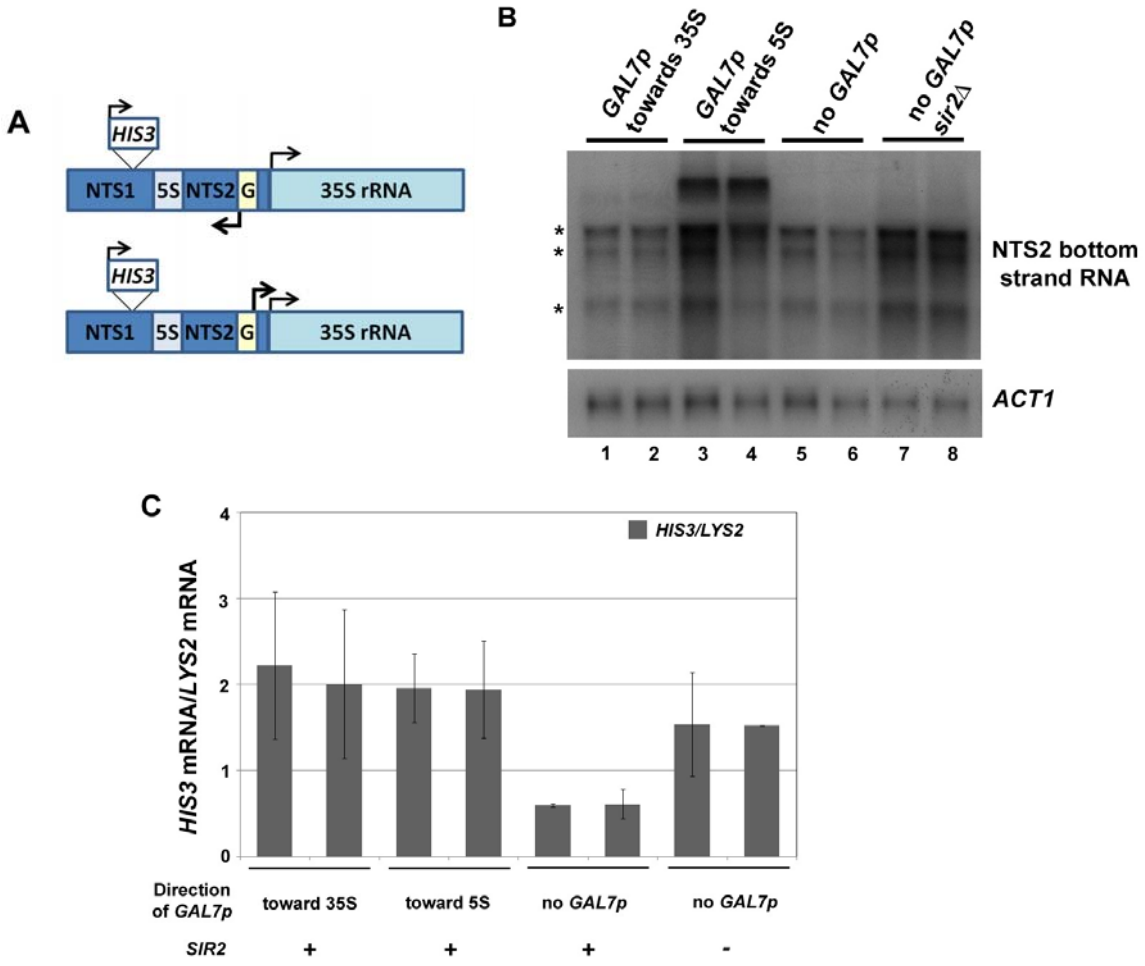

Figure 3-2. Overexpression of the NTS2 bottom strand transcripts *in cis* disrupts Pol II gene silencing at the rDNA locus. (A) Representation of the DNA fragments that were integrated into the rDNA array to overexpress the NTS2 bottom strand *in cis*. The top construct has a *GAL7* promoter inserted upstream of the NTS2 bottom strand transcription unit, with the direction in the same polarity as NTS2 bottom strand. The bottom construct has a *GAL7* promoter inserted at the same position, but it drives transcription towards the 35S rRNA gene. Both constructs contain a *HIS3* gene in the NTS1 sequences. (B) Northern blot analysis was performed to verify the overexpression of the NTS2 bottom strand transcripts *in cis*. Strains without a *GAL7* promoter inserted at the NTS2 (lane 5 and 6) were used as silent controls, and strains lacking both *GAL7* promoter and *SIR2* were used as silencing defect controls (lane 7 and 8). Labels, as in Figure 3-1. (C) Graphical representation of data from reverse transcription and quantitative real-time PCR (RT-qPCR) analyses to determine if overexpression of the NTS2 bottom strand transcripts alters transcriptional silencing of the *HIS3* gene inserted in NTS1. The signals from the *HIS3* gene were normalized to the signals from the *LYS2* gene (n=2).

First we tested if the *GAL7* promoter would drive transcription of the NTS2 bottom strand RNAs. Cells were grown in media containing 2% galactose, and the total RNA was isolated and analyzed by Northern analysis using a single-stranded probe to the NTS2 bottom strand RNA (Figure 3-2B, top panel). *ACT1* mRNA was used as a loading control (Figure 3-2B, bottom panel). As shown in Figure 3-2B, when the orientation of the *GAL7* promoter is towards 35S rRNA gene, the level of NTS2 RNA is similar to the level in the cells without *GAL7* promoter (Figure 3-2B, compare lane 1, 2 to lane 5, 6). However, when the *GAL7* promoter has the same polarity as NTS2 bottom strand sequence, we observed an average of 2-fold increase in the level of the NTS2 bottom strand RNA compared to the level from the strains without *GAL7* promoter (Figure 3-2B, compare lane 3, 4 to lane 5, 6). Significantly the levels of NTS2 bottom strand RNAs from the *GAL7* promoter- driven construct is similar to the levels observed in *sir2Δ* strains (Figure 3-2B, lane 7, 8). These data indicate that when placed in the correct orientation, the induced *GAL7* promoter can drive transcription of the NTS2 bottom strand RNAs.

Next, we examined if overexpression of NTS2 bottom strand RNAs *in cis* disrupts Pol II gene silencing at the rDNA locus. The level of mRNA from the *HIS3* gene in NTS1 was used as an indicator of Pol II gene silencing. Cells were grown in galactose-containing media, and the total RNAs were isolated. The level of mRNA from the *HIS3* gene originated from its own promoter was analyzed by reverse transcription using a *HIS3*-specific primer followed by quantitative real-time PCR analysis. As a normalization control, the level of mRNA from the *LYS2* gene was determined. The

quantity of *HIS3* mRNA obtained by RT-qPCR was normalized to the *LYS2* mRNA. As shown in Figure 3-2C, the level of normalized *HIS3* mRNA is low in cells lacking the *GAL7* promoter in NTS2 (Figure 3-2C, "no *GAL7p*/*SIR2*$^{+}$"). However, when transcription of the NTS2 bottom strand was driven by the *GAL7* promoter, there was a 3.1-fold increase in the level of the *HIS3* mRNA (Figure 3-2C, "toward 5S"). We also measured the *HIS3* mRNA level in cells lacking *SIR2* as a silencing-defect control and found that the *HIS3* mRNA level was increased about 2.5 fold (Figure 3-2C, "no *GAL7p*/*sir2Δ*"). These data suggest that overexpression of the NTS2 bottom strand RNA *in cis* disrupts Pol II gene silencing at the rDNA.

Next, we performed experiments to determine the effect of overexpression of NTS2 bottom strand transcripts *in cis* on mitotic recombination at the rDNA locus. Cells were grown in non-selective media containing galactose for 120 generations, and the rate of loss of the *HIS3* marker (integrated in the rDNA) was determined. In wild-type cells without a *GAL7* promoter, 5.7×10^{-4} to 6.14×10^{-4} His^{-} colonies were observed per generation (Table 3-3). When the *GAL7* promoter is placed in the direction towards the 35S rRNA gene, the rate of loss of the *HIS3* marker is very similar to the rate in wild-type cells. In contrast, when the *GAL7* promoter was used to drive the transcription of the NTS2 bottom strand RNAs, the rate of loss of *HIS3* gene increased 2.8 to 3.7-fold over the rate in wild-type cells. As a control, the rate of loss of the *HIS3* gene was also determined in the cells lacking *SIR2*. Consistent with the notion that Sir2 represses recombination at the rDNA locus, the rate of loss of *HIS3* in *sir2Δ* cells was 6.2 to 6.6-

Table 3-3 Mitotic stability of a *HIS3* marker when NTS2 region is overexpressed *in cis*

Strain	**Relevant genotype**	**Loss *HIS3*/generation[a], average (± SE; n)**	**Loss relative to no GAL7p strains[b]**
MBY2367	*GAL7p* towards 35S/*SIR2*$^+$	8.36 (± 0.72; 3)×10^{-4}	1.41
MBY2368	*GAL7p* towards 35S/*SIR2*$^+$	5.73 (± 1.32; 3)×10^{-4}	0.97
MBY2371	*GAL7p* towards 5S/*SIR2*$^+$	2.17 (± 0.21; 3)×10^{-3}	3.66
MBY2372	*GAL7p* towards 5S/*SIR2*$^+$	1.64 (± 0.19; 3)×10^{-3}	2.76
MBY2373	No *GAL7p* /*SIR2*$^+$	5.73 (± 0.98; 3)×10^{-4}	-
MBY2374	No *GAL7p* /*SIR2*$^+$	6.14 (± 0.49; 3)×10^{-4}	-
MBY2377	No *GAL7p* /*sir2Δ*	3.69 (± 0.12; 3)×10^{-3}	6.21
MBY2378	No *GAL7p* /*sir2Δ*	3.95 (± 0.39; 3)×10^{-3}	6.66

a. Determined after 120 generations of growth in YPAT medium containing 2% galactose.
b. The rate of loss of *HIS3* relative to no *GAL7p* strains was determined by comparing each rate of loss of *HIS3* to the average rate of loss from MBY2373 and MBY2374.

fold higher than the rate in wild-type cells. These data suggest that the act of transcription in the NTS2 region can promote mitotic recombination at the rDNA.

3.3.3. The NTS2 bottom strand RNA may encode proteins

Although my results indicate that the overexpression of the NTS2 bottom strand RNAs *in trans* has no effect on Pol II gene silencing and mitotic recombination at the rDNA locus, it is also possible that the NTS2 bottom strand RNAs may have functions that I have not tested for. One of the possible functions is to encode one or more proteins. The longest RNA transcribed from this region is about 1,700 nucleotides (Li *et al.*, 2006a). When we examined the sequence of this RNA, we identified three small potential open reading frames (ORFs). These hypothetical ORFs could encode for proteins of 71, 65 and 60 amino acids, respectively (Figure 3-3A). To determine if the ORFs are capable of encoding proteins, we prepared NTS2 bottom strand RNA by *in vitro* transcription, and then the *in vitro* transcribed RNA was used as a template for *in vitro* translation using a rabbit reticulocyte translation system in the presence of [^{35}S]-methionine. The reactions were resolved on a 15% SDS-PAGE gel. Representative results are shown in Figure 3-3B, where lane 1 contains a control translation reaction to make luciferase protein with a major product of 62 kDa. Interestingly, in the reaction using the NTS2 bottom strand RNA as the template, one band of a size less than 10 kDa was observed (Figure 3-3B, lane 2) and this band is not present in the control reaction without addition of any RNA templates (Figure 3-3B, lane3) or in the luciferase control reaction.

A

ORF1 (71 amino acids):
MQKCKIITQNINNQNQPFPHLFLCPLSTVPPNVKWPIGIHFLHPNYYKTTF
RLTFATLMVSILPPTFCPTY

ORF2 (65 amino acids):
MLIGLLLLDMYKQYSPPIFLQKKKKKHSGFVLFPPFPSLLRLILSSSSFSTP
SFSCFLFLPAFLH

ORF3 (60 amino acids):
MLLAKYHTKKLFTTETKTNGYHTLHYHHSNFTTILPSVSLFLPFSVTEIRF
RDPKGKSMP

B

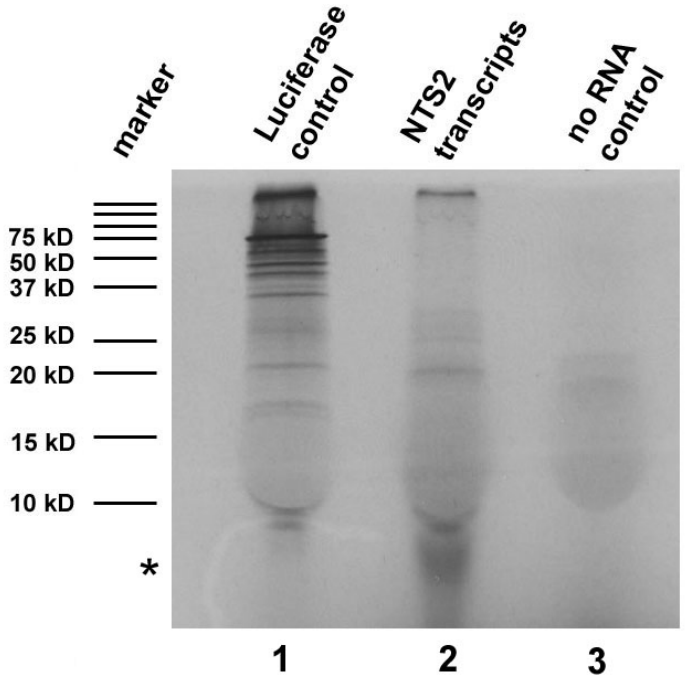

Figure 3-3. The NTS2 bottom strand RNA can be translated in vitro. (A) The amino acid sequences of 3 hypothetic ORFs from the 1.7 kb NTS2 bottom strand RNA. (B) Rabbit reticulocyte in vitro translation reactions were resolved on SDS-PAGE gel. A molecular weight marker is shown on the left. Lane 1, luciferase control; lane 2, the reaction using NTS2 transcripts as template; lane 3, no RNA control. Asterisk indicates the position of the specific band in the reaction containing NTS2 transcripts.

The results of the *in vitro* translation experiments suggest that the NTS2 bottom strand RNA may have the potential to code for a protein product, but it remains unclear if the RNA encodes proteins *in vivo*. To address this question, we used a technique called polysome profile analysis. In living cells with active protein synthesis, several ribosomes are attached to and translate a single mRNA molecule simultaneously, forming a protein synthesis unit called polysome. We reasoned that if the NTS2 bottom strand RNA encodes for proteins, it should be associated with the polysomes. To test this idea, we prepared cell extracts from wild-type and *sir2Δ* cells, separated the polysome fractions by sucrose-gradient ultracentrifugation, and the RNA from each fraction was purified and analyzed by Northern blotting. Fractions collected from lower percentage of sucrose contain free RNA and monosomes, while fractions collected from higher percentage of sucrose contain the polysomes. As shown in the top panel of Figure 3-4B, the NTS2 bottom strand RNAs from *sir2Δ* cells are present in the late fractions (lane 8-11), but not in the early fractions (lane 1, 2, 4-7), similar to the profile of the *ACT1* mRNA control (Figure 3-4B, bottom panel), indicating these RNAs are associated with polysomes. To our surprise, although the NTS2 bottom strand RNAs are much less abundant in wild-type cells compared to *sir2Δ* cells, they were also found to associate with the polysomes (Figure 3-4A). The data suggest that NTS2 bottom stand RNAs are associated with polysomes in wild-type and *sir2Δ* cells.

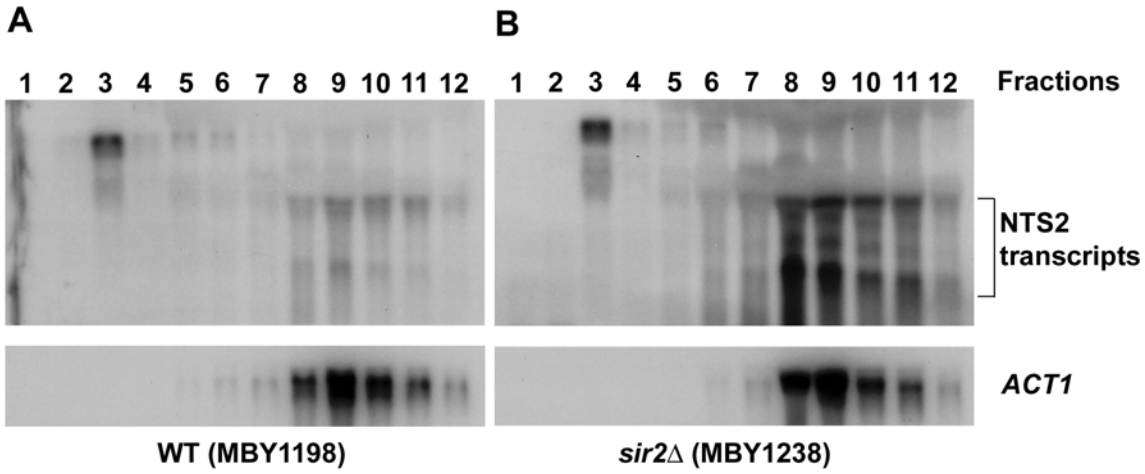

Figure 3-4. Northern blot analysis showing the distribution of the NTS2 bottom strand transcripts in polysomes from cell extracts from wild-type (A) and *sir2Δ* cells (B). Extracts were separated by sucrose gradient ultracentrifugation and total RNA was isolated from 12 fractions of the sucrose gradient. A ^{32}P-labeled strand-specific probe was used to detect the NTS2 bottom strand transcripts (top panel). Note: An uncharacterized band with a higher molecular weight than the NTS2 bottom strand RNAs is present in lane 3 of both (A) and (B).The fractionation of *ACT1* mRNA was used to identify the polysome-containing fractions (bottom panel).

3.4. DISCUSSION

The rDNA NTS region used to be known as a "nontranscribed spacer" region without any annotated genes. However, we and others recently identified Sir2-regulated endogenous Pol II transcription units from this region. In this work, the goals were to characterize the transcripts and to investigate the possible functions of the Sir2-regulated NTS2 bottom strand transcription in regulating the silent chromatin of rDNA locus. Our results showed that overexpression of the NTS2 bottom strand RNAs *in trans* did not interfere with Pol II gene silencing or mitotic recombination at the rDNA; while overexpression of the RNAs *in cis* resulted in higher levels of mitotic recombination at the rDNA locus as well as a defect in transcription silencing of a *HIS3* gene inserted within the rDNA array. The results suggest that it is the act of Pol II transcription in NTS2 disrupts the function of silent chromatin at the rDNA.

3.4.1. NTS2 bottom strand transcription and the silent chromatin at the rDNA locus

How does the transcription of NTS2 bottom strand RNA regulate genetic recombination at the rDNA locus? We know that Sir2 modulates the chromatin structure at the rDNA NTS region (Fritze *et al.*, 1997; Li *et al.*, 2006a). A simple explanation would be that the chromatin structure at the NTS region is more ready for genetic recombination in *sir2Δ* cells, but how? One possibility is that the RNAs transcribed from the NTS2 region may form RNA-DNA hybrids (R-loops) with the NTS sequence and contribute to opening the chromatin structure. This type of recombination is implicated to direct the positioning of class switch recombination during B cell maturation in

mammals (Reaban and Griffin, 1990; Yu *et al.*, 2003; Huang *et al.*, 2007). However, the overexpression of the NTS2 RNAs from a plasmid had no effect on recombination at the rDNA (Table 3-2), suggesting that the RNA itself does not have a role in regulating the silent chromatin at the rDNA. Instead, it could be the process of transcription that modulates the higher order structure of the chromatin at the rDNA, facilitating recombination events to occur. This is likely to be the answer, since placing a *GAL7* inducible promoter upstream of the NTS2 bottom strand transcriptional units at one of the rDNA repeats can disrupt Pol II gene silencing and increase the rate of loss of a marker gene located in rDNA NTS1 region, even in $SIR2^{+}$ cells (Table 3-3).

Transcription has been linked to genetic recombination by several other studies (for review, see Aguilera *et al.*, 2000). In yeast, Pol I transcription is required for the activity of a recombination hotspot called *HOT1* that is located in the rDNA NTS region (Voelkel-Meiman *et al.*, 1987; Lin and Keil, 1991; Huang and Keil, 1995) (also see Chapter I, section 3.4.1). Transcription by Pol I is believed to unwind double-stranded DNA thereby causing changes in DNA supercoiling or in the chromatin structure, which may increase the exposure of the DNA to the damaging agents. There is also evidence suggesting that Pol II transcription stimulates recombination (Dul and Drexler, 1988; Thomas and Rothstein, 1989; Nickoloff and Reynolds, 1990; Grimm *et al.*, 1991; Datta and Jinks-Robertson, 1995). One of the examples is intergenic transcription from the rDNA NTS1 region (Kobayashi and Ganley, 2005). The NTS1 region can be transcribed using a bidirectional promoter called “E-pro” (Santangelo *et al.*, 1988) in the absence of Sir2 or in cells with a reduced number of rDNA repeats (Ganley *et al.*, 2005; Kobayashi

and Ganley, 2005). Overexpression of the NTS1 RNAs using a *GAL1/10* bidirectional promoter resulted in the amplification of the rDNA repeat number, which is mediated by genetic recombination. High levels of transcription in NTS1 were found to be accompanied by dissociation of the cohesin subunits from the rDNA. Together these data contributed to the hypothesis that Pol II transcription at NTS1 region induces dissociation of the cohesin complex, increasing the chances of improper alignment of replicated sister chromatids and unequal sister-chromatid recombination, which causes change of the rDNA copy number (Kobayashi and Ganley, 2005). Our findings suggest that transcription in NTS2 has consequences that are similar to those observed in cells that have high levels of transcription in NTS1. When the NTS2 RNAs were overexpressed by placing a *GAL7* promoter upstream of the transcription start sites (TSS), we observed a hyper-recombination phenotype when transcription in NTS2 was induced. It is possible that transcription in the NTS2 region in *sir2Δ* cells also causes dissociation of the cohesin complex, which increases the opportunities for unequal-sister chromatid exchange recombination events.

We also observed a loss of silencing phenotype in cells overexpressing the NTS2 region *in cis* (Figure 3-2C). This is not due to the read through transcription by the *GAL7* promoter placed at NTS2, because the orientation of the *HIS3* marker gene is opposite to the *GAL7* promoter. The silencing defective phenotype is likely due to a more open chromatin structure caused by transcription in NTS2 which allows the access of Pol II transcription machinery. In support of this, we found elevated Pol II levels at the NTS region in cells lacking Sir2 compared to the levels in wild-type cells (Li *et al.*, 2006a).

3.4.2. The possible functions of the NTS2 bottom strand RNAs

Although our "overexpression *in trans*" experiment showed that the NTS2 bottom strand RNAs do not have a role in regulating rDNA silencing and recombination, the RNAs may still have other functions that have not been identified yet. One possibility is that they code for one or more proteins. The 1.7 kb NTS2 bottom strand RNA contains 3 hypothetical ORFs, with the potential of encoding proteins of 71, 65, or 60 amino acids. Although none of these hypothetical proteins has a significant match to known eukaryotic proteins from the non-redundant protein database, we found that NTS2 RNA could be translated into a small (<10 kDa) protein (or proteins) using an *in vitro* translation system (Figure 3-3B). The size of the *in vitro* translated product is close to the size of the predicted protein with 71 amino acids. The fact that the NTS2 bottom strand RNAs are associated with polysomes (Figure 3-4) supports the possibility that the NTS2 bottom strand RNAs are translated into proteins *in vivo*.

Alternatively, the RNAs may not be translated, but they may exert their functions by interacting with other proteins or protein complexes. An example for this is the Pol I transcribed non-coding RNAs from the rDNA spacer region of the rDNA in mouse cells, which is found to interact with NoRC, a chromatin remodeling complex that mediates silent chromatin formation and repression of a fraction of rDNA repeats (Mayer *et al.*, 2006). In our system, we did not observe a growth defect in *sir2Δ* cells or in the cells overexpressing the NTS2 bottom strand transcripts, indicating that these RNAs are not likely to repress Pol I transcription in *S. cerevisiae*. The fact that these RNAs associate with polysomes suggests that they either are translated into proteins or are involved in

the process of translation. In support of the later case, we found that the 1.7 kb RNA molecule is able to form stable a secondary structure with a free energy of -309.7 kcal/mol predicted by an RNA structure prediction method called RNAfold (Vienna RNA Secondary Structure Package, web server). More efforts are needed to test these possibilities.

3.4.3. The transcription of NTS2 region and rDNA replication

The transcription of NTS2 region may be linked to replication of the rDNA locus. It is known that each rDNA repeat contains an origin for DNA replication (ARS) (see Figure 1-5), but only 20% of the ARSs are active at a given time. One of the factors that has been shown to repress the rDNA ARS firing is Sir2 (Pasero *et al.*, 2002). Since the 5' ends of the NTS2 bottom strand RNAs map to a region near the ARS elements of the rDNA (Li *et al.*, 2006a; also see Chapter II), it is intriguing to speculate that the transcription of the NTS2 region may be involved in regulating the firing of ARS elements at the rDNA locus. In this scenario, in wild type cells, a low level of NTS2 transcription would corresponds to a low level of ARS firing; while in *sir2Δ* cells, high levels of transcription in the NTS2 region would be expected to increase ARS firing. This hypothesis could be tested by measuring the amount of nascent DNA using a single-copy marker gene located at the rDNA array in cells overexpressing NTS2 *in cis*.

In summary, we found that Sir2-regulated NTS2 bottom strand transcription is involved in regulating the silent chromatin at the rDNA locus. The data suggests that Sir2 contributes to maintaining genome stability by repressing endogenous transcription units at the rDNA "non-transcribed spacer" region. We are currently constructing a

strain with the TSS of the NTS2 transcripts deleted. We hope to eliminate the NTS2 transcription by deleting the TSS and then we will ask: 1) Does loss of NTS2 transcription affect rDNA amplification/recombination? 2) Does loss of NTS2 transcription affect rDNA replication? 3) Or does loss of NTS2 transcription disrupt Pol II gene silencing? These results of these experiments will help us better understand how silent chromatin is maintained and regulated in eukaryotic cells.

CHAPTER IV

LINKER HISTONE H1 REPRESSES RECOMBINATION AT THE RIBOSOMAL DNA LOCUS IN *Saccharomyces cerevisiae**

Several epigenetic phenomena occur at ribosomal DNA loci in eukaryotic cells, including the silencing of Pol I and Pol II transcribed genes, silencing of replication origins and repression of recombination. In *Saccharomyces cerevisiae*, studies focusing on the silencing of Pol II transcription and genetic recombination at the ribosomal DNA locus (rDNA) have provided insight into the mechanisms through which chromatin and chromatin-associated factors regulate gene expression and chromosome stability. The core histones, H2A, H2B, H3 and H4, the fundamental building blocks of chromatin, have been shown to regulate silent chromatin at the rDNA; however, the function of the linker histone H1 has not been well characterized. Here, we show that *S. cerevisiae* histone H1 represses recombination at the rDNA without affecting Pol II gene silencing. The most highly studied repressor of recombination at the rDNA is the Silent information regulator protein Sir2. We find that cells lacking histone H1 do not exhibit a premature-ageing phenotype nor do they accumulate the rDNA recombination intermediates and products that are found in cells lacking Sir2. These results suggest that histone H1 represses recombination at the rDNA by a mechanism that is independent of

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the recombination pathways regulated by Sir2.

4.1. INTRODUCTION

The fundamental unit of eukaryotic chromatin is the nucleosome, consisting of DNA wrapped around an octamer of histones containing two copies of each of the four core histones, H2A, H2B, H3 and H4. Neighbouring nucleosomes are separated by a segment of DNA that can interact with the linker histone allowing further compaction of chromatin (reviewed in Luger, 2006). In eukaryotic cells, the core histones H2A, H2B, H3 and H4 are highly conserved, while the linker histone, histone H1, is less well conserved (Wells and Brown, 1991). *Saccharomyces cerevisiae* histone H1, encoded by the *HHO1* gene, is unusual, having two globular domains instead of the one found in linker histones from other eukaryotes (Landsman, 1996; Ono *et al.*, 2003; Ali *et al.*, 2004). A number of observations are consistent with *HHO1* encoding a linker histone. First, there is a relatively high degree of sequence identity and/or structural similarity when comparing *S. cerevisiae* histone H1 to linker histones in higher eukaryotes (Landsman, 1996; Ushinsky *et al.*, 1997; Ali *et al.*, 2004). Second, studies have shown that histone H1 localizes to the nucleus, copurifies with core histones, and protects DNA located at the entry and exit point of a nucleosome from micrococcal nuclease cleavage (Ushinsky *et al.*, 1997; Patterton *et al.*, 1998; Freidkin and Katcoff, 2001). Consistent with each globular domain being functional, *S. cerevisiae* histone H1 has been shown to interact simultaneously with two four-way junctions that may have a structure similar to the point on the nucleosome where DNA enters and exits (Schafer *et al.*, 2005). This

result supports a model where a single histone H1 protein can bridge two nucleosomes and facilitate the compaction of chromatin. These properties of the *S. cerevisiae* H1 mirror those of linker histones from higher eukaryotes (reviewed in Bustin *et al.*, 2005), supporting its role as a bona fide linker histone.

The *in vivo* functions of linker histones in higher eukaryotes have been difficult to assess owing to the large number of linker histone variants. Early studies showed that deletion of any one or two of the eight known genes encoding H1 variants in mice causes no obvious mutant phenotypes (Fan *et al.*, 2001). Although, age-dependent differences in silencing of a human b-globin transgene have been observed in mice lacking a single H1 gene (Fan *et al.*, 2001; Alami *et al.*, 2003). More extensive mutant analyses have demonstrated an essential function for linker histones in mammals. Mice lacking three specific H1 variants have an embryonic lethal phenotype, with total histone H1 levels reduced to ~50% of normal, severe developmental defects, altered chromatin structure, andeither increased or decreased expression of a small number of genes (Fan *et al.*, 2003; Fan *et al.*, 2005).

In contrast to mammalian cells, in several unicellular eukaryotes, including *S. cerevisiae*, *A. nidulans* and *T. thermophila*, histone H1 is not essential for viability (Shen and Gorovsky, 1996; Escher and Schaffner, 1997; Ushinsky *et al.*, 1997; Ramon *et al.*, 2000). Although histone H1 has been shown to associate with chromatin in *S. cerevisiae*, cells lacking or overexpressing *HHO1* exhibit wild-type growth characteristics (Escher and Schaffner, 1997; Ushinsky *et al.*, 1997; Patterton *et al.*, 1998; Freidkin and Katcoff, 2001; Downs *et al.*, 2003; Veron *et al.*, 2006). Microarray analyses have revealed that

expression of very few genes is altered in cells lacking *HHO1* (Freidkin and Katcoff, 2001; Hellauer *et al.*, 2001). Moreover, defects in gene silencing at the silent mating-type loci or telomeres, phenotypes often associated with mutations that alter chromatin function, have not been observed in cells lacking histone H1 (Escher and Schaffner, 1997; Patterton *et al.*, 1998; Veron *et al.*, 2006). Evidence supporting a role for *S. cerevisiae* histone H1 in chromatin function has been provided by two reports. First, overexpression studies have indicated that histone H1 regulates chromatin barriers located between euchromatin and heterochromatin-like domains (Veron *et al.*, 2006). Second, *S. cerevisiae* cells that lack histone H1 exhibit increased tolerance to methylmethane-sulphonate and other related phenotypes, supporting a role for histone H1 in genetic recombination and/or DNA repair (Downs *et al.*, 2003).

RNA polymerase II (Pol II) transcription and genetic recombination are repressed at the ribosomal DNA locus in *S. cerevisiae* (reviewed in Moazed, 2001; Rusche *et al.*, 2003). The maintenance of silent chromatin at the rDNA requires several types of factors with known roles in chromatin function. Sir2, an NAD-dependent histone deacetylase, and Ubp10, a histone H2B deubiquitylating enzyme, are required for the repression of Pol II transcription and recombination at the rDNA (Gottlieb and Esposito, 1989; Bryk *et al.*, 1997; Smith *et al.*, 1998b; Straight *et al.*, 1999; Garcia and Pillus, 2002; Kobayashi *et al.*, 2004; Emre *et al.*, 2005; Gardner *et al.*, 2005; Calzari *et al.*, 2006; Cubizolles *et al.*, 2006). Sir2 has been shown to associate with several regions of the rDNA and deacetylate histones H3 and H4 throughout the rDNA (Bryk *et al.*, 2002; Buck *et al.*, 2002; Huang and Moazed, 2003; Li *et al.*, 2006a). A second class of

rDNA silencing factors, including Set1, a histone H3 lysine 4 methyltransferase, silences Pol II gene transcription but not recombination at the rDNA (Briggs *et al.*, 2001; Bryk *et al.*, 2002; Krogan *et al.*, 2002; Mueller *et al.*, 2006). Members of a third class of rDNA silencing factors including Sgs1 and Hpr1, repress recombination at the rDNA without affecting Pol II gene silencing (Sinclair and Guarente, 1997; Bryk *et al.*, 2001; McVey *et al.*, 2001; Merker and Klein, 2002).

Considering the role of histone H1 in compaction of chromatin and its function at barrier elements, we asked if histone H1 regulates silent chromatin at the rDNA. Our data show that histone H1 behaves similarly to Sgs1 and Hpr1, repressing recombination at the rDNA while having no effect on Pol II gene silencing. The results of genetic and molecular experiments suggest that histone H1 represses recombination at the rDNA through a mechanism that is largely independent of Sir2. For example, in *hho1*Δ cells, extrachromosomal rDNA circles (ERCs), a product of the Sir2 regulated recombination pathway, do not accumulate (reviewed in Rothstein and Gangloff, 1999; Kobayashi, 2006). Based on our findings, we propose that histone H1 acts independently of Sir2 to prevent recombination events at the rDNA that if allowed to occur would contribute to genomic instability.

4.2. MATERIALS AND METHODS

4.2.1. Media and yeast strains

Yeast media were prepared according to Rose *et al*. (1990). YPADT consists of YPD media supplemented with 20 mg/L L-tryptophan and 20 mg/L adenine sulphate.

Saccharomyces cerevisiae strains used in this study are shown in Table 4-1. MBY1198, MBY1308, MBY1311, MBY1314 and MBY1317 have been described previously (Bryk *et al.*, 2002). BY4742, BY4742 *hho1*Δ*::kan* ^{r} and BY4742 *sir2*Δ*::kan* ^{r} were purchased from Open Biosystems, Huntsville, AL. The *HHO1* gene was deleted and replaced with the *LEU2* gene from pRS405 or *KANMX4* from pRS400 (Christianson *et al.*, 1992; Brachmann *et al.*, 1998) by PCR-mediated gene disruption (Baudin *et al.*, 1993). The *sir2*Δ *hho1*Δ double mutant was made by a genetic cross. The *his3AI* gene was replaced with the *HIS3* gene by transformation of yeast cells with a 0.83 kb *Cla*I fragment from BJC38 (Curcio and Garfinkel, 1992). His^{+} transformants were verified by PCR amplification of genomic DNA. Histone H1 was tagged at the C-terminus with the myc epitope using the PCR-mediated tagging vector, pMPY-3×MYC (Schneider *et al.*, 1995). We determined that the tagged *HHO1-myc* allele behaved similarly to a wild-type *HHO1* allele by measuring the rate of marker loss from the rDNA in wild-type (MBY1445, 6.3×10^{-4}) and the *HHO1-myc* cells (MBY2191, 7.9×10^{-4}). All strains containing gene deletions or insertions were checked by restriction digest of PCR amplified genomic DNA and genetic crosses to ensure Mendelian segregation of markers.

Table 4-1 Strains and plasmids

Strain	Genotype
MBY21	*MATα ura3-167 his3Δ200 GAL*$^+$
MBY221	*MATα ura3-167 his3Δ200* GAL$^+$ Ty1*his3AI*-234 *leu2::hisG*
MBY228	*MATα ura3-167 his3Δ200 GAL*$^+$ *Ty1his3AI*-242 *leu2::hisG*
MBY320	*MATα ura3-167 his3Δ200 GAL*$^+$ *Ty1his3AI*-272 *leu2::hisG*
MBY1198	*MATα ade2Δ::his*G *his3Δ200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ0* Ty1*his3AI*-236 Ty1*ade2AI*-515
MBY1238	MBY1198 *sir2*Δ*::KANMX4*
MBY1308	*MATα his3Δ200 leu2Δ1 ura3-167 trp1Δ63 RDN1::mURA3-LEU2*
MBY1311	MBY1308 *set1Δ::TRP1*
MBY1314	*MATα his3Δ200 ura3-167 trp1Δ63 leu2Δ1::mURA3-LEU2*
MBY1317	MBY1314 *set1Δ::TRP1*
MBY1445	*MATα ade2Δ::his*G *his3Δ200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ0* Ty1*HIS3*-236 Ty1*ade2AI*-515
MBY1447	*MATα ura3-167* Ty1*HIS3*-242
MBY1653	*MATα his3*Δ*200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ0* Ty1his3AI-236 Ty1$pTEF1_b$ *kanAI-2910*
MBY1961	MBY1653 *hho1*Δ*8::LEU2*
MBY1962	MBY1653 *hho1*Δ*18::LEU2*
MBY2038	*MATa his3Δ200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ0* Ty1*HIS3*-236 Ty1$pTEF1_b$ *kanAI-2910*
MBY2039	MBY2038 *hho1Δ8::LEU2*
MBY2040	MBY2038 *hho1Δ18::LEU2*
MBY2041	MBY2038 *hho1Δ19::LEU2*
MBY2074	MBY1653 *sir2Δ::LEU2*
MBY2141	*MAT his3Δ200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ0 hho1Δ8::LEU2 sir2Δ::KANMX4* Ty1*HIS3-236*
MBY2144	MBY2038 *hho1Δ::LEU2 fob1Δ::KANMX4*
MBY2146	MBY2038 *fob1Δ::KANMX4*
MBY2156	MBY1653 *set1Δ*::*TRP1*
MBY2191	*MBY1198 HHO1-C-myc3*
MBY2238	*MATα his3Δ200 ura3Δ0 trp1Δ63* Ty1*HIS3*-242 *hho1Δ::LEU2* Ty1$pTEF1_b$ *kanAI-2910*
MBY2239	*MATa his3Δ200 ura3-167* Ty1*HIS3*-242 *hho1Δ::LEU2* Ty1$pTEF1_b$ *kanAI-2910*
MBY2290	MBY1198 *HHO1-C-myc3 sir2Δ*::*KANMX4*
MBY2296	MBY1445 *sir2Δ::LEU2*
MBY2343	MBY221 *hho1Δ::LEU2*
MBY2345	MBY228 *hho1Δ::LEU2*
MBY2347	MBY320 *hho1Δ::LEU2*
MBY2353	MBY1308 *hho1Δ::KANMX4*
MBY2354	MBY1314 *hho1Δ::KANMX4*
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*
13738	BY4742 *sir2Δ::kan*^{r}
12125	BY4742 *hho1Δ::kan*^{r}

4.2.2. Northern blot analysis

Total RNA was prepared according to Bryk *et al*. (1997), with blotting and hybridization performed as described (Swanson *et al.*, 1991). Ty1*his3AI*, total Ty1 and *PYK1* transcripts were detected with strand specific ^{32}P-labelled riboprobes (Curcio and Garfinkel, 1992). Northern blots were quantified on a Molecular Dynamics (Sunnyvale, CA) Storm 860 phosphorimager using ImageQuant software.

4.2.3. Plate assay for expression of the *LEU2* and *mURA3*

Wild-type, *hho1Δ* and *set1Δ* cultures were grown to saturation. Ten-fold serial dilutions of each culture were made in sterile water and 5 ml of each dilution was spotted onto SC, SC-Leu and SC + 5-FOA agar. Plates were photographed after 3 days of incubation at 30°C.

4.2.4. Mitotic stability of Ty1*HIS3* elements

Mitotic stability was measured according to (Bryk *et al.*, 1997) with minor modifications. His^+ strains were grown overnight at 30°C in SC-His medium and counted prior to inoculation of 10 ml of YPADT media with equal numbers of cells. Cells of the His^- auxotroph, MBY1653 with Ty1*his3AI*-236 (pregrown in YPADT) and the Ty1*HIS3*-236 derivative MBY2038 were mixed in 10 ml of YPADT to analyse the fraction of His^- cells before and after 120 generations of growth. Two-tailed Student's *t*-tests were performed to determine the significance of the data.

4.2.5. Analysis of extrachromosomal rDNA circles

DNA was prepared according to Wu and Gilbert (1995) from logarithmically growing cells (2×10^7cells ml^{-1}). Two-dimensional gel electrophoresis in the presence of

chloroquine was performed according to Sinclair and Guarente (1997) and Calzari et al. (2006). After electrophoresis, DNA was transferred to nylon membrane and blots were hybridized to a 5.4 kb probe (to the rDNA NTS and part of the 35S rRNA gene) labelled with ^{32}P-dATP by random priming (Ausubel and K., 1988). ERCs and ribosomal DNA were visualized and quantified using a Molecular Dynamics (Sunnyvale, CA) Storm 860 phosphorimager with ImageQuant software.

4.2.6. Lifespan analysis

Lifespan analyses were preformed as described previously (Kennedy *et al.*, 1994). For statistical analysis, lifespan data from *hho1Δ* and *sir2Δ* cells were compared with data from wild-type cells using the Mann-Whitney U-test.

4.2.7. Analysis of Holliday junction intermediates

DNA was prepared from logarithmically growing cultures as described (Wu and Gilbert, 1995). DNA was cleaved with BglII producing a 4577 bp fragment containing the NTS region of the rDNA with the RFB in the middle. Cleaved DNA from wild-type, *hho1Δ* and *sir2Δ* cells was separated at 1.25 V cm^{-1} for 24 h through 0.4% (w/v) agarose gels in 1×Tris-Borate-EDTA (TBE) buffer (pH 8.0) (Ausubel and K., 1988). Following electrophoresis, gels were equilibrated in 1×TBE buffer containing 5 μg ml^{-1} ethidium bromide. Individual lanes were excised from the gel and sealed with molten agarose in 0.9% (w/v) agarose gels containing 5 μg ml^{-1} ethidium bromide. DNA molecules were separated through the second dimension in 1×TBE buffer containing 5 μg ml^{-1} ethidium bromide at 4 V cm^{-1} for 18 h. Separated DNA was transferred to a nylon membrane and hybridized to a ^{32}P-labelled 4.2 kb rDNA probe. Holliday junction intermediates, Y-

shaped replication forks and linear ribosomal DNA were visualized and quantified as described for the ERC gels.

4.2.8. Chromatin immunoprecipitation analysis

ChIPs were performed as described (Li *et al.*, 2006a). Antisera used were: 20 µl of α-Sir2 (sc-6666, Santa Cruz Biotechnology, CA); 10 µl of α-K9-K14-acetylated histone H3 (06-599, Upstate Biotechnology, NY); or 10 µl of α-myc (sc-789, Santa Cruz Biotechnology, CA). Quantitative real-time PCR analysis was performed according to Li *et al*. (2006). The per cent immunoprecipitation (%IP) was calculated by dividing the signal from immunoprecipitated chromatin by the signal from input DNA. For analysis of the Sir2 ChIPs (Figure 4-3A), the value of %IP from *sir2Δ* cells was subtracted to correct for background. For analysis of the α-K9-K14-acetylated histone H3 ChIP experiments (Figure 4-3B), the value of %IP from no-antibody samples was subtracted to correct for background. Slot blot analyses were performed as described (Mueller *et al*., 2006). NTS sequences were detected by hybridization with a ^{32}P-labelled probe to the rDNA NTS. Blots were quantified on a Molecular Dynamics (Sunnyvale, CA) Storm 860 phosphorimager. Primer sequences are available upon request.

4.2.9. Statistical analysis

Student *t*-tests were performed using a two-tailed analysis.

4.3. RESULTS

The ribosomal DNA locus on chromosome XII in *S. cerevisiae* contains ~150-200 tandem direct repeats of the ribosomal rRNA genes. The rDNA is located in the

nucleolus, a substructure near the periphery of the nucleus, where transcription and processing of the ribosomal RNA (rRNA) occurs (Warner, 1990). Each 9.1 kb rDNA repeat contains a 35S rRNA gene that is transcribed by RNA polymerase I, and a 5S rRNA gene that is transcribed by RNA polymerase III, which is located in the middle of the non-transcribed spacer (NTS), splitting it into NTS1 and NTS2 (Figure 4-1A). Considering the high levels of Pol I and Pol III transcription that occur at the rDNA, it is somewhat contradictory that this locus exhibits characteristics of silent chromatin. However, silencing at the rDNA maintains genome stability and cell longevity through the repression of Pol II transcription and homologous recombination.

4.3.1. Histone H1 does not regulate transcriptional silencing at the rDNA

Previous work has shown that histone H1 does not regulate transcriptional silencing at the silent mating-type loci and telomeres in *S. cerevisiae* (Escher and Schaffner, 1997; Patterton *et al.*, 1998; Veron *et al.*, 2006). However, a function for histone H1 at the rDNA has been suggested by the results of chromatin immunoprecipitation (ChIP) experiments showing that H1 associates with the rDNA (Freidkin and Katcoff, 2001; Downs *et al.*, 2003). To determine if histone H1 is required for the silencing of Pol II genes at the rDNA, we analysed RNA from wild-type and *hho1*Δ cells that each contain a single copy of a genetically marked Ty1 element, Ty1*his3AI*, located in NTS1 (-236) or NTS2 (-272 or -234) of a single rDNA repeat (Figure 4-1). Ty1 elements are transcribed by Pol II and the level of mRNA from a Ty1*his3AI* element integrated at the rDNA is a sensitive indicator of Pol II gene silencing (Bryk *et al.*, 1997). An RNA probe complementary to the *his3* portion of

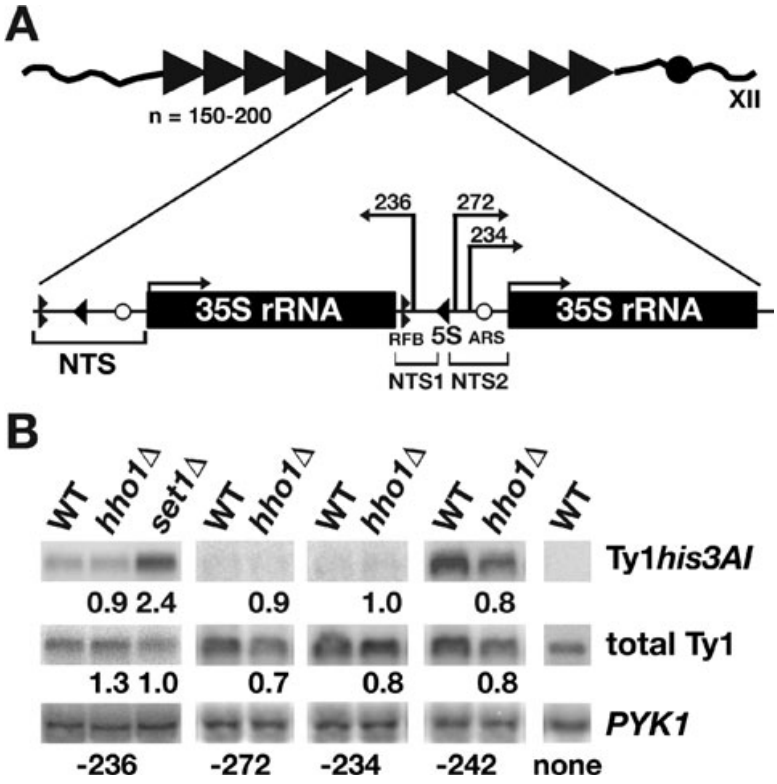

Figure 4-1. Cells lacking histone H1 do not exhibit transcriptional silencing defect. (A) The ribosomal DNA locus on chromosome XII is comprised of ~150-200 copies of the rDNA repeat. Two rDNA repeats are shown in the enlargement with each containing an intergenic spacer (NTS) and the Pol I transcribed 35S rRNA gene (35S rRNA). The NTS is divided into NTS1 and NTS2 by the Pol III transcribed 5S rRNA gene (5S). Stacked triangles, replication fork barrier (RFB); open circle, origin of replication (ARS); bent arrows with numbers, location of the Ty1*his3AI* elements. B. Northern blot analysis of total RNA isolated from wild-type, *hho1Δ* and *set1Δ* cells. Strand-specific probes were used to measure the steady-state mRNA levels of Ty1*his3AI* (top panel), total Ty1 (middle panel) or *PYK1* (bottom panel). The average ratio of Ty1*his3AI*/*PYK1* mRNA and total Ty1/*PYK1* mRNA for each strain analysed after normalization to the wild-type strain is shown below the top and middle panel respectively. The normalized values of the average ratio ± standard error for Ty1*his3AI*-236/*PYK1* mRNA were: *hho1Δ*:WT, 0.9 ± 0.1, *n* = 16; *set1Δ*:WT, 2.4 ± 0.2, *n* = 5; and for total Ty1/*PYK1* mRNA were: *hho1Δ*:WT, 1.3 ± 0.1, *n* = 14; *set1Δ*:WT, 1.0 ± 0.2, *n* = 3. Average *hho1Δ*:WT for Ty1*his3AI*-272/*PYK1* mRNA was 0.9 ± 0.04, *n* = 4; and for total Ty1/*PYK1* mRNA was 0.7 ± 0.1, *n* = 4. Average *hho1Δ*:WT for Ty1*his3AI*-234/*PYK1* mRNA was 1.0 ± 0.1, *n* = 4; and for total Ty1/*PYK1* mRNA was 0.8 ± 0.1, *n* = 4. Average *hho1Δ*:WT for Ty1*his3AI*-242/*PYK1* mRNA was 0.8 ± 0.1, *n* = 4; and for total Ty1/*PYK1* mRNA was 0.8 ± 0.1, *n* = 4. Total RNA from wild-type cells lacking the Ty1*his3AI* element (none) was analysed to provide a measure of non-specific binding of the radiolabelled probe.

Ty1*his3AI* was used to detect Ty1*his3AI* mRNA (Figure 4-1B, Ty1*his3AI*). As a control for loading, we measured the level of *PYK1* transcript (*PYK1*). In the leftmost blot of Figure 4-1B, analysis of the ratio of Ty1*his3AI*-236:*PYK1* mRNA showed that the level of Ty1*his3AI*-236 mRNA in the *hho1Δ* cells was similar to the level in wild-type cells, indicating that Pol II gene silencing at the rDNA does not require histone H1. As a positive control, we measured Ty1*his3AI*-236 mRNA in cells lacking the histone methyltransferase Set1 that is required for transcriptional silencing at the rDNA and telomeres (Briggs *et al.*, 2001; Bryk *et al.*, 2002; Krogan *et al.*, 2002; Mueller *et al.*, 2006). Consistent with the requirement for Set1 in rDNA silencing, we observed a significant increase in Ty1*his3AI*-236 mRNA in *set1Δ* cells (Figure 4-1B). We analysed pairs of wild-type and *hho1Δ* cells with Ty1*his3AI* elements in NTS2 and found that silencing of these Ty1*his3AI* elements was not affected in cells lacking histone H1 (Figure 4-1B, -272 and -234 lanes).

Most *S. cerevisiae* strains contain about 30 Ty1 elements at different locations throughout the genome. Although expression of the Ty1*his3AI* element in the rDNA was not affected by deletion of *HHO1*, we checked the level of RNA from the genomic Ty1 elements using a probe that hybridizes to all Ty1 mRNA. We did not detect a significant difference in the level of total Ty1 mRNA in wild-type and *hho1Δ* cells (Figure 4-1B, total Ty1). Likewise, we observed similar levels of Ty1*his3AI* mRNA from the Ty1*his3AI*-242 element located outside the rDNA on chromosome XII in wild-type and *hho1Δ* cells (Figure 4-1B, -242 lanes). In summary, our results indicate that histone H1 is not required for the silencing of Pol II-transcribed genes integrated in the rDNA NTS.

To determine if histone H1 is required for silencing of Pol II genes inserted in the 35S rRNA gene, we used a plate growth assay to assess silencing of a gene cassette containing a modified *URA3* gene, *mURA3* and the *LEU2* gene in one of the 35S rRNA genes in the rDNA array. Cells with the *mURA3-LEU2* cassette inserted at the *leu2Δ1* locus were also analysed (Smith and Boeke, 1997). Cultures of wild-type, *hho1Δ* and *set1Δ* cells containing the *mURA3-LEU2* cassette were spotted on synthetic complete media (SC), SC media lacking leucine (SC-Leu) or SC media containing 5-fluoroorotic acid (SC + 5-FOA) (Figure 4- 2). Wild-type and *hho1Δ* cells containing *mURA3-LEU2* at the rDNA grew less well on SC-Leu than SC media, indicating that expression of the *LEU2* gene is silenced by rDNA chromatin (Figure 4-2, middle panel). In contrast, *set1Δ* cells with *mURA3-LEU2* at the rDNA grew considerably better than wild-type cells on SC-Leu media owing to the loss of silencing at the rDNA. Wild-type, *hho1Δ* and *set1Δ* cells containing the *mURA3-LEU2* cassette at the euchromatic *leu2Δ1* locus grew well on SC and SC-Leu media, indicating that the *LEU2* gene was expressed at similar levels in these cells (Figure 4-2, top and middle panel).

Silencing of *mURA3* was measured by examining growth of cells on media containing 5-FOA. 5-FOA is converted to the toxic analogue 5-fluorouracil by the *URA3* gene product (Boeke *et al.*, 1987). Wild-type and *hho1Δ* cells with *mURA3-LEU2* at the rDNA exhibited robust growth on 5-FOA because *mURA3* was silenced (Figure 4-2, bottom panel). However, *set1Δ* cells grew poorly on 5-FOA owing to loss of silencing of the *mURA3* gene (Figure 4-2, bottom panel). When the *mURA3-LEU2* cassette was located at *leu2Δ1*, growth of wild-type, *hho1Δ* and *set1Δ* cells was severely limited on

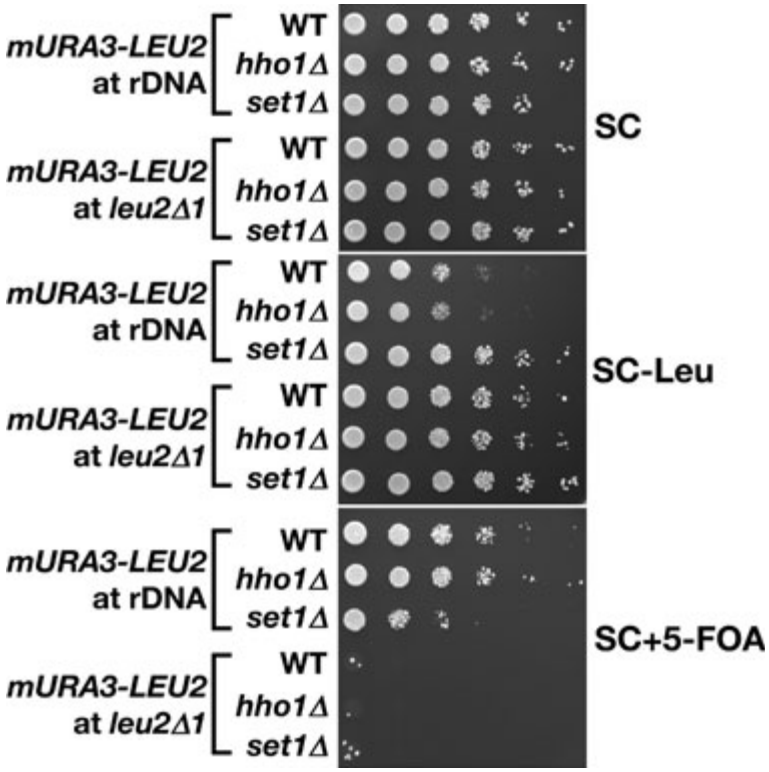

Figure 4-2. Histone H1 is not required for silencing of the *mURA3-LEU2* cassette inserted in the 35S rRNA gene. Cultures of wild-type, *hho1Δ* and *set1Δ* cells containing the *mURA3-LEU2* cassette at the rDNA or *leu2Δ1* locus were grown to saturation, serially diluted and spotted onto SC, SC-Leu and SC+5-FOA agar to evaluate Pol II gene silencing. Cells were plated on SC media to insure that similar numbers of wild-type and mutant cells were used in the assay. The results of a representative plate assay are shown ($n = 3$).

5-FOA media, owing to robust expression of the *mURA3* gene at *leu2Δ1* (Figure 4-2, bottom panel). Together, data from Northern studies and growth assays indicate that histone H1 is not required for transcriptional silencing of Pol II genes in several regions of the rDNA repeat.

4.3.2. Histone H1 represses mitotic recombination at the rDNA

In addition to silencing of Pol II-transcribed genes, silent chromatin at the rDNA represses homologous recombination between the rDNA repeats. To determine if histone H1 regulates mitotic recombination at the rDNA, we measured the rate of loss of a *HIS3* gene from the rDNA and another region of chromosome XII located outside of the rDNA array (Bryk *et al.*, 1997). Strains containing Ty1*HIS3* are histidine prototrophs with the ability to grow on media lacking histidine. Loss of the Ty1*HIS3* element from the rDNA can be caused by recombination between rDNA repeats, recombination between the Ty1 long-terminal repeats or by ectopic recombination with an unmarked Ty1 element. Cells that lose the *HIS3* marker are no longer able to grow on media lacking histidine. We measured the rate of loss of the *HIS3* marker in wild-type cells with a single copy of Ty1*HIS3* in the rDNA after 120 generations of growth in non-selective media and observed 4.60×10^{-4} His^- colonies per generation (Table 4-2). This rate of loss of the *HIS3* marker was similar to that measured in cells containing a Ty1*HIS3* element at another locus on chromosome XII outside of the rDNA (4.05×10^{-4}). To determine if the stability of the Ty1*HIS3* element was altered in cells lacking histone H1, we measured the rate of loss of Ty1*HIS3* in *hho1Δ* cells. The rate of loss of the *HIS3* marker from the rDNA was increased 3.4-fold in the *hho1Δ* cells compared with

Table 4-2 Mitotic stability of Ty1*HIS3* elements

Strain (location[a])	Relevant genotype	Loss *HIS3*/gen[b], average (± SE; n)	Loss relative to wild type
MBY2038 (in rDNA)	wild type	$4.60 (\pm 0.55; 6)\times10^{-4}$	-
MBY2039[c] (in rDNA)	*hho1Δ*	$1.58 (\pm 0.20; 12^{d})\times10^{-3}$	3.4
MBY2296 (in rDNA)	*sir2Δ*	$4.19 (\pm 0.36; 3)\times10^{-3}$	9.1
MBY2141 (in rDNA)	*hho1Δsir2Δ*	$5.80 (\pm 0.75; 2^{e})\times10^{-3}$	12.6
MBY2146 (in rDNA)	*fob1Δ*	$2.07 (\pm 0.35; 3)\times10^{-4}$	0.45
MBY2144 (in rDNA)	*hho1Δfob1Δ*	$5.62 (\pm 0.55; 3)\times10^{-4}$	1.2
MBY1447 (chr XII)	wild type	$4.05 (\pm 3.06; 2^{e})\times10^{-4}$	-
MBY2238[f] (chr XII)	*hhot1Δ*	$3.33 (\pm 1.28; 3)\times10^{-4}$	0.82

a. Location of Ty1*HIS3*: in rDNA, in NTS1 of a single rDNA repeat at position 460482; chr XII, in *YLR460C* at position 1060536.

b. Determined after 120 generations (gen) of growth in non-selective YPADT broth. Mixed cultures of isogenic His^- and His^+ cells were analysed to verify that His+ cells did not have a growth advantage. The mixed cultures contained a fraction of 787/1987 (0.467) His^- cells prior to growth in YPADT broth and 541/1223 (0.442) His^- cells after 120 generations of growth in YPADT.

c. Includes data from two additional *hho1*Δ strains, MBY2040 and MBY2041.

d. Includes data from three isogenic *hho1*Δ isolates.

e. Average (± range) determined from two independent experiments.

f. Includes data from a second *hho1*Δ strain, MBY2239.

wild-type cells ($P = 0.0016$). In contrast, the rate of loss of *HIS3* from the locus outside of the rDNA was decreased slightly in *hho1Δ* cells (Table 4-2). These results indicate that histone H1 represses recombination at the rDNA, but not at the locus outside of the rDNA on chromosome XII.

The histone deacetylase Sir2 has been shown to repress recombination at the rDNA (Gottlieb and Esposito, 1989; Bryk *et al.*, 1997; Kobayashi *et al.*, 2004; Kobayashi and Ganley, 2005). We tested if histone H1 and Sir2 act in the same pathway to regulate recombination at the rDNA by comparing the rate of loss of the *HIS3* marker from single mutants (*sir2Δ* or *hho1Δ*) and a double mutant (*hho1Δ sir2Δ*). We found that the rate of loss of Ty1*HIS3* element from the rDNA in *sir2Δ* cells was 4.19×10^{-3}, which was 9.1-fold higher than the rate in wild-type cells ($P = 0.0005$), and the rate of loss in the *hho1*Δ *sir2*Δ double mutant was 12.6-fold higher ($P = 0.0025$) (Table 4-2). Loss of Ty1*HIS3* from the rDNA in the *hho1Δ sir2Δ* double mutant was approximately equal to the sum of the rates observed for the two single mutants, suggesting that Sir2 and histone H1 act independently to regulate recombination at the rDNA.

We also addressed possible genetic interactions between histone H1 and Fob1, the rDNA replication fork barrier protein that has been shown to recruit the Sir2-containing RENT complex to NTS1 (Huang and Moazed, 2003). Consistent with previous work (Kobayashi and Horiuchi, 1996), the rate of marker loss from the rDNA was lower in *fob1Δ* cells than in wild-type cells (Table 4-2). In *hho1Δ fob1Δ* double mutants recombination rates were restored to wild-type levels, suggesting that histone H1 and Fob1 function antagonistically to regulate recombination at the rDNA.

4.3.3. Histone H1 associates with the NTS and the 35S regions of the rDNA locus

The results of experiments examining recombination (Table 4-2) suggest that histone H1 and Sir2 function in independent pathways to regulate the mitotic stability of the rDNA array. To determine if histone H1 is required for the function of Sir2 at the rDNA NTS and if Sir2 affects the association of histone H1 with the rDNA, we performed a series of ChIP experiments. First, we examined the association of Sir2 with the rDNA in cells lacking histone H1. ChIPs were performed on wild-type, *hho1Δ* and *sir2Δ* cells using antisera specific for Sir2, and a slot blot containing the immunoprecipitated and input DNA was analysed after hybridization with a radiolabelled probe to the rDNA NTS. The analysis showed that the association of Sir2 with the rDNA NTS is similar in wild-type and *hho1Δ* cells (Figure 4-3A). To assess the function of Sir2 at the rDNA in these cells, we measured the level of acetylated histone H3 at the rDNA NTS. The results showed that the level of K9-K14 acetylated histone H3 at the rDNA NTS is lower in wild-type cells than in *sir2Δ* cells (Figure 4-3B), reflecting the histone deacetylase activity of Sir2. We found that the level of K9-K14-acetylated H3 at the rDNA NTS in *hho1Δ* cells was similar to the level in wild-type cells, suggesting that histone H1 does not affect the histone deacetylase function of Sir2 at the rDNA.

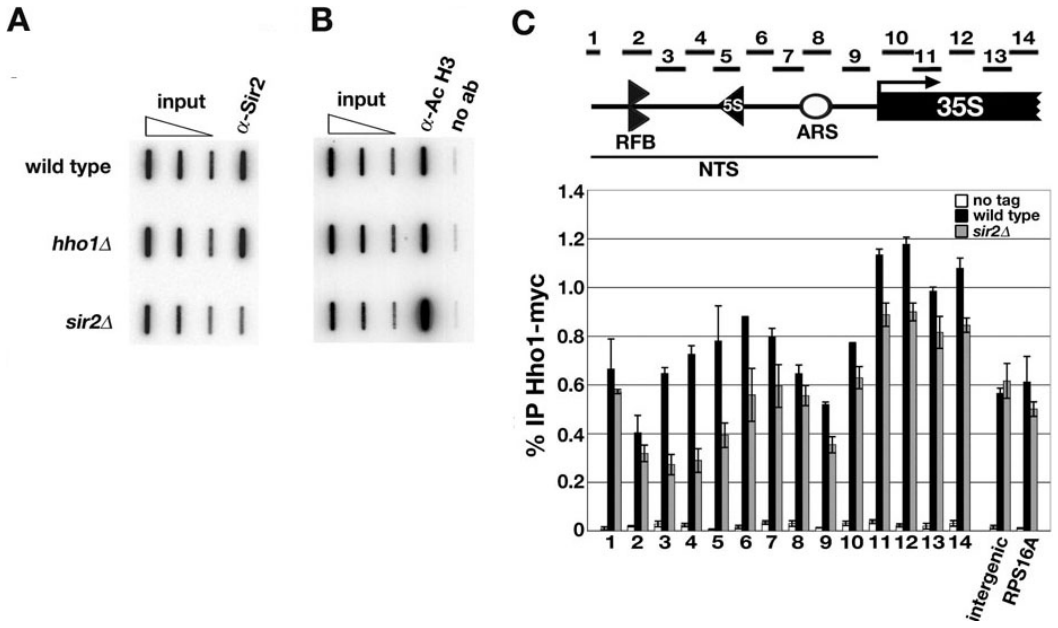

Figure 4-3. Histone H1 associates with the rDNA. (A) ChIPs were performed on wild-type (MBY1653), *hho1Δ* (MBY1961) and *sir2Δ* (MBY2074) cells using antisera that recognizes Sir2 and analysed by slot blot using a radiolabelled probe to the rDNA NTS. Data from *sir2Δ* cells provide a measure of background signal. Open triangle represents serial dilution of input DNA to verify that hybridization signal is linear with respect to the amount of DNA applied to the blot. The corrected average ratio of %IP (± range, $n = 2$) of Sir2 at the rDNA NTS for *hho1Δ*:wild-type cells was 1.2 (± 0.1). (B) ChIP analysis measuring the level of K9-K14 acetylated histone H3 at the rDNA NTS in wild-type, *hho1Δ* and *sir2Δ* cells (same strains as in A). No ab, samples to which no antibody was added. Other labels as in (A). The corrected ratio of %IP (± range, $n = 2$) of K9-K14 acetylated histone H3 at the NTS for *hho1Δ*:wild type cells, 1.10 (± 0.16); *sir2Δ* : wild type, 5.01 (± 0.46). (C) Schematic of the region of rDNA analysed by ChIP for H1 association. Numbered dashes represent the location of PCR products used for analysis of the ChIP. Other labels are as in Figure 4-1A. Bar graph shows the analysis of the ChIP using antimyc antisera and extracts from wild-type (MBY2191, filled bars) and *sir2Δ* (MBY2290, hatched bars) cells expressing myc-tagged histone H1. Extracts from cells with an untagged version of H1 (MBY1198) were evaluated to provide a measurement of background signal (open bars). Input DNA and immunoprecipitated DNA were analysed by quantitative real-time PCR using primer pairs that span the NTS and the first 1344 bp of the 35S rRNA gene, the *RPS16A* gene and an intergenic region on chromosome VIII. Values of the average %IP (± range) for two independent experiments are shown on the bar graph.

Next, we asked if Sir2 affects the ability of histone H1 to associate with the rDNA. For these ChIPs, we utilized cells containing a myc-tagged version of histone H1 and cells containing untagged histone H1 to obtain a measurement of background signal. After immunoprecipitation, we analysed immunoprecipitated and input DNAs by quantitative real-time PCR using 14 sets of primers that span the NTS and the 5′ end of the 35S rRNA gene (Figure 4-3C). Consistent with earlier work (Freidkin and Katcoff, 2001), we detected histone H1 at the 35S rRNA gene. In addition, we found that histone H1 associates with the NTS region of the rDNA repeat (Figure 4-3C, black bars). In cells lacking Sir2, we detected reduced levels of histone H1 in several regions of the NTS, with the largest effect (approximately twofold) between the RFB and 5S rRNA gene. Interestingly, the level of histone H3 is also decreased in this region in *sir2Δ* cells (Li *et al.*, 2006a), which may reflect loss of nucleosomes from, or changes in the stability or positioning of nucleosomes, at the rDNA NTS (Fritze *et al.*, 1997; Bryk *et al.*, 2002; Li *et al.*, 2006a). We also examined the association of histone H1 with the actively transcribed *RPS16A* gene and an intergenic region on chromosome VIII in wild-type and *sir2Δ* cells. We found that the association of histone H1 with these loci was similar in wild-type and *sir2Δ* cells (Figure 4-3C), suggesting that the association of histone H1 with chromatin is not regulated by Pol II activity. In summary, these data show that in cells lacking Sir2, histone H1 maintains the ability to interact with the NTS and the 5′ portion of the 35S rRNA gene, albeit at somewhat reduced levels. It is important to note that a significant amount of histone H1 remains at the rDNA in *sir2Δ* cells, a finding that is consistent with the higher level of mitotic recombination observed at the rDNA in the

hho1Δ sir2Δ double mutant in comparison to the level in either single mutant (*hho1Δ* or *sir2Δ*).

4.3.4. Extrachromosomal rDNA circles do not accumulate in *hho1Δ* cells

We wanted to determine if histone H1 acts in the same recombination pathway as Sir2 or through a different mechanism, as suggested by the additive effect on recombination that was observed in the *hho1Δ sir2Δ* double mutant (Table 4-2). ERCs, episomes containing one or more rDNA repeats, accumulate to high levels in *sir2* mutants owing to the failure to repress recombination at the rDNA. Because *hho1Δ* cells had a high level of recombination at the rDNA, we asked if these cells also accumulate ERCs. To measure ERCs, two-dimensional (2D) gel electrophoresis in the presence of chloroquine was performed. Chloroquine is a DNA intercalating agent that induces positive supercoiling in circular DNA and allows separation of circular molecules, such as ERCs, from linear molecules during 2D gel electrophoresis. We found that the level of ERCs in *hho1Δ* cells was equivalent to the level in wild-type cells (Figure 4-4A). In contrast, the level of ERCs in *sir2Δ* cells was almost sevenfold higher than the level in wild-type cells. In summary, despite an increase in mitotic recombination at the rDNA, cells lacking histone H1 do not accumulate ERCs. From this result, we conclude that reciprocal intrachromosomal recombination events that are proposed to generate ERCs are not increased in *hho1Δ* cells.

The accumulation of ERCs due to high levels of recombination at the rDNA is associated with shortened lifespan in several *S. cerevisiae* mutants (Sinclair and Guarente, 1997) (reviewed in Piper, 2006). Lifespan in *S. cerevisiae* is defined as the

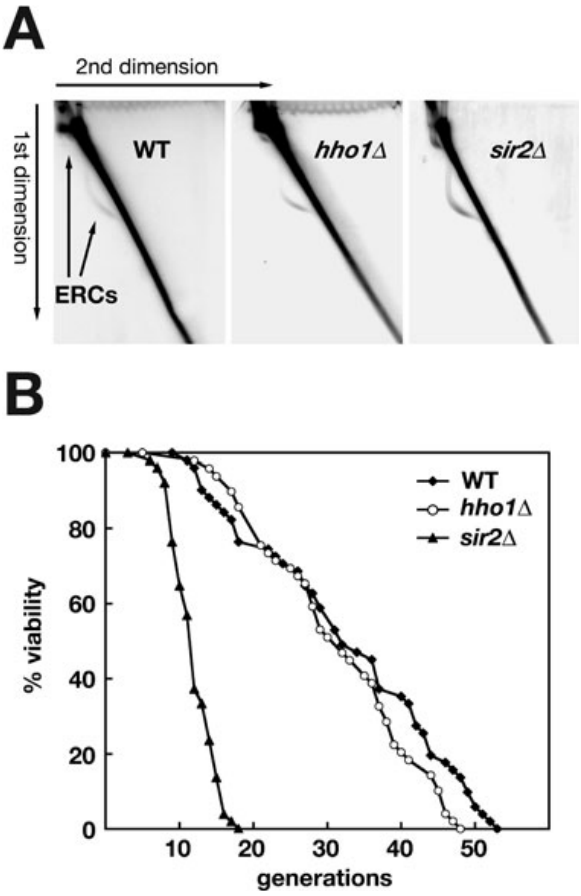

Figure 4-4. Analysis of ERCs and lifespan in *hho1Δ* cells. (A) Analysis of rDNA and ERCs isolated from wild-type (MBY2038), *hho1Δ* (MBY2039) and *sir2Δ* (MBY2296) cells. Two-dimensional gel electrophoresis in the presence of chloroquine was used to separate circular DNA molecules from linear molecules. ERCs that migrate as arcs below the linear rDNA (diagonal streak) are indicated with arrows. The average ratio (± range) of ERC DNA:linear rDNA after normalization to the wild-type strain for *hho1Δ* cells was 1.16 (± 0.03; $n = 2$) and for *sir2Δ* cells was 6.91 (± 1.86; $n = 2$). (B) Lifespan analysis was performed on wild-type (BY4742), *hho1Δ* (12125) and *sir2Δ* (13738) cells of the S288C genetic background. The average lifespan (± SE) for wild-type cells was 31.4 (±1.8; $n = 51$) with a maximum lifespan of 52; for *hho1Δ* cells was 29.8 (± 1.5; $n = 49$) with a maximum lifespan of 47; and for *sir2Δ* cells was 10.9 (± 0.4; $n = 51$) with a maximum lifespan of 17. The lifespan of *hho1Δ* cells was not significantly different from that of wild-type cells ($P = 0.55$). The lifespan of *sir2Δ* cells was significantly different from that of wild-type cells ($P = 2 \times 10^{-6}$).

number of times a mother cell divides and produces a daughter cell. A previous study that measured the lifespan of cells of the W303 genetic background, a common laboratory strain of *S. cerevisiae*, reported that cells lacking histone H1 have a shortened lifespan (Downs *et al.*, 2003). Thus, we expected that our cells lacking histone H1 would contain a high level of ERCs, yet the results presented in Figure 4-4A did not agree with that prediction. The yeast strains used in our study are from a different genetic background, S288C, another common laboratory strain of *S. cerevisiae*. Because we did not detect an accumulation of ERCs, we measured the average lifespan in our *hho1Δ* cells to address the possibility that the shortened lifespan of *hho1Δ* mutants is a strain-dependent phenotype. Different phenotypes in W303 and S288C strains with the same mutant allele have been observed previously (for example, see Rogowska-Wrzesinska *et al.*, 2001).We analysed lifespan in wild-type, *hho1Δ* and *sir2Δ* cells of the S288C genetic background (Figure 4-4B). The average lifespan of our wild-type cells was 31.4 generations and that of *hho1Δ* cells was 29.8 generations. The difference in lifespan between wild-type and *hho1Δ* cells was not significant ($P = 0.55$). As expected, *sir2Δ* cells had a shorter lifespan than wild-type cells, with an average of 10.9 generations. These data indicate that histone H1 does not regulate lifespan in S288C cells.

4.3.5. Cells lacking histone H1 form fewer Holliday junctions

Increased reciprocal recombination occurs at the rDNA in cells lacking Sir2. These recombination events involve the formation of Holliday junction intermediates that can be visualized using 2D gel electrophoresis in the presence of ethidium bromide (Figure 4-5). A schematic of the expected migration of rDNA molecules is shown in the

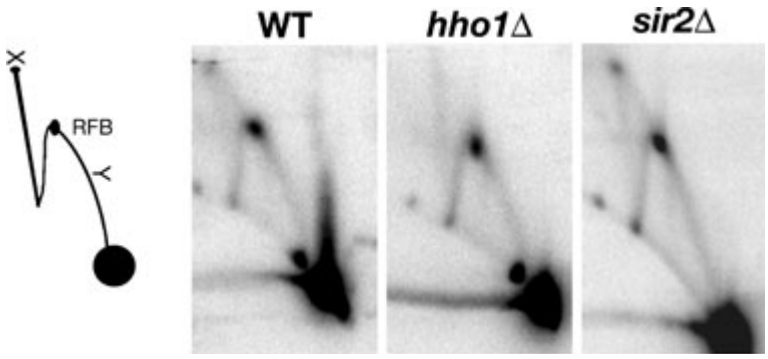

Figure 4-5. Cells lacking histone H1 form fewer Holliday junction intermediates. Analysis of recombination and replication intermediates from wild-type (MBY2038), *hho1Δ* (MBY2039) and *sir2Δ* (MBY1238) cells using 2D gel electrophoresis. Left panel, schematic of 2D gel results. Large filled circle, 1N linear rDNA; Y-arc, replicating branched DNA; RFB, stalled replication forks at the replication fork barrier; X spike, Holliday junction intermediates. Relative levels of Holliday junction intermediates were calculated by normalizing the signal in the X spike to that in the Y arc (Kobayashi *et al*., 2004). The normalized values of the average *hho1Δ*:WT ratio ± SE was 0.76 ± 0.08, $n = 4$, P = 0.02. The normalized values of the average *sir2Δ*:WT ratio ± SE was 1.16 ± 0.33, $n = 3$, $P = 0.66$.

left panel of Figure 4-5 adjacent to the analysis of DNA from wild-type, *hho1Δ* and *sir2Δ* cells. We found that the average relative intensity of recombination intermediates in *hho1Δ* cells was significantly lower than in wild-type cells, indicating that cells lacking histone H1 form fewer Holliday junctions. As shown previously (Kobayashi *et al*., 2004), the levels of Holliday junctions formed in wild-type and *sir2Δ* cells were similar. These findings add further support to our model that histone H1 functions at the rDNA by repressing a recombination pathway that is independent of Sir2.

4.4. DISCUSSION

In *S. cerevisiae*, rDNA chromatin is associated with silencing proteins as well as hypoacetylated and hypomethylated histones that contribute to the repression of Pol II transcription and homologous recombination events (Straight *et al.*, 1999; Bryk *et al.*, 2002; Buck *et al.*, 2002; Huang and Moazed, 2003; Kobayashi *et al.*, 2004; Huang *et al.*, 2006; Li *et al.*, 2006a). Recent work suggests that silencing at the rDNA requires the stable association of cohesin and condensin proteins to promote the proper alignment and pairing of the replicated sister chromatids (Laloraya *et al.*, 2000; Kobayashi *et al.*, 2004; Machin *et al.*, 2004; Kobayashi and Ganley, 2005; Huang *et al.*, 2006; Johzuka *et al.*, 2006). In the absence of Sir2, the association of proteins required for cohesion is decreased at the rDNA, and it is hypothesized that unequal sister chromatid recombination causes the overproduction of ERCs, whose accumulation is linked to a shortened lifespan in *S. cerevisiae*, coupled with changes in the length of the rDNA array (reviewed in Kobayashi, 2006). Our studies on histone H1 highlight the existence of

another rDNA silencing pathway that represses recombination at the rDNA but does not involve the accumulation of ERCs or cause premature ageing.

4.4.1. Histone H1, Sir2 and ERCs

We investigated the role of the histone H1 in silencing at the rDNA. Our results indicate that histone H1 represses mitotic recombination but is not required for silencing of Pol II-transcribed genes at the rDNA (Table 4-2; Figures 4-1 and 4-2). We determined that the rate of marker loss from the rDNA in an *hho1*Δ *sir2*Δ double mutant is equal to the sum of the levels observed in the two single mutants, suggesting that histone H1 regulates recombination at the rDNA through a Sir2-independent pathway. This conclusion is supported by the results of our ageing studies that indicate that *hho1Δ* cells of the S288C background do not exhibit a shortened lifespan (Figure 4-4B). Moreover, data from ChIP experiments revealed that cells lacking histone H1 maintain wild-type levels of Sir2 and low levels of K9, K14-acetylated histone H3 at the rDNA NTS (Figure 4-3). Previous analyses of chromatin structure in cells lacking histone H1 did not identify changes in nucleosome positioning at several regions of the genome (Puig *et al.*, 1999; Freidkin and Katcoff, 2001). Likewise, we did not observe differences in the accessibility of rDNA chromatin from wild-type and *hho1Δ* cells to MNase (data not shown). Based on our findings, we conclude that histone H1 regulates silent chromatin at the rDNA in a manner that does not involve Sir2 or nucleosome positioning.

Histone H1 can be classified with Hpr1 and Sgs1 as an rDNA silencing protein that represses mitotic recombination at the rDNA without affecting Pol II gene silencing. In contrast to several rDNA-silencing mutants, *hho1Δ* cells do not accumulate ERCs

(Figure 4-4A). Merker and Klein showed that cells lacking Hpr1, a protein that regulates recombination at the rDNA does so without an increase of ERCs (Merker and Klein, 2002). As increased recombination at the rDNA in *hho1Δ* and *hpr1Δ* mutants is not associated with the accumulation of ERCs, one possibility is that histone H1 and Hpr1 regulate the same recombination mechanism at the rDNA.

4.4.2. Regulation of recombination by histone H1

To obtain information about the effect of histone H1 on the mitotic stability of chromosomes, we measured the rate of loss of a marker from the rDNA and another locus on chromosome XII in cells lacking histone H1. Loss of the *HIS3* marker from a Ty1*HIS3* element could occur by recombination between repeated sequences at each end of the Ty1*HIS3* element or by a non-reciprocal gene conversion event with one of the 30 unmarked Ty1 elements in the *S. cerevisiae* genome. For the Ty1*HIS3* in the rDNA, intrachromosomal recombination or interchromosomal (unequal sister chromatid exchange during or after replication of rDNA) between rDNA repeats could also contribute to the loss of the *HIS3* marker. Our findings, comparing mitotic stability in wild-type and *hho1Δ* cells, indicate that there is not a significant difference in the rates of loss of the *HIS3* marker from outside of the rDNA, suggesting that histone H1 does not regulate marker loss recombination events at the locus outside of the rDNA. However, when the marked Ty1*HIS3* element is located in the rDNA array we observed a 3.4-fold increase in the rate of marker loss in cells lacking histone H1.

Our data suggest that histone H1 plays a direct role in regulating recombination at the rDNA. Consistent with previous reports (Freidkin and Katcoff, 2001; Downs *et al.*,

2003), we detect histone H1 at the rDNA at levels that are equal to or higher than levels observed at the actively transcribed Pol II gene *RPS16A* and an intergenic region on chromosome VIII (Figure 4-3C). In further support of a direct role for histone H1 at the rDNA, in affinity purification experiments performed to identify proteins involved in repressing recombination at the rDNA, histone H1 copurified with the RFB-interacting protein Fob1 (Huang *et al.*, 2006). Our recombination data (Table 4-2) suggest that the hyper-recombinogenic activities of Fob1 are offset by the action of histone H1, an interaction that is likely to promote the mitotic stability of the rDNA.

The rDNA is an unusual locus in *S. cerevisiae*; it is a highly transcribed region containing ~150-200 direct repeats, sequences that induce hyper-recombination, and silent chromatin. We expect that the factors governing recombination and the recombination events themselves are more numerous and complicated at the rDNA than at single-copy loci. Evidence suggests that several types of recombination events can occur within the rDNA, including gene conversion events, equal and unequal sister chromatid exchanges and single-strand annealing events (Gangloff *et al.*, 1996; Paques and Haber, 1999; Kraus *et al.*, 2001; Prado *et al.*, 2003; Kobayashi, 2006). At the rDNA, intrastrand recombination events that involve a cross-over are predicted to give rise to ERCs (Defossez *et al.*, 1999; Rothstein and Gangloff, 1999; Kobayashi *et al.*, 2004; Kobayashi, 2006). Given that we do not detect an increase in the level of ERCs and that we observe fewer Holliday junction intermediates in cells lacking histone H1 (Figure 4-4A and 4-5), we conclude that histone H1 does not regulate intrachromosomal recombination events that give rise to ERCs. This conclusion is supported by the finding

that we do not detect an increase in Pol II transcription at the rDNA or a loss of Sir2 from the rDNA, two events that are associated with the accumulation of ERCs (reviewed in Kobayashi, 2006).

We hypothesize that in cells lacking histone H1, there is an increase in the formation of lesions that are repaired by recombination pathways that are associated with marker loss yet do not produce ERCs. Examples of such recombination events are shown in Figure 4-6. Gene conversion events between repeated Ty1 sequences or rDNA sequences that are resolved in a non-reciprocal manner would result in marker loss without the generation of ERCs (Figure 4-6A). Likewise, single-strand annealing events used to repair a double-strand break in the rDNA could result in loss of a marker during resection of the broken strand prior to repair of the break (Figure 4-6B). Additionally, break-induced replication using an unequal rDNA repeat as template, such that the marker is not replicated in the newly made DNA, could also lead to marker loss without the production of ERCs (Figure 4-6C, pathway on right). Ongoing and future experiments that combine the *hho1Δ* mutation with mutations in factors required for specific recombination pathways, such as *RAD1*, which is required for the single-strand annealing mechanism, should provide insight into the specific recombination pathways that are regulated by histone H1.

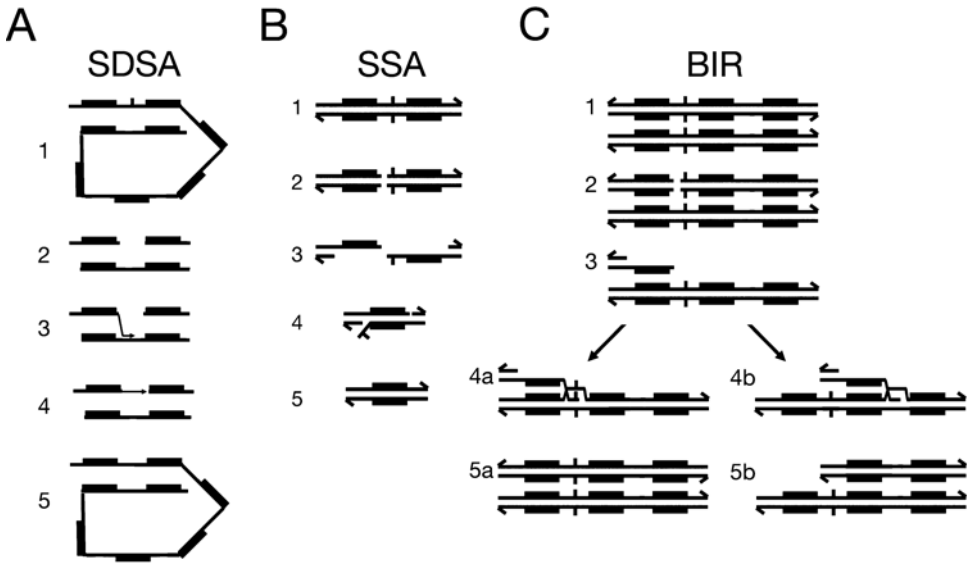

Figure 4-6. Recombination models to account for marker loss events that do not generate ERCs. (A) Intrachromosomal repair of a double-strand break (DSB) by synthesis-dependent strand annealing (SDSA). SDSA is consistent with an intrachromosomal break repair process that leads to marker loss without the generation of ERCs because Holliday junctions and cross-over products, which generate ERCs, are not formed. 1, schematic representation of eight rDNA repeats. Thick lines, 35S rRNA gene; thin lines, NTS sequences; vertical hash mark, marker gene. In 2, 3 and 4, only the rDNA repeats involved in the repair event are shown. 2, a DSB is generated near the marker gene followed by exonucleolytic removal of marker sequences. 3, the 3′ end of a broken strand invades the homologous sequences of another rDNA repeat to initiate repair synthesis (thin line with arrowhead). 4 and 5, the newly synthesized strand is released and serves as a template for synthesis of the opposite strand and repair of the DSB. (B) Repair of a DSB by the single-stranded annealing (SSA) pathway. 1, the top and bottom strands of two rDNA repeats are shown with half arrows representing the 3′ ends. Other labels as in (A). 2, a DSB is generated near the marker gene. 3, 5′ exonucleolytic degradation results in removal of the marker gene from the top strand and the generation of substantial 3′ extensions on the top and bottom strands. 4 and 5, homologous pairing and nucleolytic processing leads to repair of the DSB, loss of the marker gene and a decrease in the number of rDNA repeats. (C) Break-induced replication (BIR). 1, the top and bottom strands of three rDNA repeats on two sister chromatids are shown. Labels, as in (A) and (B). 2, a DSB is generated near the marker gene on one chromatid. 3, 5′ exonucleolytic processing leads to loss of the chromosomal fragment. 4a, if sister chromatids are equally aligned, invasion and annealing of a 3′ end from the broken duplex with the homologous sister chromatid leads to the initiation of repair synthesis. 5a, completion of repair maintains the marker gene on both chromatids. 4b and 5b, if sister chromatids are unequally aligned the repair process leads to an unequal sister chromatid exchange and loss of the marker gene from the broken chromatid.

CHAPTER V

SUMMARY AND CONCLUSIONS

The major function of the ribosomal DNA cluster (rDNA) on chromosome XII in *S. cerevisiae* is to encode the ribosomal RNAs. Despite a high level of RNA polymerase I activity at the rDNA, it is a region of the yeast genome where Pol II gene transcription, mitotic recombination and DNA replication are silenced. Many factors that are involved in regulating chromatin structure regulate the repressive state of the rDNA locus, supporting the model that aspects of chromatin play an essential role in regulating the heterochromatin-like nature of the rDNA. During my PhD thesis research, I investigated how the basic building blocks of chromatin, histones, and their modifying enzymes contribute to the specialized silent chromatin at the rDNA locus.

5.1. Sir2 represses endogenous Pol II transcription units in the rDNA NTS region

Silent chromatin domains are enriched with hypoacetylated and hypomethylated histones, while euchromatin is associated with hyperacetylated and hypermethylated histones. A key enzyme that regulates histone acetylation and protects silent chromatin from euchromatinization is Sir2. Sir2 is a NAD^+-dependent histone deacetylase that is required for silencing at the three known silent chromatin domains in *S. cerevisiae*: the rDNA, telomeres and the *HM* loci. The known targets of Sir2 include the acetylated lysine residues on histone H3 (at K9 and K14) and histone H4 (at K16). Interestingly, deletion of *SIR2* not only causes an increase in the levels of acetylated histone H3, but

also allows an elevation in the levels of K4-methylated histone H3 at the rDNA (Bryk *et al.*, 2002). This result suggested that Sir2 controls the composition of rDNA chromatin by regulating the levels of modified histones that are not direct substrates of its deacetylase activity.

My first project was to determine how the histone deacetylase Sir2 affects the levels of modified histones at the rDNA. First, I examined the distribution of several modified histones at the rDNA in wild-type cells and *sir2Δ* cells. Using chromatin immunoprecipitation analyses (ChIP) and antisera specific for K4-mono-, K4-di- or K4-trimethylated H3, I found that in cells lacking Sir2, the level of K4-di- and trimethylated H3 did not increase uniformly across the rDNA repeat. Instead, these two types of K4-methylated H3 were increased primarily at the NTS2 region of the rDNA repeat. High levels of K4-trimethylated histone H3 are known to be associated with genes that are being transcribed by Pol II. The increased level of K4-trimethylated H3 at NTS2 region suggested to us that Pol II transcription might be occurring in NTS2, despite the fact that there were no known Pol II-transcribed genes in this region. In subsequent ChIP experiments, I found that the level of Pol II was increased approximately two-fold over the NTS region in *sir2Δ* cells. I also identified and characterized several Pol II transcription units in the rDNA whose expression was upregulated in *sir2Δ* cells. My model is that the increased level of K4-methyalted H3 is the result of the loss of silent chromatin at the rDNA and derepression of Pol II transcription in the NTS region in *sir2Δ* cells. Consistent with my model, the peak of K4-di- and trimethylated H3 at the NTS2 region in *sir2Δ* cells was not observed in the cells lacking both Sir2 and Paf1, a

Pol II-associated factor that is required for efficient transcription elongation (data not shown). Taken together, my data suggest that Sir2 prevents the euchromatinization of the rDNA by restricting Pol II access to the rDNA sequences.

5.2. Functional analysis of the endogenous Pol II transcription units in the rDNA NTS region

Next, I investigated the possibilities that transcripts from or transcription of the NTS2 region play a role in the maintenance of silent chromatin at the rDNA. Because deletion of *SIR2* causes a Pol II gene silencing defect and hyperrecombination at the rDNA, we wanted to know if transcription of the NTS2 region is involved in regulating the silent chromatin at the rDNA locus. I decided to study the NTS2 bottom strand transcripts because these transcripts were resolved as three well defined bands by Northern blot analysis (see Chapters 2 and 3).

To determine if transcripts from NTS2 are required for the formation of silent chromatin, I expressed the transcripts *in trans* from an inducible promoter or *in cis* by driving transcription of the NTS2 region using a galactose-inducible promoter. When the NTS2 transcripts were overexpressed *in trans* (from a plasmid), no silencing or hyperrecombination defect was observed at the rDNA. In contrast, when the NTS2 transcripts were overexpressed *in cis* (at the rDNA array), the silencing of a Pol II gene inserted at the rDNA locus was disrupted and the marker gene was lost more frequently by recombination. The loss-of-silencing phenotypes are probably due to changes in chromatin accessibility caused by galactose-induced transcription in the NTS2 region.

During the course of my studies, it was reported that deletion of *SIR2* causes the dissociation of cohesin from the rDNA (Kobayashi and Ganley, 2005). Thus, one possible mechanism to explain the hyperrecombination phenotype observed in the cells where the NTS2 region was overexpressed *in cis* is that transcription in NTS2 prevents or reduces the association of cohesin with the rDNA. Failure in proper cohesin binding allows misalignment of sister chromatids, and increases the probability of unequal sister chromatid exchanges that lead to expansion and contraction events in repeated sequences, such as the rDNA array. My data support a model where Sir2 represses mitotic recombination at the rDNA by preventing Pol II transcription in the NTS region, thereby allowing the stable association of cohesin, which keeps the rDNA repeats in proper register and to ultimately stabilize the rDNA locus.

I have found that overexpression of the NTS2 region causes hyperrecombination at the rDNA. One question I am currently addressing is what will happen to the silent chromatin at the rDNA if the transcription of the NTS2 region is hindered? My hypothesis is that if the hyperrecombination at the rDNA that is observed in *sir2Δ* cells depends on Pol II transcription in the NTS region, then preventing transcription at the NTS region should repress the rDNA hyperrecombination defect. Toward completing this goal, I am constructing a yeast strain that will be unable to transcribe the NTS2 bottom strand. As shown in Figure 2-9C, the major 5' end of the NTS2 bottom strand transcripts colocalizes with a consensus Pol II transcription start site (TSS). My goal is to delete the TSS from each rDNA repeat and to prevent transcription of the NTS2 bottom strand by Pol II. Because the rDNA array contains more than 150 rDNA repeat,

and each rDNA repeat contains a TSS in NTS2, it would be impossible to delete each TSS in the rDNA array. Instead, I subcloned one rDNA repeat on a plasmid, and deleted the TSS in NTS2 by PCR-mediated site-directed mutagenesis. The mutagenized rDNA repeat was then introduced back to chromosome XII of a strain that lacks the entire chromosomal rDNA array (*rdnΔΔ* strain, see Oakes *et al.*, 2006a). Growth of the *rdnΔΔ* strain is supported by a helper plasmid that has the rRNA genes under the control of a *GAL7* promoter. This strain is only viable in galactose-containing media and not in media containing glucose.

After growing the transformants for several generations, I observed that the cells lacking the rDNA TSS were able to grow on glucose-containing media, suggesting that the rDNA repeats had expanded to at least a minimum copy number that allows cells growth (~20 copies, Takeuchi *et al.*, 2003). In addition, the helper plasmid was no longer needed for viability and could be segregated from the cells. In the future, we are going to use this strain to test if the NTS2 bottom strand transcripts are eliminated in the presence and absence of Sir2. If transcription of the NTS2 bottom strand is abolished by deleting the TSS, we will measure the level of Pol II transcription and recombination in the rDNA to see if it is altered in cells that lack transcription of the NTS (see below). I will also examine the extent to which the rDNA array is expanded in this strain. Failure to expand the locus to the wild-type copy number could imply that loss of transcription has imparted a replication defect at the rDNA.

Each rDNA repeat contains an autonomous replication sequence (ARS) at the NTS2 region (see Figure 1-5). However, only 20% of the rDNA ARSs are fired during

each cell cycle, and the rest of the ARSs are repressed in a Sir2-dependent manner that is not well understood (Pasero *et al.*, 2002). Given that the transcription start site of the NTS2 bottom strand transcripts is about 50 bp from one of the ARS elements, it is possible that transcription across NTS2 is required for rDNA ARS firing. In this scenario, firing of the rDNA ARS will be hindered in the "ΔTSS" strains due to lack of transcription in NTS2, and replication of the rDNA locus will depend on the ARS elements flanking the rDNA array. We expect that the ARS elements flanking the rDNA locus will not be able to support replication of the full length rDNA array (~ 1.4 - 1.8 Mb). Therefore, defects in rDNA replication will limit the extent of rDNA repeat expansion. This experiment will tell us if the transcription in NTS2 region has a role in regulating rDNA ARS firing and help us understand how Sir2 represses rDNA replication.

Two other important characteristics of silent rDNA chromatin that need to be examined using the strains that lack the TSS in NTS are Pol II gene silencing and mitotic recombination. This will be accomplished by measuring the mRNA level using Northern blotting analysis and by measuring the rate of loss of a marker gene inserted in one of the rDNA repeats in cells lacking the NTS2 TSS. The mRNA level of the reporter gene will indicate the degree of silencing and the rate of loss of the marker gene will reflect the mitotic recombination at the rDNA. If the transcription of the NTS2 region does regulate the silent chromatin at the rDNA, deletion of *SIR2* in strains lacking the NTS2 TSS will not cause Pol II silencing defect or hyperrecombination as observed in the stains with an intact NTS2 TSS. This will allow us to confirm the previous conclusion

that Sir2 regulates the silent chromatin rDNA locus by repressing Pol II transcription at the rDNA NTS region.

In addition, I found that the NTS2 bottom strand transcripts can be translated *in vitro*, and that they associate with polysomes. These data suggest that the NTS2 bottom strand transcripts may encode proteins. To investigate if these transcripts encode proteins *in vivo*, the sequences encoding an epitope tag should be introduced into the possible ORFs found within the NTS2 bottom strand transcripts. With these strains, Western blotting analysis using antibodies against the tags could be used to test if the transcripts have the potential to be translated. Alternatively, the NTS2 bottom strand transcripts may not encode any proteins but serve as a regulator in ribosome functions. In support of this, the NTS2 bottom strand transcripts were predicted to form stable secondary structures using an RNA secondary structure prediction method called "RNAfold".

5.3. Linker histone H1 represses recombination at the rDNA locus in *S. cerevisiae*

As the basic components of the chromatin structure, histones play direct roles in regulating the silent domains of the genome. Many studies have been conducted to understand how the core histones (including H2A, H2B, H3 and H4) regulate silent chromatin, yet little is known about the linker histone H1. Different from the core histones, histone H1 binds and protects the entry and exit point of the DNA wrapping around a nucleosome and facilitates the compaction of the chromatin (reviewed in Bustin *et al.*, 2005). It was found to be present at several regions of the yeast genome,

including the subtelomeric region and the rDNA repeats (Freidkin and Katcoff, 2001; Downs *et al.*, 2003). However, whether H1 regulates the silent chromatin at the rDNA locus remained unclear. In my study, we examined the role of histone H1 on Pol II gene silencing and mitotic recombination at the rDNA locus. Our results showed that histone H1 represses mitotic recombination without affecting Pol II gene silencing at the rDNA. Since Sir2 contributes to repression of rDNA recombination by inhibiting intrachromosomal recombination events, we next tested if H1 acts in the same pathway as Sir2. All the evidence that we have found suggest histone H1 and Sir2 act to repress different recombination pathways at the rDNA. First, the recombination rate in the *hho1Δ sir2Δ* double mutant is equal to the sum of recombination rate from the two single mutants. Secondly, the *hho1Δ* cells do not accumulate extrachromosomal rDNA circles (ERCs) or the Holliday junction intermediates, which accumulate in cells lacking Sir2. In addition, the association of Sir2 or H1 with the rDNA repeats does not depend on each other. These data suggest that histone H1 regulates recombination at the rDNA through a Sir2-independent pathway.

The ongoing and future experiments are focusing on identifying the recombination pathway(s) that are repressed by histone H1. There are several recombination pathways that do not involve in formation of the Holliday junction intermediates, such as synthesis-dependent strand annealing pathway (SDSA), the single-stranded annealing pathway (SSA), and the break-induced repair pathway (BIR). Different recombination proteins are involved in each of the three pathways. For example, SDSA requires Rad51 and Rad52, but not Rad1 nor Rad10. SSA requires

Rad1and Rad10, but not Rad51. SSA requires Rad52 when DNA repeat lengths are short, but not when DNA repeat lengths are long (~10 kb) as in the rDNA. Finally, BIR requires Rad52, but not Rad1, Rad10 nor Rad51. Dr. John Mueller and other members of our lab are using the single and double deletion mutants lacking *HHO1*and one the Rad factors mentioned above to measure the rates of rDNA recombination, and to ultimately identify the recombination pathway(s) that histone H1 represses at the rDNA.

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APPENDIX I

ISW2 REGULATES GENE SILENCING AT THE RIBOSOMAL DNA LOCUS IN *Saccharomyces cerevisiae*

Since the discovery of rDNA silencing in 1997 (Bryk *et al.*, 1997; Smith and Boeke, 1997), a number of studies have identified factors required for silencing at the rDNA including, but are not limited to, histones, histone-modifying enzymes and chromatin remodelers (Bryk *et al.*, 1997; Fritze *et al.*, 1997; Smith and Boeke, 1997; Smith *et al.*, 1999; Straight *et al.*, 1999; Roy and Runge, 2000; Bryk *et al.*, 2002; Dror and Winston, 2004; Clarke *et al.*, 2006; Mueller *et al.*, 2006; Mueller and Bryk, 2007). Chromatin remodeling proteins have been shown to regulate silencing at the rDNA. Deletion of *SNF2* or *SNF5*, which encode members of the Swi/Snf complex, causes loss of silencing at the rDNA and telomeres (Dror and Winston, 2004). The chromatin remodeling protein Isw1 is also required for silencing at the rDNA (Mueller and Bryk, 2007). Isw1 is a member of the Imitation Switch (ISWI) family of chromatin remodelers (Saha *et al.*, 2006). Given that Isw1 associates with the rDNA and that deletion of *ISW1* causes changes in rDNA chromatin structure, it has been proposed that Isw1 functions directly at the rDNA to regulate silencing (Mueller and Bryk, 2007). A second ISWI family gene in S. cerevisiae, *ISW2*, is required for gene repression (Mellor and Morillon, 2004). Cells lacking *ISW2* overexpress a-specific genes in *MATα* cells and fail to repress early meiotic genes (Goldmark *et al.*, 2000; Ruiz *et al.*, 2003). Isw2 has been shown to repress individual genes as well, including *INO1*, *PHO3* and *CLB2* (Fazzio *et al.*, 2001; Kent *et al.*, 2001; Sugiyama and Nikawa, 2001; Sherriff *et al.*, 2007). Here we used Northern analysis to determine if Isw2 is required for transcriptional silencing at the rDNA.

To determine if Isw2 regulates Pol II gene silencing at the rDNA, we constructed a strain with the *ISW2* gene replaced with *KANMX4* gene. All the strains used this experiment contains a genetically marked Ty1 element, Ty1*his3AI*, in one of the rDNA repeats. In wild-type cells, the Ty1*his3AI* is transcriptionally silenced at the rDNA array (Bryk *et al.*, 1997). However, if rDNA silencing is disrupted, it will be transcribed by Pol II. The level of Ty1*his3AI* mRNA is inversely proportional to the degree of silencing at the rDNA locus in these strains.

We measured the steady-state Ty1*his3AI* transcript levels in wild-type cells and

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isw2Δ cells. Ty1*his3AI* mRNA was detected by hybridization with a probe specific to *his3* sequence (Figure A-1, upper panel). The low level of Ty1*his3AI* mRNA observed in wild-type cells is consistent with transcriptional silencing at the rDNA. In *isw2Δ* cells, Ty1*his3AI* mRNA levels were increased 4.3-fold relative to the level in wild-type cells, indicating that Isw2 regulates transcriptional silencing at the rDNA. As a control, we performed Northern analyses on RNA from cells lacking *ISW1*, a chromatin remodeling protein that is closely related to Isw2 and is required for silencing at the rDNA and *HM* loci (Cuperus and Shore, 2002; Mueller and Bryk, 2007). As expected, Ty1*his3AI* RNA levels were increased in *isw1Δ* cells.

Approximately 30 endogenous Ty1 elements have been mapped in the *S. cerevisiae* genome and all of these are located outside of the rDNA. To determine if deletion of *ISW2* causes a general increase in transcription of endogenous Ty1 elements, we measured total Ty1 mRNA (Figure A-1, middle panel). Consistent with previous studies (Kent *et al.*, 2001; Gelbart *et al.*, 2005), no significant difference in steady-state total Ty1 mRNA levels were observed in wild-type and *isw2Δ* cells. In contrast to the regulation of the Ty1*his3AI* element in the rDNA, cells lacking either *ISW1* or *ISW2* individually do not exhibit defects in transcription of genomic Ty1 elements (Kent *et al.*, 2001; Mueller and Bryk, 2007). However, previous work has shown that cells lacking both *ISW1* and *ISW2* have higher levels of Ty1 transcripts than wild-type or single *ISW1* or *ISW2* deletion mutants, suggesting that Isw1 and Isw2 act redundantly to repress transcription of genomic Ty1 elements(Kent *et al.*, 2001). Consistent with transcriptional silencing of Pol II genes at the rDNA being a result of specialized silent chromatin, data presented here and elsewhere (Mueller and Bryk, 2007) suggest that Isw1 and Isw2 alter the function of silent chromatin at the rDNA. However, in contrast to the regulation of endogenous Ty1 elements located outside of the rDNA (Kent *et al.*, 2001), our results suggest that *ISW1* and *ISW2* do not act redundantly in the silencing of Pol II-transcribed genes in the rDNA.

There is precedence for the regulation of the rDNA locus by the ISWI family chromatin remodelers. In *S. cerevisiae*, Isw1 associates with the rDNA and is required for the silencing of Pol II-transcribed genes at the rDNA (Mueller and Bryk, 2007). In mammals, Snfh2, an ISWI chromatin remodeling protein and member of the nucleolar remodeling complex NoRC, is required for silencing of Pol I transcription (Strohner *et al.*, 2004; Li *et al.*, 2006b). Here, we show that Isw2 plays a role in the regulation of Pol II transcription at the rDNA in *S. cerevisiae*, however, the lack of an effect of deletion of *ISW2* on total rRNA levels or cell-growth properties (data not shown) suggests that Isw2 does not regulate transcription of the ribosomal RNA genes by Pol I in *S. cerevisiae*.

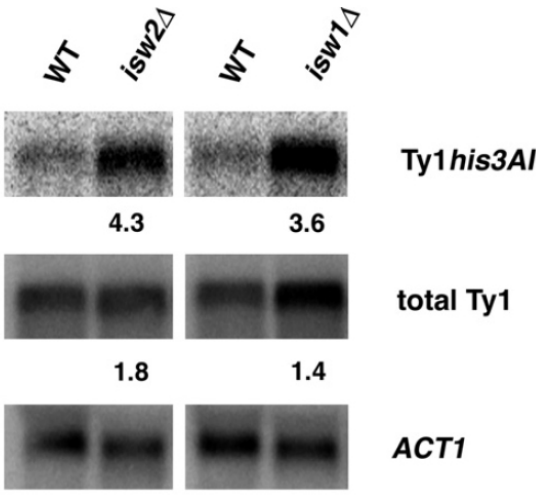

Figure A-1. Isw2 is required for transcriptional silencing at the rDNA. Total RNA from wild-type, *isw2Δ* and *isw1Δ* cells were analyzed to determine the levels of Ty1*his3AI* mRNA (upper panel), total Ty1 mRNA (middle panel) and *ACT1* mRNA (lower panel). Transcript levels for Ty1*his3AI* and total Ty1 were normalized to levels of *ACT1* or *PYK1* (not shown) mRNA. The average ratios of normalized Ty1*his3AI* mRNA in mutants relative to that in wild-type cells are shown below each panel (n=3). These values ±SE were: *isw2Δ*, 4.3±0.7, p=0.011; *isw1Δ*, 3.6±0.8, p=0.036. The average ratios of normalized total Ty1 transcript in mutants relative to wild-type cells are shown below each panel (n=3). These values ±SE were: *isw2Δ*, 1.8±0.6, p=0.24; *isw1Δ*, 1.4±0.2, p=0.12.

APPENDIX II

AN INCREASE IN MITOCHONDRIA DNA EXCLUDES SIR2 ASSOCIATION AT THE RIBOSOMAL DNA LOCUS

Coordination between cellular metabolism and DNA replication determines when cells initiate division. It has been assumed that metabolism only plays a permissive role in cell division. While blocking metabolism arrests cell division, it is not known whether an up-regulation of metabolic reactions accelerates cell cycle transitions. To determine if metabolism can actively promote cell division it is important to identify gain-of-function mutations in metabolic pathways that also accelerate cell proliferation. Such mutations have not been described in the yeast *S. cerevisiae*. In eukaryotic cells, the mitochondria are membrane-bound organelles which produce energy and regulate cell metabolism. Each mitochondrion contains a mitochondria-specific genome (also referred as mtDNA) that is evolutionarily different from the nuclear DNA. Abf2 is a conserved mtDNA maintenance protein (Diffley and Stillman, 1991; Bonawitz *et al.*, 2006), which directly binds to, bends and compacts mtDNA (Friddle *et al.*, 2004; Stigter, 2004). Moderate over-expression of Abf2 by 2-3 fold elevates the amount of mtDNA by 50-150% (Zelenaya-Troitskaya *et al.*, 1998). The consequences of an increase in mtDNA in cell proliferation have not been explored.

In the study to determine if up-regulation of metabolism could promote cell division, Heidi Blank (2008) from Dr. Michael Polymenis' lab found that cells with increased amount of mitochondria DNA by moderately overexpressing *ABF2* proliferate and increase in size more rapidly than wild-type cells, indicating that increasing the amount of mtDNA may promote nuclear DNA replication. One of the connections between mitochondria function and the nuclear DNA replication is Sir2. Sir2 is a class III histone deacetylase whose activity depends on NAD^+, an important coenzyme involved in cell metabolism (reviewed in Blander and Guarente, 2004). It is known that Sir2 not only represses Pol II gene expression at several silent loci in *S. cerevisiae*, but also appears to negatively impact on rDNA replication. In *sir2Δ* cells, twice as many origins are activated within the rDNA array (Pasero *et al.*, 2002). My contribution to this work was to determine if overexpressing the mitochondrial protein Abf2 can down-regulate the level of Sir2 at the rDNA locus hence promote DNA replication at the rDNA locus.

To test if increased amount of mtDNA negatively regulates the level of Sir2 at the rDNA, I performed chromatin immunoprecipitation (ChIP) with $ABF2^+$ or $3\times ABF2^+$ cells using antisera against Sir2. $ABF2^+/sir2\Delta$ cells and $3\times ABF2^+/sir2\Delta$ cells were examined to provide a measurement of background. Immunoprecipitated DNA was analyzed by real-time PCR using primers that span the ARS elements in the rDNA. We found that the level of Sir2p at the rDNA ARS elements was reduced about two-fold in $3\times ABF2^+$ cells, compared to the level in $ABF2^+$ cells (Figure A-2). The level of Sir2 at *RPS16A*, a locus that does not contain an ARS element,

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was not altered by over-expression of Abf2. These data indicate that increasing the amount of mtDNA reduces Sir2 association with the rDNA. In support of this, John Mueller also showed that the levels of K9-, K14-diacetylated histone H3 (a known target of Sir2) were increased at the rDNA in the 3×*ABF2*$^{+}$ strain (see Figure 9C in Blank *et al.*, 2008). And in contrast to the reduced level of Sir2 in 3×*ABF2*$^{+}$ strain, cells lacking the mtDNA (ρ^{-}) have increased level of Sir2 at the rDNA locus (see supplemental Figure S6 in Blank *et al.*, 2008), suggesting the level of Sir2 bound at the rDNA ARS elements is inversely related to the amount of mtDNA.

In conclusion, our data suggest the positive effect of increasing mtDNA on cell division may be mediated by lowering the level of Sir2 bound at the rDNA ARS region. The inhibitory effects of Sir2 on DNA replication extend beyond rDNA. Loss of Sir2 suppresses replication defects of mutants that cannot assemble a pre-replicative complex of proteins (pre-RC) at origins of DNA replication in the G1 phase of the cell cycle (Pappas *et al.*, 2004). Recently, another study showed that Sir2 represses five DNA replication origins on chromosome III and VI in *S. cerevisiae* by inhibiting the loading of the MCM (minichromosome maintenance) complex (Crampton *et al.*, 2008). Hence, Sir2 could possibly act as a common sensor to coordinate cell metabolism and DNA replication.

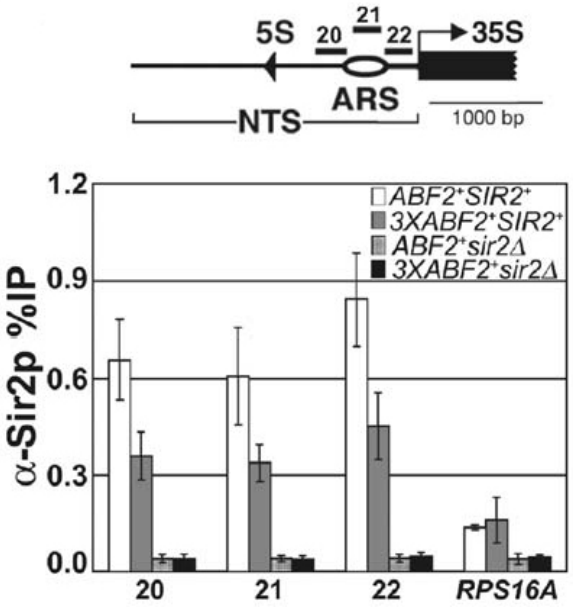

Figure A-2. Cells over-expressing *ABF2* have less Sir2 at the rDNA ARS elements. ChIP experiments analyzed by real-time PCR show that the level of Sir2p (%IP) at the rDNA ARS elements is reduced in 3×*ABF2*$^{+}$ cells. Part of one rDNA repeat is shown above the graph indicating the location of the rDNA ARS elements, the PCR products analyzed (20, 21, 22); the nontranscribed spacer (NTS); and the 35S and 5S rRNA genes. The values shown in the bar graph are the average %IPs (± SD) of three independent experiments.

APPENDIX III

THE HISTONE VARIANT H2A.Z DOES NOT REGULATE RIBOSOMAL DNA SILENCING

The nucleosome is the fundamental building block of chromatin in eukaryotic cells. The canonical nucleosome is composed of the four core histone proteins, H2A, H2B, H3 and H4. These canonical histones can be modified by adding different chemical groups to distinguish different chromatin states. In addition, there are histone variants that are used as an alternative means of marking chromatin domains (reviewed in Sarma and Reinberg, 2005). One of the most well characterized histone variants is H2A.Z, the variant for histone H2A. The H2A.Z is found in nearly all eukaryotes, and its functions have been linked to antagonizing gene silencing and activation of gene expression. In *S. cerevisiae*, Htz1 (H2A.Z in *S. cerevisiae*) has been found to act redundantly with SWI/SNF complex in activation of the *GAL1* and *PHO5* genes upon induction (Santisteban *et al.*, 2000). It is also enriched at regions near telomeres and in regions flanking the *HMR* silent loci to prevent spreading of the silent chromatin (Meneghini *et al.*, 2003). Deletion of *HTZ1* was found to mildly derepress a reporter gene inserted at the silent *HMR* loci (Dhillon and Kamakaka, 2000), which is probably due to the spreading of the limited amount of Sir proteins into euchromatin (Meneghini *et al.*, 2003).

H2A.Z has been localized to chromatin flanking the rDNA locus (Dhillon and Kamakaka, 2000). However, the role of Htz1 in regulating rDNA silencing remains unclear. To address this question, I replaced the endogenous *HTZ1* gene with *KANMX4* in a strain carrying a Ty1*his3AI* element inserted in one rDNA repeat, and measured the steady-state Ty1*his3AI* mRNA levels in wild-type cells, *htz1Δ* cells and the silencing-defective control strain *set1Δ* cells by Northern blotting. As shown in Figure A-3, the level of Ty1*his3AI* mRNA in wild-type cells is low (top panel, lane 1), consistent with transcriptional silencing at the rDNA locus. As a silencing-defective control, we looked at the level of Ty1*his3AI* mRNA in *set1Δ* cells. Set1 is a histone methyltransferase and it is absolutely required for rDNA silencing (Nislow *et al.*, 1997; Briggs *et al.*, 2001; Bryk *et al.*, 2002; Nagy *et al.*, 2002; Krogan *et al.*, 2003a). As expected, deletion of *SET1* results in a 3.3-fold increase in the Ty1*his3AI* mRNA compared to the level in wild-type cells (top panel, lane2). We also measured the levels of Ty1*his3AI* mRNA in three isolates of *htz1Δ* strains, and found that deletion of *HTZ1* causes mild increase of the level of Ty1*his3AI* mRNA (top panel, lane 3-5). However, the increase of the level of Ty1*his3AI* mRNA from the Ty1*his3AI* element at the rDNA locus is not likely to reflect loss of rDNA silencing in *htz1Δ* cells, because we also observed that the transcription of other genomic Ty1 elements is elevated slightly in cells lacking Htz1 (middle panel, lane 3-5). The data suggest that histone H2A variant Htz1 in *S. cerevisiae* represses transcription of the all genomic Ty1 elements regardless of their position in the genome.

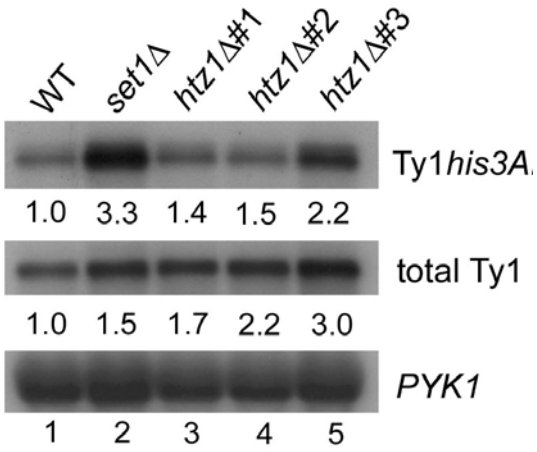

Figure A-3. Htz1 is not required for Pol II gene silencing at the rDNA. Total RNA from wild-type (MBY1198), *set1Δ* (MBY1217) and three *htz1Δ* (MBY2071, 2072 and 2073) strains were analyzed to determine the levels of Ty1*his3AI* mRNA (upper panel), total Ty1 mRNA (middle panel) and *PYK1* mRNA (lower panel). Transcript levels for Ty1*his3AI* and total Ty1 were normalized to levels of *PYK1* mRNA. The average ratios of normalized Ty1*his3AI* mRNA in mutants relative to that in wild-type cells are shown below each panel (n=2, except *set1Δ*, n=1). These values ±range were: *set1Δ*, 3.3; *htz1Δ*#1, 1.4 ±0.05; *htz1Δ*#2, 1.5±0.12; *htz1Δ*#3, 2.2±0.02. The average ratios of normalized total Ty1 transcript in mutants relative to wild-type cells are shown below each panel (n=2, except *set1Δ*, n=1). These values ± range were: *set1Δ*, 1.5; *htz1Δ*#1, 1.7±0.03; *htz1Δ*#2, 2.2±0.06; *htz1Δ*#3, 3.0±0.01.

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