Introduction
I.1 Chromatin domains in Eukaryotes

DNA in Eukaryotes is organized into chromatin with the nucleosome being the basic unit (Fig. I-1). Two copies each of the four core histones, H2A, H2B, H3 and H4, are assembled into an octamer around which 147 bp of DNA are wrapped to form a nucleosome core particle (Fig. I-1) (Luger et al., 1997). The repeating nucleosome cores are further organized into a higher-order structure which is stabilized by linker histone H1 located in the interior folding structure where DNA enters and exits the nucleosome core (Graziano et al., 1994). Oligonucleosome arrays can in turn be folded into secondary chromatin structures such as 30 nm diameter chromatin fibers and higher order tertiary structures (Hansen, 2002; Woodcock and Dimitrov, 2001). Chromatin can be roughly divided into condensed heterochromatin and decondensed euchromatin based on its cytological and molecular properties. Euchromatic domains are in general accessible to DNA binding factors and allow genes to be expressed. Whereas heterochromatic domains are inaccessible and transcriptionally silent (Grewal and Moazed, 2003).

Eukaryotic chromatin plays a crucial role in regulating DNA transactions including transcription, replication, recombination and repair. Heterochromatic domains surround centromeres and telomeres, and are also interspersed throughout the genome (Grewal and Moazed, 2003). Heterochromatin in general is relatively refractive to DNA modifying agents, has reduced levels of histone acetylation, replicates late in S phase, and represses transcription in its vicinity leading to position effect on gene expression (Braunstein et al., 1993; Loo and Rine, 1994; Reynolds et al., 1989). An extreme example of heterochromatin mediated position effect is X-chromosome inactivation in female mammals in which the genes on one of the two X chromosomes become coordinately inactivated, and this inactive
state is stably maintained and inherited through subsequent cell divisions for the life of the animal (Gartler and Riggs, 1983; Grant and Chapman, 1988). Another well-documented example of position effect is position effect variegation (PEV) in *Drosophila* (Henikoff, 1990). PEV describes the phenomenon that an active gene translocated to the vicinity of heterochromatin is repressed in some clusters of cells but expressed in others. This variegation is the result of stable maintenance of the repressed state through multiple cell divisions by an epigenetic mechanism, but with switching between the repressed and expressed states in a detectable number of cell divisions.

![Schematic illustration of chromatin structure and composition](image)

**Fig. 1-1. Schematic illustration of chromatin structure and composition.** Shown are the steps involved in the folding of extended nucleosomal arrays into maximally folded chromatin fibers.
I.2 Transcriptionally silent chromatin in yeast

Transcriptionally silent chromatin in *Saccharomyces cerevisiae* is akin to heterochromatin in higher organisms such as maize, flies, and mammals. First, DNA in transcriptionally silent chromatin in yeast is relatively inaccessible to various modifying agents and replicates late in S phase (Gottschling, 1992; Reynolds et al., 1989; Singh and Klar, 1992; Terleth et al., 1989). Second, nucleosomes from the silent *HM* loci and telomeres have reduced acetylation compared to nucleosomes from active regions of the genome (Braunstein et al., 1993), and the pattern of acetylation of histone H4 at the *HM* loci is identical to that in centric heterochromatin in *Drosophila* (Braunstein et al., 1993). Third, DNA within transcriptionally silenced HM loci is more negatively supercoiled than that in derepressed *HM* loci indicating that silent chromatin is more compact (Bi and Broach, 1997; Cheng et al., 1998). Finally, high-resolution chromatin mapping of *HML*, *HMR* and *MAT* revealed that silent HM loci have a uniquely organized chromatin structure (Ravindra et al., 1999; Weiss and Simpson, 1998).

Transcriptional silencing in *S. cerevisiae* plays an important role in mating-type determination, mating-type switching, telomere maintenance, as well as cellular senescence (Rusche et al., 2003). The mating-type of *S. cerevisiae* is governed by the type of information (a or α genes) present at the *MAT* locus which is constitutively expressed. The same genes also exist at the homothallic mating loci *HML* and *HMR*, but are transcriptionally silenced. These silent *HM* loci serve as donors of information in a mating-type switching event. Silencing is generally region-specific, but sequence-nonspecific as evidenced by the finding that relocating the mating-type genes away from the silenced loci to other loci activates them while other heterologous genes inserted at the silent loci are repressed.
Notably, the silenced state at *HM* loci is stably inherited in mitotically dividing cells representing a good example of epigenetic inheritance (Pillus and Rine, 1989). Silent chromatin is also found at telomeres and the rDNA locus.

Transcriptional silencing at the *HM* loci is established and maintained by the combined actions of *cis*-acting DNA elements and *trans*-acting proteins (Rusche et al., 2003). The *cis*-acting sites, known as the *E* and *I* silencers, are small negative regulatory sequences flanking each of the *HM* loci (Fig. I-2) (Abraham et al., 1984; Feldman et al., 1984). They are composed of various combinations of binding sites for proteins Rap1p (Repressor/Activator Protein 1) and Abf1p (ARS Binding Factor 1), and ORC (the origin recognition complex for DNA replication) whose binding is required for silencing (Fig. I-2) (Rusche et al., 2003).

**Fig. I-2. Schematic representation of the *MAT* and *HM* loci on chromosome III.** *HML* and *HMR* on chromosome III in *S. cerevisiae* (not drawn to scale). *HML*-E and -I are shown as filled arrows with white letters, and *HMR*-E and -I are shown as open arrows with black letters. Note the directions of silencers are drawn pointing toward the inside of *HML* or *HMR* but are not necessarily the functional directions. The sites for Abf1p, Rap1p, and ACS (ARS consensus sequence) for ORC recognition in silencers are indicated by stippled, filled, and open rectangles, respectively. They are variants of motifs 5’-RTCRYNNNNACG-3’ (for Abf1p), 5’-ACACCCRYACAYM-3’ (Rap1p), and 5’-WTTTAYRTTTW-3’ (ORC) (M, A/C; N, A/C/T/G; R, A/G; Y, C/T; W, A/T) (Breier et al. 2004, Lied et al. 2001; Rhode et al. 1992). Arrows above the factor-binding sites correspond to the 5’-3’ directions of these consensus sequences. The *α1* and *α2* genes at *HML* and *α1* and *α2* genes at *HMR* are also indicated. *CEN*, centromere. Tandem arrowheads indicate telomeric repeats.
The trans-acting factors involved in silencing include silencer-binding proteins (ORC, Rap1p and Abf1p), histones, and the four silent information regulator (SIR) proteins, Sir1p through Sir4p. Sir2p, Sir3p and Sir4p are the structural components of silenced chromatin and essential for silencing, but non-essential for growth (Rine and Herskowitz, 1987). The silencer-binding proteins are believed to promote the initiation of silencing by recruiting the Sir complex through physical interactions (Rusche et al., 2003). Rap1p interacts with Sir3p and Sir4p, whereas ORC interacts with Sir1p that binds Sir4p. Besides its role in silencing, Rap1p and Abf1p have essential functions as transcriptional activators of diverse genes (Lieb et al., 2001; Miyake et al., 2002), and the ORC complex has a well-conserved function in replication initiation (Bell, 2002).

The formation of silenced chromatin is mediated by polymerizing SIR complex that interacts with nucleosomes and thereby spreading outward from its nucleation site at the silencers. Sir3p/Sir4p interact with the N-terminal tails of histones H3 and H4 which is essential for the establishment and maintenance of silencing (Hecht et al., 1995) and there is evidence that Sir3p (hence the SIR complex) has a higher affinity for hypoacetylated histones. Sir2p is a histone deacetylase that is responsible for reducing histone acetylation in silent chromatin (Moazed, 2001b). The current model for silencing propose that Sir2p recruited to the silencer deacetylates histones in an adjacent nucleosome enabling it to bind another SIR complex with high affinity (Fig. I-3). The nucleosome-bound SIR complex in turn deacetylates the neighboring nucleosome allowing it to recruit a new SIR complex. In this manner, the SIR complex promotes its own propagation along an array of nucleosomes (Moazed, 2001a).
Fig. I-3. Model for the establishment of silent chromatin at *HM* loci in yeast. (a and b) A silencer is bound by silencer-binding proteins which in turn recruit Sir1p and the SIR complex (consisting of Sir2p, Sir3p and Sir4p). Sir2p deacetylates a nearby nucleosome (blue arrow). The first SIR complex recruits another SIR complex, which binds to the deacetylated nucleosome and then deacetylates the adjacent nucleosome. (c) SIR complex (silent chromatin) propagates (black arrow) by repeating the process in (b). (d) Symbols are shown for Rap1p-, Abf1p- and ORC-binding sites. Yellow nucleosomes represent those in silent chromatin (hypoacetylated); Black nucleosomes represent those in active chromatin (hyperacetylated). Other symbols are shown in the figure.
Although silencers have similar functions in establishing the silent state of the *HM* loci, they differ in potencies. At *HML*, either *E* or *I* individually is sufficient to maintain the silencing state (Mahoney and Broach, 1989). In contrast, at *HMR*, *E* is essential but *I* is dispensable for silencing (Brand et al., 1985). The difference in the potency of silencing of different silencers could reflect any combination of a number of possible factors including the differences in the spacing of the protein-binding sites, the affinities of individual sites for their respective factors, or the sequence context of the sites.

The genomic context of a silencer may harbor regulatory elements that can influence the silencing function of the silencer on either side. Two silencers separated by a distance of up to 4 kb can cooperate to bring about silencing in the region they bracket that is stronger than that promoted by either silencer alone (Boscheron et al., 1996; Feldman et al., 1984; Rivier et al., 1999; Sekinger and Gross, 1999). Moreover, a single binding site for any of the silencer-binding proteins can enhance the activity of a distant silencer without acting as a silencer on its own, and is therefore referred to as a protosilencer (Boscheron et al., 1996; Feldman et al., 1984; Rivier et al., 1999; Sekinger and Gross, 1999). In known cases of silencer-protosilencer interactions, silencing of genes located in the region flanked by the silencer and protosilencer was found to be increased (Boscheron et al., 1996; Cheng et al., 1998; Lebrun et al., 2001). Silencers and protosilencers are collectively called silencing elements. How two silencing elements collaborate to induce stronger silencing has not been resolved. Moreover, several proto-silencers are dispersed across the *HML* locus but their roles in *HML* silencing are not clear.
Like the silent mating loci, telomeres can also repress genes inserted in their vicinity (Gottschling et al., 1990). Moreover, silencing by telomeres also requires most of the genes needed for silencing at *HML* and *HMR* indicating that they share a common repression mechanism (Aparicio et al., 1991). Telomeric repeats consist of multiple Rap1p sites. Rap1p and Ku70/80 that binds chromosomal ends recruit the Sir complex, which propagates along subtelomeric chromatin (Fig. I-4) (Gottschling et al., 1990; Luo et al., 2002). The fact that silent chromatin at telomeres and that at *HM* loci shares the Sir complex suggests a competition for limiting factors between them. Consistent with this idea, increased telomeric silencing is associated with decreased silencing at *HMR* (Buck and Shore, 1995). Sir2p, but not the other *SIR* proteins, is also involved in a special form of transcriptional silencing at the rDNA locus (Straight et al., 1999).
Fig. I-4. Model for the establishment of silent chromatin at telomeres in yeast. (a) Rap1p binds to telomeric repeats and in turn recruits SIR complex (consisting of Sir2p, Sir3p and Sir4p). (b) Sir2p deacetylates a nearby nucleosome (blue arrow). (c) The Rap1p-bound SIR complex recruits another SIR complex, which binds to the deacetylated nucleosome and then deacetylates the adjacent nucleosome. SIR complex (silent chromatin) propagates (black arrow) by repeating the process in (b and c). Note that Sir1p is not involved in telomeric silencing. Yellow nucleosomes represent those in silent chromatin (hypoacetylated); Black nucleosomes represent those in active chromatin (hyperacetylated). Other symbols are shown in the figure.
I.3 Roles of chromatin in DNA replication

In *S. cerevisiae*, DNA replication is initiated at defined origins named autonomous replicating sequences (*ARSs*) across the genome (Bell, 2002). Each *ARS* element contains an essential, conserved, 11 bp elements known as *ARS* consensus sequence (*ACS* or “A element”) and multiple 10-15 bp “B elements” that also contribute to origin function (Fig. I-5). The origin recognition complex (ORC) plays a key role in replication initiation. ORC is composed of six subunits (Orc1p-Orc6p), all of which are required for cell survival. ORC associates with replication origins throughout the cell cycle, but its main function is to nucleate an initiation competent pre-replication complex (pre-RC) at origins in the G1 phase (Bell, 2002; Sasaki and Gilbert, 2007). Defects in pre-RC formation caused by ORC mutations may result in incomplete DNA replication and increased chances of replication fork stalling/breakage that triggers DNA-damage and spindle assembly checkpoint pathways leading to mitotic arrest (Sasaki and Gilbert, 2007). There is also evidence that ORC participates in sister-chromatid cohesion, which is distinct from its role in DNA replication (Shimada and Gasser, 2007).

**Fig. I-5. The structure of a yeast origin.** Note that not all ARSs have the three B elements.
Similar to other DNA transactions such as transcription, DNA replication is subject to regulation by chromatin modifying/remodeling activities. Histone acetylation is thought to enhance nucleosome mobility and improve the access of replication factors to origins. In S. cerevisiae, deletion of the histone deacetylase (HDAC) Rpd3p has been shown to increase global histone acetylation and to make a late-firing origin to fire earlier. Targeting the HAT Gcn5p to a normally late-firing origin causes its early activation (Vogelauer et al., 2002). Transiently acetylation of histon H3 K56 by the HAT Rtt109p during S phase is implicated in the regulation of replication (Han et al., 2007). The HAT Hat1p was recently shown to physically interact with ORC, and deletion of HAT1 exacerbated the temperature sensitivity of orc mutants (Suter et al., 2007). These studies suggest that HATs play positive roles, and HDACs play negative roles in the process of DNA replication. However, interestingly, there is also evidence suggesting that the HAT Sas2p negatively regulate ORC function (Ehrenhofer-Murray et al., 1997; Weinberger et al., 1999).

During replication elongation, progression of the replication fork leads to disrupt nucleosomes ahead of the fork into (H3-H4)2 tetramers and H2A-H2B dimmers. The parental histones are thought to be transferred randomly to the two daughter DNA strands (Gruss et al., 1993). Nucleosome disruption is thought to be facilitated by ATP-dependent chromatin remodeling enzymes (Groth et al., 2007). The IWSI chromatin-remodeling complex has been implicated in heterochromatin replication (Bozhenok et al., 2002; Collins et al., 2002). Chromatin is rapidly reassembled after DNA duplication with the deposition of H3-H4 followed by H2A-H2B on the DNA. Chromatin assembly factor-I (CAF-I) and Asf1 both have the ability to deposit H3 and H4 on DNA in a replication-coupled fashion in vitro (Verreault et al., 1996). CAF-I is targeted to replicated DNA by directly interacting with
PCNA (Proliferating Cell Nuclear Antigen) which is associated with the replication fork (Moggs et al., 2000). In addition to nucleosome reassembling, histone modifications are also perpetuated during DNA replication to maintain cell identity (Corpet and Almouzni, 2009). The HAT complex SAS-I interacts with both Asf1p and CAF-I (Meijsing and Ehrenhofer-Murray, 2001). Moreover, HAT HAT1 complex also interacts with Asf1p (Fillingham et al., 2008). However, the roles of the HATs in chromatin replication have not been resolved.

*S. cerevisiae* shares many similarities in the structure and function of heterochromatin at the molecular level with higher eukaryotic cells. The goal of this study was to use *S. cerevisiae* as the model system to delineate the mechanism underlying the function of silencers that initiate the formation of silent chromatin in yeast and the role of histone acetyltransferase (HAT) Sas2p in regulating transcriptionally silencing and DNA replication.
Chapter 1

Asymmetric positioning of nucleosomes and directional establishment of transcriptionally silent chromatin by *Saccharomyces cerevisiae* silencers
1.1 Abstract

The current model for the formation of heterochromatin in eukaryotes invokes an initiation process at specific nucleation centers, or silencers, that promote the propagation of silencing complex along the chromation. In *Saccharomyces cerevisiae*, silencers flanking the *HML* and *HMR* loci consist of various combinations of binding sites for Abf1p, Rap1p and the origin recognition complex (ORC) that serve to recruit the Sir silencing complex thereby initiating the establishment of transcriptionally silent chromatin. There have been seemingly conflicting reports concerning whether silencers function in an orientation-dependent or – independent manner, and what determines the directionality of a silencer has not been explored. I demonstrate that chromatin plays a key role in determining the potency and directionality of silencers. I show that nucleosomes are asymmetrically distributed around the *HML-I, HMR-E* or *HML-E* silencer so that a nucleosome is positioned close to the Abf1p or Rap1p side but not the ORC side of the silencer. This coincides with preferential association of Sir proteins and transcriptional silencing on the Abf1p or Rap1p side of the silencer. Asymmetric nucleosome positioning in the immediate vicinity of a silencer is independent of its orientation and genomic context indicating that it is the inherent property of the silencer. Moreover, it is also independent of the Sir complex and thus precedes the formation of silent chromatin. I conclude that the silencers promote asymmetric positioning of nucleosomes leading to unequal potentials of transcriptional silencing on their sides, and hence directional silencing.
1.2 Introduction

The establishment of condensed and transcriptionally silent heterochromatin in the eukaryotic genome is achieved via an initiation process that includes recruitment of silencing/repressor complexes to specific regulatory sites and propagation to nearby sequences along the chromatin. In the yeast *S. cerevisiae*, transcriptional silencing of the *HML* and *HMR* loci as well as regions near the telomeres is mediated by a silent chromatin that share many similarities with metazoan heterochromatin at the molecular level (Grewal and Moazed, 2003). Silent chromatin at the *HM* loci is initiated by silencers, known as *HML-E* and -I and *HMR-E* and -I, which flank the *HM* loci (Fig. 1-2 and 1-1A). Each silencer is composed of a unique combination of binding sites for proteins Abf1p, Rap1p and ORC (origin recognition complex for DNA replication) (Fig. 1-2 and 1-1A) whose binding is required for silencing (Rusche et al., 2003). The *trans*-acting factors include silencer-binding proteins, histones, and Sir1p through Sir4p. Sir2p is an NAD-dependent histone deacetylase that is believed to be responsible for the reduction of histone acetylation in silent chromatin (Moazed, 2001b; Rusche et al., 2003). Sir2p-Sir4p interact with each other and form the Sir complex that can interact with the silencer-binding proteins, as well as with histones H3 and H4. Moreover, the Sir complex has a higher affinity for histones H3 and H4 with reduced acetylation, which is important for the establishment and maintenance of silent chromatin (Carmen et al., 2002; Liou et al., 2005; Rusche et al., 2003)

The silencer-binding proteins are believed to promote the initiation of silencing by recruiting the Sir complex through physical interactions (Rusche et al., 2003). Rap1p interacts with Sir3p and Sir4p and ORC interacts with Sir1p that binds Sir4p. Once recruited
to the silencer, the Sir complex is thought to deacetylate nearby nucleosomes. As the Sir complex self interacts and preferentially binds hypoacetylated histones (Carmen et al., 2002; Liou et al., 2005), additional Sir complexes are recruited to the newly deacetylated nucleosomes. The nucleosome-bound Sir complex then deacetylates the neighboring nucleosome which in turn binds a new Sir complex. Interactions between Sir complexes also help recruit the incoming Sir complex (Liou et al., 2005; Rudner et al., 2005). In this manner, the Sir complex is thought to promote its own stepwise (nucleosome-by-nucleosome) propagation along a continuous array of nucleosomes thereby establishing an extended region of silent chromatin (Grewal and Moazed, 2003). This model is supported by the finding that disrupting the regularity of nucleosomes by nucleosome-excluding structures hinders the spread of silent chromatin (Bi et al., 2004).

Although yeast silencers promote silencing by a common mechanism, they exhibit different efficiencies of silencing. The HML-E, HML-I and HMR-E silencers have the ability to initiate silencing on their own, whereas HMR-I only plays an auxiliary role in silencing at the HMR locus (Brand et al., 1985; Mahoney and Broach, 1989; Rivier et al., 1999; Rusche et al., 2003). There is evidence that HMR-E is stronger than HML-E that is stronger than HML-I in silencing (Shei and Broach, 1995). This likely reflects the structural differences among the silencers. For instance, the binding sites for Abf1p, Rap1p or ORC in the silencers are actually distinct variants of a consensus sequence (see Fig. 1-1A legend) that may have different affinities for the corresponding protein (Boscheron et al., 1996; Buchman et al., 1988). Moreover, the arrangement of the factor-binding sites in each silencer concerning the distance and intervening sequence between any pair of adjacent sites is unique. These factors
may collectively determine the efficiency of a specific silencer in recruiting Sir proteins thereby determining its strength of silencing.

The model for silencer function discussed above implies that initiation of silencing is bidirectional. This is consistent with an early report that *HMR-E* could function in either orientation to silence the *a1* gene at the *HMR* locus (Brand et al., 1985). However, equal silencing on the two sides of *HMR-E* was observed in the presence of *HMR-I* that could also contribute to silencing at *HMR* (Brand et al., 1985; Rivier et al., 1999). In the absence of *HMR-I*, silencing by *HMR-E* was detectably stronger on its Abf1p side than the ORC side (Brand et al., 1985). Moreover, *HMR-E* ectopically inserted at the *MAT* locus or the structurally similar *HML-I* in its native location also preferential silences genes on its Abf1p side (Bi et al., 1999; Shei and Broach, 1995). Therefore, the *HMR-E* or *HML-I* silencer per se seems more active in promoting silencing on its Abf1p side. The directionality of *HML-E* has not been directly examined but it has been shown that *HML-E* could silence *URA3* inserted to the left (telomere-proximal side) (Bi, 2002). The directionality of a silencer could be determined by its intrinsic structural characteristics such as the number and arrangement of factor-binding sites and their affinity for the factors. In addition, such a directionality may be masked by other silencing elements in the genomic context (Brand et al., 1985; Zou et al., 2006b). Moreover, the observed directionality in a specific experiment is also dependent on the sensitivity/resolution of the assay used (Zou et al., 2006b).

In this report, I have further examined the directionality of silencing by *HMR-E* both in its native *HMR* location and in the context of *HML*, using three different reporter genes *URA3*, *a1* and *α1*, respectively. The results provide further support for the notion that for
both *HMR-E* and *HML-I* silencing is generally robust on the Abf1p side but diminished on the ORC side. Importantly, I then show that this is correlated with asymmetric positioning of nucleosomes on the two sides of the silencer. Specifically, a nucleosome is stably positioned close to the Abf1p site but not the ORC site of the silencer. And I also examined that *HML-E* placed in the context of *HML-I* promoted moderate silencing on its Rap1p side but no silencing on the ORC side, which is also correlated with asymmetric positioning of nucleosomes on the two sides of the silencer. Moreover, asymmetric positioning of nucleosomes in the immediate vicinity of a silencer is not dependent on its orientation or genomic context and is therefore the inherent property of the silencer. It is also independent of Sir proteins and therefore precedes the formation of silent chromatin. On the other hand, the Sir complex served to stabilize the specific chromatin configuration at each silencer and organize nucleosomes in its path of propagation into a compact array.
1.3 Materials and Methods

1.3.1 Plasmids and strains

The sequences of “full-length” *HML-I* and *HMR-E* shown in this thesis were those defined in the original deletion analyses of *HML* and *HMR* (Abraham et al., 1984; Feldman et al., 1984). Specifically, *HML-I* and *HMR-E* correspond to coordinates 14555-14839, and 291245-291559 of chromosome III, respectively. Every silencer contains a core element consisting of binding sites for the silencer-binding proteins and flanking sequences as illustrated in Fig. 1-1A.

Plasmid pUC26 was made by inserting the BamHI-*HML*-BamHI fragment (9666-16263 of chromosome III) into pUC19. A 1.1 kb *URA3* sequence (Bi et al., 1999) was inserted at the EcoRV site (15411) of pUC26 to make pYZ1-01. The HpaI-*HML-I*-HindIII sequence (14555-14839) in pYZ1-01 was replaced by a HindIII site to make pYZ1-07. *HML-I* was inserted at the HindIII site of pYZ1-07 in the opposite direction (as compared to that in pYZ1-1) resulting in pYZ1-02. The *HMR-E* sequence in either direction was inserted at the HindIII site of pYZ1-07 resulting in pYZ1-03 and -04. The *HML-E* sequence in either direction was inserted at the HindIII site of pYZ1-07 resulting in pYZ1-05 and -06. Plasmid pUC18-HMR was made by inserting HindIII-*HMR*-HindIII (289227-294210) into pUC18. pYZ1-08 was made by inserting *URA3* at the SpeI site (291110) of pUC18-HMR. *HMR-E* in pYZ1-08 was replaced by a BamHI site resulting in pYZ1-08m. An inverted *HMR-E* was inserted at the BamHI site of pYZ1-08m making pYZ1-09. *URA3* was inserted at the XbaI site (291731) of pUC18-HMR making plasmid pYZ1-10m. The *HMR-I* silencer was deleted from pYZ1-10m resulting in pYZ1-10. *HMR-E* in pYZ1-10 was replaced by a BamHI site resulting in pYZ1-11m. An inverted *HMR-E* was inserted at the BamHI site of pYZ1-11m
making pYZ1-11. Plasmids pYZ1-12 and 1-13 were derived from pYZ1-08 and 1-09 by deleting $HMR-I$, respectively. Plasmids pYZ1-14 and 1-15 were derived from pYZ1-03 and 1-04 by deleting $HML-E$, respectively. pMB21 is an integration plasmid containing $SIR3$-$TRP1$ and $SUP4-o$ (Bi et al., 1999).

The names and relevant genotypes of yeast strains were listed in Table 1. Strains 1-01s – 1-11s were made by transforming Y729 to Ura$^+$ with BamHI-digested pYZ1-01 – 1-07 and pYZ1-14 – 1-15, respectively. Strains 1-08s – 1-11s were made by transforming Y729 to Ura$^+$ with HindIII-digested pYZ1-08 – 1-11, respectively. These strains were rendered $SIR3^+$ by integrating pMB21 at $TRP1$ in the genome, resulting in strains 1-01 ~ 1-08 and 1-11 ~ 1-12. Strains 1-12 and 1-13 were made by transforming W303$\alpha$ to Ura$^+$ with HindIII-digested pYZ1-12 and 1-13, respectively. $SIR3$ in strains 1-12 and 1-13 was replaced by kanMX resulting in strains 1-12s and 1-13s, respectively. $SIR1$, $SIR2$ and $SIR4$ in strain 1-04 were replaced by kanMX resulting in strains 1-04sir1, 1-04sir2 and 1-04sir4, respectively. Strains 1-03’ and 1-04’ was made by transforming 1-03 and 1-04 to Geneticin-resistant with a PCR-produced fragment encoding 9-myc linked to kanMX embedded in a sequence spanning the 3’ region of $SIR3$ ORF. The relevant genotypes of all strains were confirmed by Southern blotting and/or PCR.

### 1.3.2 Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from cells grown to an optical density of about 0.6 and diluted to a concentration of 1 $\mu$g/ml. The RNA sample was treated with DNase I at room
temperature for 40 minutes. DNase I was then heat inactivated at 70°C for 10 minutes. The sample was then used as template for RT-PCR with the SuperScript III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen) in a 25 µl reaction. Fivefold serial dilutions were used in PCR as follows: for measuring ACT1 expression, 0.05 and 0.25 µl of RNA were used in a 25-cycle PCR. For URA3, HMRa1 and HMLα1, 0.5 and 2.5 µl were used in a 32-cycle PCR. PCR products were fractionated on 2.0% agarose gels. The intensity of each band was determined using NIH-image. No-RT control PCR was performed by using Platinum Taq DNA polymerase instead of the RT/ Platinum® Taq Mix.

The sequences of PCR primers used in RT-PCR (Fig. 1-4 and 1-5) were listed in table 2.

1.3.3 Chromatin immunoprecipitation (ChIP)

ChIP was carried out as described (Chiu et al., 2003; Sandmeier et al., 2002) except for a few minor modifications. SC cultures (50 ml) of strain 1-03’ or 1-04’ were grown to log phase (0.8–1.2 OD600) and then fixed for 20 min at room temperature (RT) in 1% formaldehyde. Cells were harvested and washed twice with dH2O and FA-lysis 140 buffer (50 mm HEPES-KOH pH 7.5, 140 mm NaCl, 1 mm EDTA, 1% Triton X-100, 0.1% deoxy-cholic acid). Cell pellets were resuspended in 500µl FA-lysis 140 (with 10µl Sigma P-8215) and then 600µl acid-washed glass beads (425–600 µm) were added. A BioSpecMini-Beadbeater was used to make the extracts (eight 30-sec-cycles) that were then transferred to new Eppendorf tubes. With the use of a Branson Sonifier 450 the extracts were sonicated 12 times for eight pulses each time at 90% duty cycle and output setting 4. The lysate was then clarified by centrifugation at 14,000 rpm for 20 min. 150µl whole-cell
extract was added to each immunoprecipitation (IP) for a final volume of 250 µl in FA-lysis 140 with Sigma P-8215 at 16 µl/ml. Serum antibodies against c-myc tag (Roche mouse monoclonal antibody (clone 9E10), 0.4 mg/ml) were added at 2 µl/IP. Incubation of IP reactions was done at 4°C overnight.

Bound chromatin was precipitated with 20 µl of Protein A Sepharose beads (50% slurry in 1 x TE/0.1% BSA/0.1% Na Azide) for 2 hr at 4 °C. The beads were washed extensively and the immune complexes were eluted twice with 200 µl 1% SDS/0.1 m NaHCO₃ at RT. The cross-links were then reversed at 65°C for 5 hr in the presence of NaCl and ethanol precipitated overnight at -20°C. The recovered material was RNase A and Proteinase K treated and phenol:chloroform extracted. Purified DNA was resuspended in 200 µl 1 x TE. 3 µl of each sample was used in 25-µl PCR reactions where the $T_A$ was 50°C for 28 cycles. In PCR reactions the proper amount of input and IPed chromatin DNA used was predetermined to be in the linear range by serial dilutions. Input chromatin was added to PCR reactions as a 1:20 dilution. PCR products were separated on a 1.3% agarose gel. Images were captured with VisionWorks 32 software from UVP (San Gabriel, CA) and bands were quantified using NIH-image.

The sequences of PCR primers used in ChIP (Fig. 1-6) were listed in table 3.

1.3.4 Chromatin mapping by MNase digestion and indirect end labeling

Chromatin analysis was carried out as described (Bi et al., 2004; Sandmeier et al., 2002). Spheroplasts made from ~10⁹ log-phase cells by using zymolyase were permeabilized with NP-40 as described (Kent et al., 1993). About 2 x 10⁸ permeabilized spheroplasts were
treated with MNase at 75, 150, and 300 units/ml, respectively, at 37°C for 4 minutes. The reaction was stopped by 0.5% sodium dodecyl sulfate and 25 mM EDTA, and the DNA was isolated. An aliquot of permeabilized spheroplasts not treated with MNase was used to isolate naked DNA, which was digested with MNase at 7.5 U/ml at 37°C for 1 min. The DNA in each sample was then digested with a restriction endonuclease (AvaII, BamHI, BsrGI or XbaI) and run on a 1.0% agarose gel. The relevant fragments were visualized by an appropriate probe (see Fig. 1-7 to 1-11) after Southern blotting.

The sequences of PCR primers used for probes in chromatin mapping (Fig. 1-7 to 1-11) were listed in table 4.
1.4 Results

1.4.1 Directional silencing by *S. cerevisiae* silencers

It was puzzling that *HML-I* initiate silencing only on its Abf1p side at the *HML* locus whereas the structural similar *HMR-E* apparently functions in an orientation-independent manner at the *HMR* locus (Bi et al., 1999; Brand et al., 1985). To avoid the effect of the genomic context on the silencer directionality, I tested the functions of the *HMR-E*, *HML-I* and *HML-E* silencers in the same genomic location.

![Fig. 1-1. Directional silencing by yeast silencers.](image)

(A) *HML* and *HMR* on chromosome III in *S. cerevisiae* (not drawn to scale). *HML-E* and –*I* are shown as filled arrows with white letters, and *HMR-E* and –*I* as open arrows with black letters. Note the directions of silencers are drawn as pointing toward the inside of *HML* or *HMR*, but are not necessarily the functional directions. The sites for Abf1p, Rap1p, and ACS (ARS consensus sequence) for ORC recognition in silencers are indicated by stippled, filled and open rectangles, respectively. They are variants of motifs 5’-RTCRRNNNNACG-3’ (for Abf1p), 5’-ACACCCRYACAYM-3’ (Rap1p), and 5’-WTTTAYRTTTW-3’ (ORC) (M=A/C, N=A/C/T/G, R=A/G, Y=C/T, W=A/T) (Breier et al., 2004; Lieb et al., 2001; Rjode et al., 1992). Arrows above the factor-binding sites correspond to the 5’→3’ directions of these consensus sequences. The α1 and α2 genes at *HML* and α1 and α2 genes at *HMR* are also indicated. *CEN*, centromere. Tandem arrow heads, telomeric repeats. (B) Silencers promote Sir-dependent directional silencing. Left, modified *HML* locus in strains 1-01 – 1-07. Cells of each strain were grown to late log phase and serial 10-fold dilutions were spotted on test plates and allowed to grow for 3 days. SC, synthetic complete medium. FOA, SC plus 1 mg/ml of 5-fluoroorotic acid (FOA). Note that *HMLα* (two white arrows in the black box) shown in stain 1-01 is also present in strains 1-02 - 1-07 where it is shown as a line.
To further examine the directionality of silencing by HMR-E. I tested its function in the context of HML-I. HMR-E in either orientation was used to replace HML-I and tested for its ability to silence a URA3 gene inserted nearby (Fig. 1-1B, strains 1-03 and 1-04). The URA3 gene is a widely used reporter for studying transcriptional silencing (van Leeuwen and Gottschling, 2002). Ura3p is involved in pyrimidine biosynthesis and can convert 5-fluoroorotic acid (FOA) to a toxic metabolite thus its expression renders cells sensitive to FOA (Boeke et al., 1987). Therefore, URA3 silencing could be measured by FOA-resistance of the cell. URA3 inserted to the right (centromere-proximal) side of HML-I was not silenced (Fig. 1-1B, lack of growth of strain 1-01 on FOA medium). However, it was silenced when HML-I was inverted (Fig. 1-1B, strain 1-02), confirming that HML-I acts in a directional fashion (Bi et al., 1999). Strain 1-03 was sensitive, whereas 1-04 was resistant, to FOA (Fig. 1-1B, 1-03 and 1-04 on FOA). Therefore, like HML-I, HMR-E in the context of HML-I also preferentially initiates silencing on its Abf1p side.

I next tested whether HML-E also promoted directional silencing when placed in the context of HML-I. To this end, I replaced the HML-I silencer in strain 1-01 with HML-E in either direction (Fig. 1-1B, strains 1-05 and 1-06). URA3 silencing was weaker but detectable when HML-E was pointing away from URA3, but was barely detectable when HML-E was in the opposite direction (Fig. 1-1B, compare 1-05 and 1-06). Therefore, HML-E in the context of HML-I preferentially promotes silencing on its Rap1p side, but its silencing efficiency is significantly lower than that of HML-I or HMR-E.

It was formally possible that in trains 1-01 – 1-06, URA3 silencing resulted from the action of the resident HML-E silencer on the left of HMLα, and the silencers present on the
right side of HMLα served as orientation-dependent barriers to the spreading of silencing initiated from the resident HML-E. I tested this by examining URA3 silencing in strain 1-07 that was derived from strain 1-01 by deletion the HML-I silencer (Fig. 1-1B). The lack of URA3 silencing in strain 1-07 (Fig. 1-1B) indicated that silencing initiated at the resident HML-E silencer was not able to propagate to URA3. Therefore, URA3 silencing in strains 1-01 – 1-06 was mediated by silencers present in the position of HML-I and HML-E, HML-I and HMR-E all promote directional silencing at this genomic context.

![Figure 1-2](image)

**Fig. 1-2. HMR-E promotes directional silencing in its native location.** Left, modified HMR locus in strains 1-08 – 1-11. [Δ] indicates the deletion of HMR-I. Note that HMRα (two black arrows in the white box) shown in strain 1-08 is also present in strains 1-09 - 1-11 where it is shown as a line.

I also re-examined the directionality of HMR-E in its native location using the URA3 gene as the reporter for silencing. URA3 was not silenced when inserted to the left (centromere-proximal) of the resident HMR-E silencer (Fig. 1-2, strain 1-08), but was silenced when the direction of HMR-E was inverted (Fig. 1-2, compare strains 1-09 and 1-08). On the other hand, URA3 was silenced when inserted to the right of the resident HMR-E silencer in the absence of HMR-I, but was not silenced when HMR-E was inverted (Fig. 1-2, compare strains 1-10 and 1-11). These results demonstrate that HMR-E also preferentially
initiate silencing on its Abf1p side in its endogenous context when *URA3* is used as the reporter. Note that silencing of *URA3* expression in strains 1-01 – 1-11 and other strains used in this work was *SIR*-dependent as it was completely abolished by deleting *SIR3* (Fig. 1-1B, Fig. 1-2 and Fig. 1-3 compare strains 1-01s – 1-11s with 1-01 – 1-11, respectively, on FOA; data not shown).

**Fig. 1-3. Silencers promote Sir-dependent directional silencing.** Left, modified *HML* and *HMR* loci in strains 1-01s – 1-06s and 1-08s – 1-11s.

To directly compare the directionality of *HMR-E* in silencing *URA3* and that in silencing the native *HMRα* genes we made strains 1-12 and 1-13 in which *HMR-E* was in opposite directions (Fig. 1-4A). These *MATα* strains both have *URA3* inserted to the left of
HMR and have HMR-I deleted (Fig. 1-4A), and would therefore allow simultaneous measurement of silencing of URA3 and HMRa by HMR-E alone. The abundance of the transcripts of URA3 and HMRa1 as well as the control ACT1 were measured by quantitative RT-PCR in strains 1-12 and 1-13 as well as their sir3 derivatives 1-12s and 1-13s. Three independent experiments were performed and a representative gel picture is shown in Fig. 1-4B. Data from all three experiments were quantified and the mean of the relative abundance of URA3 or a1 in each strain (together with standard deviation) was graphed in Fig. 1-4C. The abundance of URA3 in the SIR3+ strain 1-13 was only ~1/5 of that in the sir3 strain 1-13s demonstrating that HMR-E silences URA3 inserted on its Abf1p side. On the other hand, the level of URA3 in strain 1-12 was not significantly different from that in strain 1-12s demonstrating that HMR-E fails to silence URA3 inserted on its ORC side.

The HMRa1 message was barely detectable in strain 1-12 but was abundant in strain 1-12s (Fig. 1-4C, compare shaded bars 1-12 and 1-12s), demonstrating that HMRa1 is fully silenced by HMR-E. In strain 1-13, HMRa1 was detectable and its abundance was ~1/3 of that in the sir3 strain 1-13s (Fig. 1-4C, compare shaded bars 13 and 13s). These data indicate that silencing of HMRa1 is partial in strain 1-13 compared to that in strain 1-12 (Fig. 1-4C, compare shaded bars 1-13 and 1-12), and therefore HMR-E preferentially silences HMRa1 on its Abf1p side. This is consistent with the early S1 mapping result shown by Brand et al. on a1 expression showing that although HMR-E in either orientation could silence the a1 gene, the efficiency of silencing is nevertheless orientation-dependent (a1 transcript was not detected when HMR-E was orientated toward a1, but was present, albeit with low abundance, when HMR-E was in the opposite direction) (Brand et al., 1985).
Fig. 1-4. Analysis of the directionality of HMR-E silencing at its native locus using quantitative RT-PCR. (A) Modified HMR locus in strains 1-12 and 1-13. [Δ] indicates the deletion of HMR-I. (B) Measurement of the abundance of URA3 and HMRα1 transcripts in strains 1-12, 1-13, 1-12s and 1-13s by qRT-PCR. Three independent experiments were performed and the products were analyzed by agarose gel electrophoresis. Shown were results from one of these experiments. In each lane, the template concentration for URA3 and HMRα1 was the same, but that for ACT1 was 10 fold lower (see Materials and Methods 1.3.2). The template concentration in the even-numbered lanes was one fifth of that in the odd-numbered ones. The RNA template for No-RT control was of strain 1-12s. (C) Quantification and analysis of qRT-PCR data. Each open or shaded bar represents the ratio of intensity of URA3 or HMRα1 product over that of the ACT1 product in a strain. The means of data from all three experiments together with standard deviation are presented.
I also examined whether \textit{HMR-E} ectopically placed at \textit{HML} silenced \textit{HML}\textit{\alpha} in an orientation dependent manner. The \textit{MAT\alpha} strains 1-14 and 1-15 were deleted for \textit{HML-E}, and had \textit{HML-I} replaced by \textit{HMR-E} in opposite directions, and \textit{URA3} inserted to the right of \textit{HML} (Fig. 1-5A). I examined the expression of \textit{URA3} and \textit{HML}\textit{\alpha}1 in strains 1-14 and 1-15 as well as their \textit{sir3}' derivatives 1-14s and 1-15s simultaneously by qRT-PCR. Note that when \textit{HML}\textit{\alpha} is derepressed in a \textit{MAT\alpha} strain (e.g., by disrupting the \textit{SIR} genes), the \textit{\alpha}1 and \textit{\alpha}2 proteins form a complex that negatively regulates the expression of \textit{HML}\textit{\alpha}1 and \textit{\alpha}2 (Klar et al., 1981; Palacios DeBeer and Fox, 1999; Siliciano and Tatchell, 1984). However, \textit{\alpha}1 mRNA is detectable in such a strain especially when methods with high sensitivity like qRT-PCR are used (Chi and Shore, 1996; Matecic et al., 2006; Wyrick et al., 1999). Matecic et al. has demonstrated that the transcriptional state of \textit{HML} could be examined quantitatively by measuring the abundance of \textit{HML}\textit{\alpha}1 message (Matecic et al., 2006). As shown in Fig. 1-5B and C, \textit{URA3} expression in strain 1-15 was about 1/5 of that in 1-15s and was therefore silenced by \textit{HMR-E} in strain 1-15. On the other hand, \textit{URA3} was not silenced by \textit{HMR-E} in strain 1-14 (Fig. 1-5B and C, compare 1-14 and 1-14s). \textit{HML}\textit{\alpha}1 in strain 1-15 was about 1/5 of that in strain 1-15s, whereas it was not detectable in strain 1-14 (Fig. 1-5B and C, compare 1-15 to 1-15s and 1-14 to 1-14s). Therefore, while \textit{HML}\textit{\alpha} was completely silenced by \textit{HMR-E} in strain 1-14, it was partially silenced in strain 1-15. These results support the notion that silencing by \textit{HMR-E} is stronger on its Abf1p side, and is therefore directional.

The above results of measuring messengers RNA confirmed that the \textit{HMR-E} silencer is a unidirectional silencer. It preferentially promotes silencing on its Abf1p side but not the ORC side.
Fig. 1-5. Analysis of the directionality of HMR-E silencing at HML-I locus using quantitative RT-PCR. (A) Modified HMR loci in strains 1-14 and 1-15. [Δ] indicates the deletion of HML-E. (B) Measurement of the abundance of URA3 and HMLα1 transcripts in strains 1-14, 1-15, 1-14s and 1-15s by qRT-PCR. In each lane, the template concentration for URA3 and HMLα1 was the same, but was 10 fold lower for ACT1. The template concentration in the even-numbered lanes was one fifth of that in the odd-numbered ones. The RNA template for No-RT control was of strain 1-14s. (C) Quantification of qRT-PCR data. The ratio of intensity of URA3 or HMLα1 product over that of the ACT1 product in each strain is listed in the table.
1.4.2 Directional establishment of transcriptionally silent chromatin by the \textit{HMR-E} silencer

To test whether directional silencing by a silencer is correlated with directional formation of silent chromatin, we used chromatin immunoprecipitation (ChIP) to examine the association of Sir3p with \textit{HMR-E} in either orientation as well as adjacent sequences. I tagged Sir3p with the myc epitope in strains 1-03 and 1-04 (Fig. 1-1B) resulting in 1-03’ and 1-04’, respectively (Fig. 1-6A). This had no detectable effect on the silencing of \textit{URA3} or the directionality of \textit{HMR-E} (Fig. 1-1B and 1-6A, compare 1-03’ and 1-04’ to 1-03 and 1-04, respectively). ChIP was performed with a myc antibody (\(\alpha\)-myc). The abundance of sequences a (corresponding specifically to \textit{HMR-E} in the context of \textit{HML-I}), b-d (Fig. 1-6A, left), a control sequence spanning the \textit{HMR-I} silencer, and another control from the \textit{ACT1} locus, in the immunoprecipitated chromatin fragments were measured by PCR. All the sequences measured are between 250 to 350 bp in length. Three independent ChIP experiments were performed and a representative gel picture was presented (Fig. 1-6B). The intensity of each band was quantified and normalized against input control. The relative abundance of Sir3p-myc associated with a sequence in a strain was estimated as the ratio of the intensity of the corresponding fragment over that of the \textit{HMR-I} control (taken as 1.0) in the same strain. The mean of data from all three experiments (together with the standard deviation) was graphed in Fig. 1-6C. For both strains 1-03’ and 1-04’, there was little or no Sir3p-myc associated with the \textit{ACT1} sequence (Fig. 1-6C, \textit{ACT1}), which was consistent with the fact that \textit{ACT1} is normally not subject to transcriptional silencing, and not associated with Sir proteins. On the other hand, the abundance of Sir3p-myc at sequence a was high in both strains 1-03’ and 1-04’ (Fig. 1-6C, a), indicating that \textit{HMR-E} in either orientation is able to
recruit Sir3p-myc. In strain 1-04’, the Sir3p-myc abundance at b-d was also high (Fig. 1-6C, b-d, shaded bars), which was indicative of Sir3p-myc spreading from HMR-E toward URA3. On the other hand, Sir3p-myc abundance at b-d was significantly less abundant in 1-03’ than in 1-04’ (Fig. 1-6C, compare strain 1-03’ and 1-04’ at b-d) indicating that there was reduced spreading of Sir3p-myc from HMR-E, which was in accord with diminished silencing of URA3 in strain 1-03’. These results demonstrate that HMR-E initiates the spread of Sir proteins preferentially on its Abf1p side, which is consistent with previous data obtained by Loo and Rine indicating that Sir-dependent silent chromatin extends more efficiently on the Abf1p side than the ORC side of HMR-E (Loo and Rine, 1994).

Fig. 1-6. Directional spread of Sir3p promoted by HMR-E. (A) Myc-tagged Sir3p is functional in silencing. Strains 1-03’ and 1-04’ were identical to 1-03 and 1-04, respectively, except that 9 tandem copies of a sequence encoding the myc tag were fused in frame to the 3’ end of the SIR3 ORF. Sequences a-d to be tested by PCR in ChIP were indicated (roughly proportional). Growth phenotypes of strains 1-03’ and 1-04’ were shown. (B) Analysis of Sir3p-myc abundance near HMR-E by ChIP. The abundance of a-d as well as control sequences from HMR-I and ACT1 was measured by PCR before (input) and after (α-myc) chromatin IP. No Ab, samples from mock ChIP without using antibody. Data from three independent ChIP experiments were quantified and plotted in (C). See text for more descriptions.
1.4.3 Unidirectional silencing by *HML-I* or *HMR-E* coincides with an asymmetric distribution of nucleosomes

The directional nature of *HML-I* or *HMR-E* function might result from the fact that it consists of an asymmetric DNA sequence, and perhaps more importantly, that its factor-binding sites are arranged in a directional fashion: ORC-Rap1p-Abf1p (Fig. 1-1A). How might the sequence/structural asymmetry of a silencer be translated into functional asymmetry? Propagation of Sir complexes along the chromatin depends on their association with nucleosomes and presumably requires a continuous array of closely positioned nucleosomes (Bi et al., 2004; Rusche et al., 2003). Given that Abf1p, Rap1p and ORC all have the ability to modulate local chromatin structure upon binding to DNA (Bi et al., 2004; Lipford and Bell, 2001; Rusche et al., 2003), nucleosomes might be organized differently (asymmetrically) on the flanks of *HML-I* and *HMR-E* resulting in unequal potentials for the spread of Sir proteins on the two sides. To test this hypothesis, I mapped nucleosomes around *HML-I* and *HMR-E* using micrococcal nuclease (MNase) digestion and indirect end labeling (Ryan et al., 1999). MNase introduces double-stranded breaks mainly in chromosomal DNA not assembled into nucleosomes (i.e., linker DNA), and therefore the presence of a nucleosome could be inferred from a MNase-resistant region of about 147 bp (the length of DNA in a nucleosome) bordered on both sides by MNase-sensitive sites (Ryan et al., 1999). To determine the putative positions of nucleosomes, the chromatin is subjected to partial digestion with MNase yielding a series of fragments associated with various numbers of nucleosomes. DNAs from these fragments are then examined by indirect end labeling to reveal the positions of MNase cleavage sites relative to a reference restriction site,
thereby allowing a tentative assignment of sequences protected by nucleosomes in the region of interest (Ryan et al., 1999).

I first mapped chromatin around HML-I in its original orientation in strain 1-01. DNA purified from MNase-treated chromatin was digested with AvaII 300 bp downstream from HML-I, and was then subjected to electrophoresis and Southern blotting using a probe corresponding to a 200 bp sequence abutting the AvaII site (Fig. 1-7, bar labeled probe). I found that the HML-I core (consisting of the binding sites for Abf1p, Rap1p and ORC) coincided with three MNase-sensitive sites that were likely caused by the binding of Abf1p, Rap1p and ORC (Fig. 1-7, lanes 1-3). Notably, the patterns of MNase cleavage on the two sides of the core sequence were different. Specifically, the region adjacent to ACS (ORC recognition site) contained a MNase-hypersensitive region (labeled by a dot) and two moderately sensitive sites that were indicative of the absence of a stably positioned nucleosome (Fig. 1-7, compare lanes 1-3 with N1; the dashed ellipse indicates the lack of a stably positioned nucleosome). In contrast, on the Abf1p side there were two MNase-sensitive sites bracketing a region of ~150 bp that was relatively resistant to MNase cleavage, a pattern indicative of the presence of a positioned nucleosome (Fig. 1-7, lanes 1-3, filled ellipse labeled 1’). Therefore, the organization of nucleosomes around HML-I in strain 1-01 was asymmetric, and the absence of a stably positioned nucleosome near ACS coincided with the lack of URA3 silencing on this side of HML-I as shown in Fig. 1-1B. To examine if the Sir complex was involved in the asymmetric positioning of nucleosomes at HML-I, I disrupted SIR3 in strain 1-01 making 1-01s. The MNase cleavage pattern around HML-I in strain 1-01s was similar to that in 1-01 (Fig. 1-7, compare lanes 4-6 with 1-3) indicating that an intact Sir complex is not required for asymmetric nucleosome positioning at HML-I.
I next mapped chromatin around the inverted \textit{HML-I} in strain 1-02. The pattern of MNase digestion within the inverted \textit{HML-I} sequence mirrored that of \textit{HML-I} in its original direction indicating that the structure of \textit{HML-I} was not affected by its inversion (Fig. 1-7, compare lanes 10-12 with 1-3). Strikingly, the pattern of nucleosome positioning on either side of the inverted \textit{HML-I} in strain 1-02 was dramatically different from that of \textit{HML-I} in strain 1-1 (Fig. 1-7, compare lanes 10-12 with 1-3). On the Abf1p side, three consecutive nucleosomes could be inferred (Fig. 1-7, right, filled ellipses labeled 1-3), which is consistent with data of chromatin mapping on the Abf1p side of native \textit{HML-I} obtained by Weiss and

\textbf{Fig. 1-7. Asymmetric positioning of nucleosomes around the \textit{HML-I} silencer.} DNA isolated from MNase-treated chromatin in strains 1-01 and 1-02 were digested with AvaiI, and then subjected to gel electrophoresis and Southern blotting using a probe abutting the AvaiI site. The positions of the \textit{HML-I} silencer and \textit{URA3} in each strain are shown. Filled ellipse, inferred stably positioned nucleosome. Dashed ellipse, lack of stably positioned nucleosome. N1-N2, MNase-treated naked genomic DNA from strains 1-01 and 1-02, respectively. See text for detailed descriptions.
Simpson (Weiss and Simpson, 1998). On the other hand, no stable nucleosomes were located immediately adjacent to ACS (Fig. 1-7, right, the dashed ellipse). Therefore, flipping HML-I led to an apparent inversion of the pattern of nucleosome positioning around the silencer core (Fig. 1-7, compare lanes 10-12 with 1-3, note the apparent two-fold rotational symmetry between sites a and a’, between the two dashed ellipse, and filled ellipses 1 and 1’). The presence of nucleosomes 1-3 on the Abf1p side of HML-I coincided with robust URA3 silencing on this side (Fig. 1-1B and 1-7, strain 1-02). To examine if the Sir complex played any role in forming the asymmetric structure of chromatin around the inverted HML-I, I deleted SIR3 in strain 1-02 making 1-02s. In strain 1-02s, site b marking the border of nucleosome 3 disappeared, and certain sites within the region spanning nucleosomes 2 and 3 became sensitive to MNase (Fig. 1-7, compare lanes 7-9 with 10-12). Therefore, it could be inferred that nucleosomes 2 and 3 were no longer stably positioned there in strain 1-02s. This suggested that the positioning of nucleosome of 2 and 3 was the result of the spread of the Sir complex in strain 1-02. On the other hand, nucleosome 1 remained positioned there in 1-02s (Fig. 1-7, compare lanes 7-9 to 10-12, note that band a marking the border of the putative nucleosome 1 was retained). Similar results regarding the effect of SIR3 deletion on nucleosomes near the Abf1p side of HML-I was obtained by Weiss and Simpson (Weiss and Simpson, 1998). In summary, I conclude that asymmetric positioning of nucleosomes on the Abf1p and ORC sides of HML-I is independent of the direction of the silencer and of intact Sir complexes.

HMR-E is similar to HML-I in that it also contains binding sites for Abf1p, Rap1p and ORC in the same order (Fig. 1A). We examined if HMR-E organized chromatin in a similar way as HML-I by mapping chromatin around HMR-E in strains 1-03 and 1-04, as well as
their \textit{sir3}' derivatives 1-03s and 1-04s. The distribution of MNase-sensitive sites around 
\textit{HMR-E} in strain 1-03 was reminiscent of that around \textit{HML-I} in strain 1-01 (compare lanes 1-3 in Fig. 1-8 with 1-3 in Fig. 1-7). A stable nucleosome was inferred as positioned near the Abf1p site but not the ORC site of \textit{HMR-E} (Fig. 1-8, left, filled ellipse \textbf{1}' and dashed ellipse). The pattern of nucleosome positioning was largely unchanged by the deletion of \textit{SIR3} (Fig. 1-8, compare lanes 4-6 with 1-3), which was similar to the situation regarding \textit{HML-I} (Fig. 1-7, lanes 1-6). Chromatin configuration around inverted \textit{HMR-E} in strain 1-04 was again very similar to that around inverted \textit{HML-I} in strain 1-02 (compare Fig. 1-8 and Fig. 1-7). Three consecutive nucleosomes (labeled \textbf{1}-\textbf{3}) were positioned near the Abf1p sites (Fig. 1-8, lanes 10-12), a pattern similar to chromatin mapping data of native \textit{HMR-E} obtained by Ravindra et al. (Ravindra et al., 1999). There were no stably positioned nucleosomes adjacent to ACS (lanes 10-12 in Fig. 1-8). \textit{SIR3} deletion had no significant effect on the positioning of nucleosome \textbf{1} (Fig. 1-8, compare lanes 7-9 with 10-12, note the presence of site \textbf{a} inferred as the border of nucleosome \textbf{1} in lanes 7-9). On the other hand, site \textbf{b} inferred as the border of nucleosome \textbf{3} became blurred indicating that nucleosomes \textbf{3} and \textbf{2} were no longer as stably positioned in strain 1-04s as in 1-04 (Fig. 1-8, lanes 7-9), which was again similar to the situation concerning \textit{HML-I} (Fig. 1-7, lanes 7-9). Nucleosomes \textbf{2} and \textbf{3} were likely positioned/stabilized by the Sir complex during the formation of silent chromatin on the Abf1p side of \textit{HMR-E} in strain 1-04. In summary, these results demonstrate that, similar to \textit{HML-I}, \textit{HMR-E} also asymmetrically positions nucleosomes in an orientation- and Sir complex-independent fashion, and a stably positioned nucleosome coincides with the presence of Sir3p and robust silencing on the Abf1p side (Fig. 1-1B and 1-6C, strains 1-04.
and 1-04’), whereas the lack of a positioned nucleosome adjacent to ACS coincides with the absence of silencing on the ACS side (Fig. 1-1B and 1-6C, strains 1-03 and 1-03’).

**Fig. 1-8.** Asymmetric positioning of nucleosomes around the *HMR-E* silencer. DNA isolated from MNase-treated chromatin in strains 1-03 and 1-04 were digested with AvaII, and then subjected to gel electrophoresis and Southern blotting using a probe abutting the AvaII site. The positions of the *HMR-E* silencer and *URA3* in each strain are shown. Filled ellipse, inferred stably positioned nucleosome. Dashed ellipse, lack of stably positioned nucleosome. N3-N4, MNase-treated naked genomic DNA from strains 1-03 and 1-04, respectively. See text for detailed descriptions.
1.4.4 Nucleosome positioning in the immediate vicinity of a silencer is independent of the Sir proteins

I have shown above that asymmetric nucleosome positioning in the immediate vicinity of a silencer was not significantly affected by the deletion of SIR3 (Fig. 1-7 and 1-8). However, the Sir2p/Sir4p complex can be recruited to silencers in a Sir3p-independent manner (Hoppe et al., 2002). This may be mediated by Rap1p-Sir4p interaction and/or Sir1p-Sir4p interaction (Rusche et al., 2003). To test whether Sir2p/Sir4p, as well as Sir1p play a role in nucleosome positioning at silencers, we deleted SIR1, SIR2, and SIR4, respectively, from strain 1-04 resulting in 1-04sir1, 1-04sir2 and 1-04sir4 (Fig. 4C). I mapped nucleosomes near HMR-E in strains 1-04 (SIR⁺), 1-04sir1 (sir1⁻), 1-04sir2 (sir2⁻), 1-04sir3 (sir3⁻) and 1-04sir4 (sir4⁻). The profile of MNase-sensitive sites in strain 1-04s indicated that deletion of SIR3 had no effect on the positioning of nucleosome 1 near the Abf1p site and the exclusion of a nucleosome adjacent to the ORC site of HMR-E (Fig. 1-9, compare lanes 10-12 to 1-3; see also lanes 7-9 in Fig. 1-8). The profiles of MNase-sensitive sites in strains 1-04sir2 (sir2⁻) and 1-04sir4 (sir4⁻), were both very similar to that in strain 1-04s (sir3⁻) (Fig. 1-9, compare lanes 7-9 and 13-15 to 10-12), which suggested that like Sir3p, Sir2p and Sir4p also did not play a significant role in nucleosome positioning on either side of HMR-E. The pattern of MNase-sensitive sites in strain 1-04sir1 (sir1⁻) was nearly identical to that in strain 1-04 (SIR⁺) (Fig. 1-9, compare 4-6 to 1-3), demonstrating that Sir1p was not involved in nucleosome positioning at HMR-E. In summary, the above data demonstrate that asymmetric nucleosome positioning in the immediate vicinity of HMR-E is generally independent of Sir proteins.
1.4.5 Unidirectional silencing by \textit{HML-E} also coincides with an asymmetric distribution of nucleosomes

The \textit{HML-E} silencer differs from \textit{HML-I} and \textit{HMR-E} in that it lacks the Abf1p-binding site (Fig. 1-1A). I have shown that \textit{HML-E} in the context of \textit{HML-I} preferentially promotes silencing on its Rap1p side, but its efficiency of silencing was significantly lower.
than that of \textit{HML-I} and \textit{HMR-E} (Fig. 1-1B strains 1-05 and 1-06). I next examined whether \textit{HML-E} also asymmetrically organized nucleosomes. Chromatin mapping was performed on strains 1-05 and 1-06 and their \textit{sir3} derivatives 1-05s and 1-06s. As shown in Fig. 1-10, the core sequence of \textit{HML-E} in strain 1-05 or 1-06 composed of the binding sites for Rap1p and ORC was flanked by two MNase-hypersensitive sites (Lane 1, 2, 7 and 8; the dot indicates the MNase site outside of \textit{HML-E}). In both strains 1-05 and 1-06, a nucleosome could be inferred as positioned close (about 50 bp) to the Rap1p binding site (Fig. 1-10, Lane 1, 2, 7 and 8, filled ellipses 1 and 1'), while another about 100 bp away from ACS (Fig. 1-10, Lane 1, 2, 7 and 8, filled ellipses 2 and 2'). Such a pattern of nucleosome positioning at \textit{HML-E} was largely not affected by the deletion of \textit{SIR3} (Fig. 1-10, compare lanes 3 and 4 to 1 and 2, as well as 4 and 6 to 7 and 8). Therefore, \textit{HML-E} also promotes asymmetric positioning of nucleosomes in an orientation and Sir-independent fashion, which is correlated with preferential silencing on its Rap1p side (Fig. 1-1B strain 1-05 and 1-06).

Combining data on silencing and nucleosome positioning promoted by silencers (Fig. 1-1B, 1-7, 1-8 and 1-10), it was clear that silencing occurred on the Abf1p or Rap1p side of a silencer where a nucleosome is stably positioned nearby ($\leq$ 50 bp) but not on the ACS side where there was a sizable gap ($\geq$ 100 bp) between ACS and the nearest nucleosome. This is consistent with the belief that to initiate the formation of silent chromatin, the Sir complex recruited to a silencer has to deacetylate the nearby nucleosome which then serves as a platform for recruiting more Sir complexes. I think that a sizable gap between the silencer-bound Sir complex and the nearest nucleosome would hinder this first step in the propagation of silent chromatin.
Fig. 1-10. Asymmetric positioning of nucleosomes around the HML-E silencer. DNA isolated from MNase-treated chromatin in strains 1-05 and 1-06 were digested with AvaII, and then subjected to gel electrophoresis and southern blotting using a probe abutting the AvaII site. The positions of the HML-E silencer and URA3 in each strain are shown. Filled ellipse, inferred stably positioned nucleosome. Dashed ellipse, lack of stably positioned nucleosome. N5 and N6, MNase-treated naked genomic DNA from strain 1-05 and 1-06. See text for detailed descriptions.
1.4.6 Asymmetric nucleosome positioning by a silencer is not dependent on its genomic context

In the above experiments on chromatin organization around the silencers, \textit{HMR-E} and \textit{HML-E} were placed in the ectopic position of \textit{HML-I} (Fig. 1-8, 1-9 and 1-10). While this enabled us to directly compare the properties of different silencers in the same genomic context, it also raised the question of whether the observed configuration of chromatin around \textit{HMR-E} or \textit{HML-E} was the same as that in its native location. Moreover, \textit{URA3} inserted near silencers (Fig. 1-7, 1-8 and 1-10) might contribute to/alter the chromatin structure at the silencers. To address these questions, I mapped chromatin around \textit{HML-E}, \textit{HML-I} and \textit{HMR-E} in their unmodified native locations in strain Y728 (\textit{SIR}^+) and its \textit{sir3} derivative Y729 (the parent of all the strains used in this work). As shown in Fig. 1-11, the pattern of nucleosome positioning around \textit{HML-E}, \textit{HML-I}, or \textit{HMR-E} in its unmodified genomic context in strain Y728 (\textit{SIR}^+) resembled that around its counterpart in strain 1-05, 1-01 or 1-03, respectively (compare lanes 1, 2 and 3 in Fig. 1-11A to 1 and 2 in Fig. 1-10; compare lanes 7, 8 and 9 in Fig. 1-11 to 1, 2 and 3 in Fig. 1-7; compare lanes 16, 17, 18, 22, 23, and 24 in Fig. 1-11 to 1, 2, 3, 10, 11 and 12 in Fig. 1-8; note the filled and dashed ellipses). \textit{SIR3} deletion had little or no effect on the arrangement of nucleosomes in the vicinity of the native \textit{HML-I} and \textit{HMR-E} silencers (Fig. 1-11, compare lanes 4-6 to 1-3, 10-12 to 7-9, 16-18 to 13-15, as well as 21-24 to 19-21, respectively). These results suggest that the ability of \textit{HML-E}, \textit{HML-I} or \textit{HMR-E} to asymmetrically organize chromatin in its immediate vicinity is its intrinsic property that does not depend on its genomic context.
Fig. 1-11. Chromatin organization around native silencers. DNA from chromatin of strain Y728 (SIR3+) or Y729 (sir3−) treated with MNase was digested with SpeI (Lanes 1-6), AvaII (lanes 7-12), Xbal (13-18) and BsrGI (19-24), respectively. Relevant DNA fragments were revealed by probes a (lanes 1-6), b (7-12), c (13-18) and d (19-24) that are 200 bp in length and are indicated as black bars near the SpeI, AvaII, Xbal and BsrGI sites, respectively, in the diagrams of HML and HMR. The structures of the silencers are shown on the left of the four panels. N, naked DNA control. Other symbols are as described in Fig. 1-7 – 1-10.
1.4.7 ORC plays a critical role in asymmetric chromatin organization and unidirectional silencing by the HMR-E silencer

The above results demonstrated that the absence of a stably positioned nucleosome on the ACS side in HMR-E or HML-I, which was correlated with a deficiency in silencing on this side of the silencer (summarized in Fig. 1-12). It was possible that ORC bound to the silencer prevented nucleosome positioning near the 3’ of ACS (the direction of ACS is taken as 5’-WTTTAYRTTTW-3’). ORC is also involved in the initiation of DNA replication at ARSs (autonomous replication sequences) that are composed of the conserved ACS and Abf1p site, as well as the poorly conserved B1 and B2 elements (Fig. 1-12, ARSI). Interestingly, a unique chromatin configuration is maintained around ARSI with two nucleosomes stably positioned near both the Abf1p-binding site and the ACS sequence, respectively (Fig. 1-12, ARSI) (Lipford and Bell, 2001). This configuration is important for ARS function, and Abf1p and ORC are responsible for its creation/maintenance (Lipford and Bell, 2001; Venditti et al., 1994). Taken together, the features of chromatin organization at silencers and ARSI (Fig. 1-12) suggest that ORC allows the positioning of a nucleosome near the 5’, but hinders nucleosome positioning near the 3’ end of ACS. If this were the case, then inverting ACS in HMR-E should lead to the positioning of a nucleosome near the ACS side of the silencer, which is predicted to allow URA3 silencing to occur on this side.

Another graduate student in our lab, Qun Yu, performed chromatin mapping for mutated HMR-E (either deleting or inverting the ORC-binding site in HMR-E) (Zou et al., 2006a). The results provided direct evidence for a role of ORC in the establishment of asymmetric nucleosome positioning and directional silencing by HMR-E. We think that at HMR-E, ORC helps the initiation of silencing on the Abf1p side by recruiting Sir proteins,
but at the same time hinders silencing on the ORC side by preventing the positioning of a nucleosome. When ACS is inverted or deleted from \textit{HMR-E}, the resultant silencer becomes bi-directional (Zou et al., 2006a). Therefore, ORC plays a critical role in making \textit{HMR-E} function in a unidirectional fashion.

\textbf{Fig. 1-12. Roles of ORC and Abf1p in asymmetric nucleosome positioning and directional silencing by yeast silencers.} Shown are configurations of chromatin at silencers and the replication origin \textit{ARS1}. Illustrated is a summary of data presented in Fig. 1-7, 1-8 and 1-10, and of Lipford and Bell (2002). Abf1p and Rap1p sites are drawn as stippled and filled arrows, and ACS open arrow. B1 and B2 elements in \textit{ARS1} are indicated. Filled ellipse, stably positioned nucleosome. Dashed ellipse, absence of stably positioned nucleosome.
1.4.8 Roles of Abf1p in nucleosome-positioning and transcriptional silencing

Of the three silencer-binding proteins, Abf1p is the least studied concerning its role in the initiation of silencing. A comparison of the configurations of chromatin in the vicinities of \textit{HMR-E}, \textit{HML-I} and \textit{ARS1} revealed that a nucleosome was always positioned near the Abf1p site, which coincided with strong silencing on this side of the silencers (Fig. 1-12). Like ACS, the sequence of the Abf1p-binding site is also asymmetric (Fig. 1-1A legend) and its direction might also be important for its function in silencers. Qun’s chromatin mapping assay indicates that Abf1p contributes to silencer function by, at least in part, positioning nucleosomes, which is independent of the orientation of its binding (Zou et al., 2006a). This is reminiscent of its role in the initiation of DNA replication at \textit{ARS1} (Lipford and Bell, 2001).
1.5 Discussion

The silencers in *S. cerevisiae* present an excellent model system for studying the mechanisms of the establishment of transcriptionally silent chromatin in eukaryotes (Grewal and Moazed, 2003; Rusche et al., 2003). In this work I have investigated the directionality of silencer function and how it is determined. Bi et al. 1999 found previously that the *HML-I* silencer establishes silencing at the HML locus in an Orientation-dependent fashion. I showed here that the *HMR-E* or *HML-E* silencer ectopically placed in the context of *HML-I* also promotes directional silencing. For both *HML-I* and *HMR-E* that are structurally similar, silencing was found to preferentially occur on the Abf1p sides of the silencers (Fig. 1-7 and 1-8). This is in accord with data from previous studies demonstrating unequal silencing on the two sides of *HML-I* or *HMR-E* (Bi et al., 1999; Shei and Broach, 1995). A comparison of data obtained here and previous studies indicates that the difference in silencing efficiency between the two sides of *HMR-E* may vary depending on the assay employed (Bi et al., 1999; Brand et al., 1985; Shei and Broach, 1995; Zou et al., 2006b). This is because the apparent strength of silencing on either side of a silencer may be affected by non-silencer factors including its genomic location (Zou et al., 2006b). Different genomic loci may contain distinct sets of elements that could influence silencer function (e.g., other silencers and protosilencers) (Fourel et al., 2002; Palacios DeBeer and Fox, 1999; Zou et al., 2006b). Along this line, the presence of *HMR-I* at *HMR* likely masked the directionality of *HMR-E* in silencing *HMRα* observed in an early experiment done by Brand et al. (Brand et al., 1985). Consistently, silencing of *α1* by *HMR-E* alone was stronger on the Abf1p side than the ORC side (Brand et al., 1985; van Leeuwen and Gottschling, 2002). The efficiency of silencing on either side of a silencer is also dependent on the promoter strength of the
reporter gene (Brand et al., 1985; van Leeuwen and Gottschling, 2002). For example, I found that on the ORC side of \textit{HMR-E}, \textit{URA3} was completely depressed whereas $a1$ or $\alpha1$ was still silenced albeit partially (Fig. 1-4 and 1-5). As for \textit{HML-E} that lacks an Abf1p-binding site, silencing is modest on the Rap1p side but barely detectable on the ORC side. Therefore, silencers prefer the Abf1p or Rap1p side to the ORC side for initiation of silencing (summarized in Fig. 1-12).

Chromatin plays an important role in the regulation of cellular transactions involving DNA. It has been well established that correct nucleosome positioning at promoters and replication origins are crucial for the initiation of transcription and replication, respectively. I have demonstrated in this work that chromatin also plays a key role in determining the directionality of a silencer. I found that nucleosomes are asymmetrically positioned around the silencers. Specifically, a nucleosome is stably positioned close to (< 50 bp) the Abf1p-binding site but not the ORC recognition site of the \textit{HML-I} or \textit{HMR-E} silencers, which coincides with robust silencing on the Abf1p side and little or no silencing on the ORC side (Fig. 1-12). As for \textit{HML-E}, the distance between the nearest nucleosome and the Rap1p site is about 50 bp, whereas that for ACS is about 100 bp, which is correlated with moderate silencing on the Rap1p side and barely detectable silencing on the ORC side (Fig. 1-12). Importantly, asymmetric nucleosome positioning at a silencer is largely independent of its orientation and genomic location. It is also independent of the Sir proteins and therefore happens before the establishment of silent chromatin. We propose that the asymmetry in chromatin structure at a silencer determines its directional nature of silencing. Specifically, the nucleosome positioned close to the Abf1p/Rap1p side of the silencer allows/facilitates the Sir complex to start spreading on this side, whereas a sizable gap between ORC site and the
nearest nucleosome (e.g. $\geq 100$ bp) reduces the efficiency of initiation of Sir propagation on this side. This hypothesis is based on the fact that nucleosomes are both the substrates for the histone deacetylase activity of the Sir complex and also the platform for Sir recruitment according to the current view of the nucleosome-by-nucleosome propagation of Sir complexes along chromatin (Bi et al., 2004; Grewal and Moazed, 2003; Rusche et al., 2003). In support of this contention, I have demonstrated that Sir3p preferentially associates with sequences on the Abf1p side of the \textit{HMR-E} silencer (Fig. 1-6).

How is asymmetric nucleosome positioning established at silencers? To answer this question, we turned to known examples of chromatin organization at gene promoters and replication origins for clues. At promoters, transcriptional activators can induce changes in chromatin structure either via their own nucleosome disrupting/positioning activities and/or by recruiting chromatin remodeling complexes. Of particular interest is chromatin reorganization by Rap1p and Abf1p at the UASs (upstream activating sequences) of the genes they regulate (Lieb et al., 2001; Miyake et al., 2004). Rap1p or Abf1p is believed to facilitate the access of primary transcriptional activators such as Gcn4p to their binding sites located nearby by disturbing nucleosomes bearing its binding sites thereby “opening” chromatin (Yarragudi et al., 2004; Yu and Morse, 1999). Note that by preventing nucleosome formation on the sequence containing its binding site, Abf1p/Rap1p may help define the boundary of an array of nucleosomes thereby serving to position nucleosomes. Chromatin also plays an important role in the initiation of DNA replication. The \textit{ARS} sequences consisting of ACS, B1, B2 and Abf1p site are free of nucleosomes, whereas two nucleosomes are stably positioned near the Abf1p and ORC sites of \textit{ARS} (Fig. 1-12) (Lipford and Bell, 2001). This pattern of nucleosome arrangement is accomplished by combined
actions of Abf1p and ORC, and its perturbation affects the formation of the pre-replication complex and disrupts replication initiation (Lipford and Bell, 2001).

In light of the roles of Abf1p, Rap1p and ORC in chromatin organization at gene promoters and replication origins, these factors may also be responsible for the establishment of asymmetric nucleosome positioning at silencers. A comparison of chromatin configurations around silencers and \textit{ARS1} suggests that ORC allows the positioning of a nucleosome near the 3' of ACS but excludes one near the 5' end (Fig. 1-12). In accordance with this, Qun Yu showed that inverting ACS in \textit{HMR-E} so that it was orientated toward the inside of the silencer led to the positioning of a nucleosome near the ACS side of the silencer, and a concomitant significant increase in silencing on this side. As a result, there appeared to be symmetric positioning of nucleosomes and comparable silencing on the two sides of the modified \textit{HMR-E} (Zou et al., 2006a). On the other hand, inversion of ACS in an \textit{ARS} disrupts \textit{ARS} function (Holmes and Smith, 1989), likely because it disrupts the nucleosome normally positioned near ACS outside of \textit{ARS} (see Fig. 1-12) that is important for \textit{ARS} function. These results suggest that ACS (via ORC) asymmetrically positions nucleosomes on its sides, which is likely the reason why its direction is important for its function. Qun Yu has also found that removing ACS from \textit{HMR-E} led to symmetric positioning of nucleosomes and comparable silencing on both sides, which further demonstrated that ORC in its native orientation serves as an obstacle to silencing on the ACS side (Zou et al., 2006a).

The fact that the Abf1p site in a silencer or \textit{ARS} is associated with a stably positioned nucleosome nearby led us to propose that Abf1p-binding is responsible for nucleosome positioning. Consistent with this hypothesis, Qun Yu demonstrated that mutating the Abf1p-
binding site in *HMR-E* caused clear changes in nucleosome positioning as well as a significant reduction in silencing on the Abf1p side of the silencer (Zou et al., 2006a). However, inverting the direction of the Abf1p site in *HMR-E* caused little or no change in chromatin configuration or silencing (Zou et al., 2006a). Therefore, we think that Abf1p can position nucleosomes on both sides of its site. We conclude that both ORC and Abf1p play major roles in promoting asymmetric positioning of nucleosomes on the two sides of *HMR-E* or *HML-I*, which determines the directional nature of silencing by the silencer.

In summary, results presented in this work demonstrate that a yeast silencer (via the silencer-binding proteins) promote asymmetric positioning of nucleosomes, which is independent of its orientation or genomic location. We think such a structural asymmetry results in distinct potentials for the initiation of transcriptional silencing on the two sides of the silencer.
Chapter 2
Position effect on the directionality of silencer function in *Saccharomyces cerevisiae*
2.1 Abstract

In *Saccharomyces cerevisiae*, silencers flanking the *HML* and *HMR* loci initiate the establishment of transcriptional silencing. I demonstrate that the activity of a silencer pertaining to its potency and directionality is dependent on its genomic position. The context of the *HML-E* silencer is more permissive to silencer function than that of *HML-I* or *HMR-E*, despite that *HML-E* and *HML-I* are only 3.3 kb apart. The apparent strength and directionality of a silencer in a particular location is affected by other silencing elements (silencers and protosilencers) present in its context. I show that at the *HML* locus, at least four silencing elements engage in multiple functional interactions that contribute to the activities of the silencers. Notably, these dispersed silencing elements can synergize to silence genes located not only inside, but also outside the *HML* sequence that harbor them. Moreover, the relative positions and orientations of these elements are important for silencing indicating that they belong to an intricate silencing network.
2.2 Introduction

In the yeast *S. cerevisiae*, the *HML* and *HMR* loci and regions near the telomeres are transcriptionally silenced via the formation of a repressive chromatin structure that is akin to metazoan heterochromatin (Grewal and Moazed, 2003; Moazed, 2001a; Rusche et al., 2003). Silencers flanking the *HML* and *HMR* loci serve to recruit the Sir complex thereby initiating the establishment of transcriptionally silent chromatin (Rusche et al., 2003). Sir2p in the Sir complex associated with the silencer deacetylates histones in adjacent nucleosomes that then bind additional Sir complexes. The nucleosome-bound Sir complex then deacetylates the neighboring nucleosome which in turn binds a new Sir complex. In this manner, the Sir complex is proposed to promote autonomous stepwise propagation along an array of nucleosomes leading to the formation of transcriptionally silent chromatin across the *HM* loci (Grewal and Moazed, 2003; Moazed, 2001a; Rusche et al., 2003). The telomeric repeats contain multiple binding sites for Rap1p that can recruit Sir proteins, and propagation of the Sir proteins inward along the chromosome leads to the formation of silent chromatin in regions near the telomeres (Rusche et al., 2003).

Although yeast silencers function through a common mechanism, they exhibit different efficiencies in silencing. At the *HMR* locus, *HMR-E* can silence the *HMRα* genes on its own, whereas *HMR-I* plays no, or at most an auxiliary, role (Brand et al., 1985; Rivier et al., 1999). At the *HML* locus, on the other hand, either *HML-E* or *HML-I* alone is sufficient to silence the *HMLα* genes (Mahoney and Broach, 1989). When ectopically inserted near the *MAT* locus, *HMR-E* was shown to be stronger than *HML-E*, which itself was stronger than *HML-I* in silencing activity (Shei and Broach, 1995). A possible explanation for the hierarchy of the silencers concerning their potencies of silencing is that
each silencer consists of a unique sequence. Although silencers are all composed of combinations of binding sites for Abf1p, Rap1p and ORC, the actual sites for a specific factor in different silencers are variants of a consensus sequence, and therefore may have distinct affinities for the corresponding factor. In fact, it has been shown that Rap1p binds *HMR-E* more tightly than *HML-I* (Boscheron et al., 1996). Moreover, the organization of the factor-binding sites regarding the spacing of the sites is unique in each silencer. These may collectively contribute to the difference in the potency of silencing among silencers.

The *HML* and *HMR* loci are located relatively close (about 11 and 23 kb) to the left and right telomeres of chromosome III, respectively. Yeast telomeres tend to cluster at the nuclear periphery, thereby forming subnuclear compartments/foci that are rich in Sir proteins (Taddei and Gasser, 2004). Consequently, the *HM* silencers are believed to be in the vicinity of Sir-rich foci due to their proximity to telomeres. In accord with this, moving *HM* loci to locations far away from the telomeres, or placing them in plasmids, generally results in a reduction or elimination of silencing (Feldman et al., 1984; Maillet et al., 1996; Shei and Broach, 1995; Thompson et al., 1994). Moreover, there is evidence for a direct interaction between *HML* and the left telomere of chromosome III, which requires the *HML* silencers (Lebrun et al., 2003).

Silencers or protosilencers can act cooperatively. A silencer’s activity can be enhanced by another silencer located up to 4kb away (Boscheron et al., 1996; Feldman et al., 1984; Rivier et al., 1999; Sekinger and Gross, 1999). And it has been shown that silencing of genes located in the region flanked by the silencer and protosilencer was found to be increased (Boscheron et al., 1996; Cheng and Gartenberg, 2000; Lebrun et al., 2001; Lieb et
al., 2001). How two silencing elements (silencers and protosilencers) collaborate to induce stronger silencing has not been resolved.

In chapter 1, I demonstrated that inherent structural characteristics of a silencer determine its directionality in silencing. Every silencer is clearly asymmetric with an order of ORC-Rap1p-Abf1p sites for both \textit{HML-I} and \textit{HMR-E}, and Rap1p-ORC sites for \textit{HML-E} (Fig. 2-1A). Such a structural asymmetry leads to an asymmetric organization of nucleosomes around the silencer (Zou et al., 2006a). Consistent with the notion that a continuous array of nucleosomes is required for the spreading of silent chromatin (Bi et al., 2004; Rusche et al., 2003), I showed that the different patterns of nucleosome distribution on the two sides of a silencer coincide with unequal potentials for silencing (Zou et al., 2006a).

In this chapter, I explored why silencers appeared to exhibit distinct directionalities and potencies in silencing as reported in different studies. To avoid potential complications that might result from comparing data obtained using different assays, I examined the activities of silencers using the same \textit{URA3} reporter gene in the same context of \textit{HML-E}, \textit{HML-I} or the \textit{HMR-E} silencer. The results revealed that the function of a silencer regarding both its directionality and potency is dependent on its genomic context, as a consequence of functional interactions between the silencer and other silencing elements present in its surroundings. I found that the context of the \textit{HML-E} silencer was significantly more permissive to the function of a silencer than that of \textit{HML-I} and \textit{HMR-E}. I also showed that at the \textit{HML} locus, at least four dispersed silencing elements could synergize to silence genes located not only inside, but also outside the \textit{HML} sequence. Moreover, the relative positions
and orientations of these elements were important for silencing indicating that they formed an intricate silencing network.
2.3 Materials and Methods

2.3.1 Plasmids and strains

URA3 was inserted at the Bsu36I site (10979) of pUC26 (see chapter 1) resulting in pYZ2-05. The AflII-HML-E-EcoRI fragment of pYZ2-05 was inverted to make pYZ2-06, and replaced by HML-I or HMR-E in either direction to make pYZ2-01 – 2-04, respectively. The PstI-HML-URA3-Pst fragment in pYZ1-01 or pYZ1-02 was inverted making pYZ2-07 or pYZ2-08, respectively.

Plasmid pYZ2-09 was derived from pUC26 by inserting URA3 at its PvuII site (14441). The HML-I sequence of pYZ2-09 was replaced by a HindIII site resulting in pYZ2-11. An inverted HML-I was inserted at the HindIII site of pYZ2-11 making pYZ2-10. The HML-E sequence in pYZ2-09 and 2-10 was replaced by an AflII site making pYZ2-12 and 2-13, respectively. The HMR-E sequence in either direction was inserted at the HindIII site of pYZ2-11 making pYZ2-16 and pYZ2-17, respectively. The HML-I silencer in pYZ2-12 was replaced by HMR-E in either direction making pYZ2-18 and 2-19, respectively. The UASα sequence (12985-13279) of pYZ2-18 and pYZ2-19 was replaced by a HindIII site making pYZ2-20 and pYZ2-21, respectively. Renamed pYZ1-10m from chapter one as pYZ2-14. HMR-E in pYZ2-14 was replaced by a BamHI site resulting in pYZ2-15m. An inverted HMR-E was inserted at the BamHI site of pYZ2-15m making pYZ2-15.

The HML-E sequence in pYZ1-01 – pYZ1-04 was replaced by an AflII site making pYZ2-22, pYZ2-23, pYZ2-24 and pYZ2-25, respectively. The HML-I sequence in pYZ2-03 or pYZ2-04 was replaced by a HindIII site making pYZ2-26 and pYZ2-27, respectively. UASα was deleted from pYZ1-02, pYZ1-04 and pYZ2-25 making pYZ2-28, pYZ2-29, pYZ2-30, respectively. The Rap1p-binding site R2 (14538-14550) was deleted from pYZ1-
01-pYZ1-04 making pYZ2-32, pYZ2-33, pYZ2-35 and pYZ2-36, respectively. R2 was deleted from pYZ2-30 making pYZ2-31. *HML-I* plus R2 was inserted at the HindIII site of pYZ1-07 making pYZ2-34. The AflIII-*HML*-HindIII sequence in pYZ1-01 and pYZ102 was inverted making pYZ2-37 and pYZ2-38, respectively.

The names and relevant genotypes of yeast strains used in this study are listed in Table 1. Strains 2-01s – 2-13s and 2-16 – 2-38s were constructed by transforming strain DMY2 to Ura+ with BamHI-digested plasmids pYZ2-01 – pYZ2-13 and pYZ2-16 – pYZ2-38, respectively. Strains 2-14s and 2-15s were constructed by transforming DMY2 to Ura+ with HindIII-digested plasmids pYZ2-14 and pYZ2-15, respectively. These strains were rendered *SIR3*+ by integrating pMB21 at *TRP1* in the genome, resulting in strains 2-01 – 2-38, respectively. Strains 1-01r-1-04r, 1-07r and 2-24r – 2-25r were derived from 1-01 – 1-04, 1-07 and 2-24 – 2-25, respectively, by replacing the coding sequence of *PPRI* with kanMX. The relevant genotypes of all strains were confirmed by Southern blotting.

**2.3.2 RT-PCR.**

Cells were grown at 30°C to log phase in SC. Total RNA was isolated from ~ 5 x 10⁷ cells and 0.4 µg of RNA was used for multiplex RT-PCR with SuperScript III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase (Invitrogen). The proper concentration of RNA template for RT-PCR had been determined to be in the linear range by serial dilutions. Oligo dT(20) was used for the cDNA synthesis step and additional dNTP’s were added to accommodate multiple PCR products. *URA3* and *ACT1* ORF primers (the sequences of primers were listed in table 2) were used to generate PCR products of 300-550 bp that were fractionated on 2.0% agarose gels.
2.4 Results

2.4.1 The apparent directionality of a silencer measured in an experiment is dependent on the sensitivity of the assay used

I used *URA3* as the marker to measure the silencing abilities of silencers. In the presence of uracil, *URA3* is expressed at a low basal level, whereas uracil depletion activates the Ppr1p transactivator thereby increasing the expression of *URA3* to a higher activated level (Losson and Lacroute, 1981). Therefore, while cell growth on FOA-containing medium (which also contains uracil) indicates the repression of the basal level transcription of *URA3*, lack of growth on medium depleted of uracil (-Ura) reflects the silencing of activated expression of *URA3* (van Leeuwen and Gottschling, 2002). In the absence of Ppr1p, *URA3* cannot be expressed at the higher activated level even when uracil is depleted from the medium. Therefore, for *ppr1* cells, growth on FOA and lack of growth on -Ura media would both indicate the silencing of basal expression of *URA3*.

By measuring the repression of basal expression of *URA3* inserted near *HML-I* in chapter 1, I showed that *HML-I* initiated silencing in only one direction (Fig 1-1 B and Fig. 2-1B, compare growth phenotypes of strains 1-01 and 1-02 on FOA). *HMR-E* ectopically placed in the position of *HML-I* also functioned unidirectionally (Fig. 1-1B and Fig. 2-1B, growth phenotypes of strains 1-03 and 1-04 on FOA) (Zou et al., 2006a). And *HML-E* inserted in the position of *HML-I* only weakly silenced basal *URA3* expression in one direction (Fig 1-1B and Fig. 2-1B, 1-05 and 1-06 on FOA).
Fig. 2-1. Directional silencing by yeast silencers in the genomic context of HML-I. (A) Schematics of the HML and HMR loci on chromosome III in S. cerevisiae. The HML-E and HML-I silencers are shown as filled arrows with white letters, and the HMR-E and HMR-I silencers are shown as open arrows with black letters. The direction of each silencer sequence is drawn as pointing toward the HMLα or HMRα genes, but is not necessarily the functional direction. The binding sites for Abf1p, Rap1p and ORC in the silencers as well as their 5’→3’ directions are indicated. In HML-E, 16 bp centromere-proximal to the ORC site is a putative Sum1p-binding site whose deletion has been shown to reduce the activity of a weakened HML-E silencer (Irlbacher et al., 2005). The α1 and α2 genes at the HML locus and the a1 and a2 genes at HMR are also indicated. CEN, centromere. Tandem arrowheads, telomeric repeats. The region spanning the HMR locus and flanking sequence are indicated by thin shaded lines. (B) Effects of the genomic contexts of HML-I on the directionality of silencers. Left, the modified HML locus in strains 1-01 – 1-06 (see Materials and Methods 1.3.1 for their construction). The silencers, HM genes and the URA3 reporter gene are indicated. Cells of each strain were grown to late log phase and serial 10-fold dilutions were spotted on test plates and allowed to grow for 3 days. SC, synthetic complete medium. FOA, SC supplemented with 1 mg/ml 5-fluoroorotic acid (FOA). –Ura, SC depleted of uracil. Growth phenotypes of each strain were shown on the right.
Fig. 2-2. Sir-dependent *URA3* silencing in representative strains. (A). The abundance of *URA3* mRNA in strains 1-01 – 1-04, 1-07 and 2-24 – 2-25, and their *sir3*− derivatives 1-01s – 1-04s, 1-07s and 2-24s – 2-25s grown to log phase in SC medium was measured by RT-PCR. The abundance of *ACT1* mRNA in each strain was simultaneously measured as an internal control. (B) Shown were growth phenotypes of the *sir3*− strains 1-01s – 1-04s, 1-07s and 2-24s – 2-25s on SC, FOA and –Ura.
To complement the assay of *URA3* silencing by monitoring cell growth on FOA, I also directly measured the level of *URA3* mRNA using RT-PCR in a representative set of strains used in this work. *URA3* mRNA was abundant in strains 1-01, 1-03, 1-07 and 2-24, but was barely detectable in strains 1-02, 1-04 and 2-25 (Fig. 2-2A). These results are consistent with the growth phenotypes of strains 1-01 – 1-04, 1-07 and 2-24 – 2-25 on FOA (Fig. 2-1B, 1-01 – 1-04 on FOA; Fig. 2-9, 1-07, 2-24 and 2-25 on FOA). Note that *URA3* silencing measured in this work was strictly Sir-dependent as deletion of *SIR3* completely abolished *URA3* silencing (Fig. 2-2B, compare *URA3* RNA in 1-02, 1-04 and 2-25 to that in their *sir3*- derivatives 1-02s, 1-04s and 2-25s, respectively; Fig. 2-2B, lack of growth of the *sir3*- strains on FOA; and data not shown).

I also examined the directionality of silencing of silencers using activated expression of *URA3* as the reporter. Interestingly, none of the silencers in either direction (in the context of *HML*-I) was able to silence the activated expression of *URA3* as indicated by the robust growth of all strains 1-01 – 1-06 on -Ura (Fig. 2-1B). Since the activated expression of *URA3* is mediated by the trans-activator Ppr1p, it should not occur if *PPR1* is deleted. Therefore, silencing of *URA3* under non-inducing conditions could be better examined in the absence of *PPR1*. We deleted *PPR1* from the aforementioned set of seven representative strains (Fig. 2-2), resulting in strains 1-01r – 1-04r, 1-07r and 2-24r – 2-25r (Fig. 2-3, left). *URA3* silencing in these strains was measured by monitoring their growth phenotypes on FOA and –Ura, and compare to that in their *PPR1* parents (Fig. 2-3). As shown in Fig. 2-3, unlike strains 1-02 and 1-04, their *ppr1*- derivatives 1-02r, and 1-04r were not able to grow on –Ura, which confirmed that Ppr1p-independent basal expression, but not Ppr1p-dependent activated
expression of \textit{URA3}, could be silenced by \textit{HML-I} or \textit{HMR-E} orientated toward the \textit{URA3} gene.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure.png}
\caption{Examination of silencer function by measuring basal \textit{URA3} silencing in the absence of \textit{PPRI}. The growth phenotypes of strains 1-01 – 1-04, 1-07 and 2-24 – 2-25, and their \textit{pprI} derivatives 1-01r – 1-04r, 1-07r and 2-24r – 2-25r on SC, FOA and –Ura were shown.}
\end{figure}

Results from the above experiments suggest that the outcome of a silencing experiment depends on the sensitivity of the reporter gene to the silencing machinery. Throughout this report, both the basal and activated expressions of \textit{URA3} were used as silencing reporters to examine the potency and directionality of silencers.
2.4.2 The apparent potency and directionality of a silencer are dependent on its genomic context

To investigate whether the directionality of a silencer is influenced by its genomic context, I compared the behaviors of a silencer in the contexts of the HML-I, HML-E and HMR-E silencers (Fig. 2-1B and Fig. 2-4). The basal expression of URA3 inserted to the left (telomere-proximal) of HML was strongly silenced by HML-E in either orientation (Fig. 2-4B, strains 2-05 and 2-06 on FOA). This was in contrast to the weak and unidirectional silencing by HML-E transplaced in the position of HML-I (Fig. 2-1B, 1-05 and 1-06 on FOA). HML-I and HMR-E in the context of HML-E also efficiently silenced URA3 in an orientation-independent manner (Fig. 2-4, 2-01 – 2-06 on FOA). Therefore, the context of HML-E, opposite to that of HML-I, allows/facilitates silencers to function robustly and bidirectionally. Notably, whereas HML-E or HML-I in either direction was not able to silence the activated expression of URA3 (Fig. 2-4, growth of 2-01, 2-02, 2-05 and 2-06 on –Ura), HMR-E was able to do so in one orientation (toward URA3) but not the other (Fig. 2-4, compare 2-03 and 2-04 on -Ura). Therefore, the directional nature of HMR-E silencing was revealed only when activated expression of URA3 was used as the reporter. These results suggest that, compared to the context of HML-I, the context of HML-E increases the efficiency of silencing on both sides of a silencer so that basal URA3 expression could be silenced independent of the orientation of the silencer. However, the strengths of silencing on the two sides of the silencer were likely still unequal. This was manifested by the fact that HMR-E silenced activated URA3 expression in only one direction. In chapter 1, I have examined the function of HMR-E in its native position at HMR using URA3 as the reporter. URA3 inserted to the left of HMR-E was not silenced (Fig 1-2 and Fig. 2-4, lack of growth of
strain 1-08 on FOA). However, *URA3* was silenced when *HMR-E* was inverted to face *URA3* (Fig. 1-2 and Fig. 2-4, growth of strain 1-09 on FOA). On the other hand, *HMR-E* in either direction was not able to silence activated expression of *URA3* (Fig. 2-4, 1-08 and 1-09 on -Ura).

![Diagram showing growth phenotypes of strains with different genomic contexts](image)

**Fig. 2-4.** The apparent directionality of a silencer observed in a specific experiment depends on the genomic location of the silencer as well as the sensitivity of the silencing assay. Effects of the genomic contexts of *HML-E* on the directionality of silencers are shown. Left, the modified *HML* and *HMR* loci in the strains assayed. Growth phenotypes of each strain were shown on the right.

In summary, the above results suggest that each silencer is inherently unidirectional (presumably due to its asymmetric structural features, see chapter 1), but may promote bidirectional silencing if placed in a context that can increase the efficiency of silencing on the “disfavored” side of the silencer to a level comparable to that on the other side. They also show that the apparent directionality of a silencer measured in a particular experiment depends on the sensitivity/resolution of the assay employed.
2.4.3 High activity of a silencer in the context of HML-E is correlated with its relative proximity to the telomere

Results presented in Fig. 2-1B and 2-4 demonstrated that the context of HML-E is clearly more permissive to silencer function than those of HML-I and HMR-E (e.g., compare 1-03 and 1-08 to 2-03 on FOA). It was possible that the flanking sequences of HML-E acted to enhance silencing by HML-E or any other silencer ectopically inserted there, whereas the flanking sequences of HML-I or HMR-E failed to do so. In an attempt to test this hypothesis, I inverted the sequence encompassing the entire HML locus plus ~1 kb flanking sequence on each side (Fig. 2-5, zigzag and thick lines), together with the URA3 gene inserted to the right of HML-I in strains 1-01 and 1-02 (Fig. 2-5, large shaded arrow), resulting in strains 2-07 and 2-08 (Fig. 2-5). Interestingly, although this inversion did not change the local context of HML-I, or the position and orientation of URA3 relative to HML-I (Fig. 2-5, left, compare 2-07 and 2-08 to 1-01 and 1-02, respectively), it greatly improved URA3 silencing by HML-I in either orientation (Fig. 2-5, compare 2-07 to 1-01 on FOA, as well as 2-07 and 2-08 to 1-01 and 1-02, respectively, on –Ura). Because the telomere-proximal flanking sequence of the resident HML-E (Fig. 2-5, zigzag line) was not in the vicinity of HML-I in strains 2-07 or 2-08, it was probably not involved in the enhancement of the activity of HML-I.

Inversion of HML and its flanking sequences (indicated by the large shaded arrow in Fig. 2-5) shortened the distance between HML-I and the left telomere of chromosome III (TEL III-L) by ~3.3 kb. It is possible that TEL III-L enhances silencer function at HML by directly interacting with the silencer (Fig. 2-5, bottom), which is in line with the evidence for physical contact between HML and TEL III-L (Lebrun et al., 2003). However, if such a looping model were correct, then it would be hard to imagine why TEL III-L enhanced the
function of the endogenous $HML-E$ but not $HML-I$, despite that the two silencers were only 3.3 kb apart. On the other hand, it is also possible that a signal that could positively regulate silencing (e.g., the Sir complex) propagates from $TEL \, III-L$ toward $HML$ (Fig. 2-5, bottom, large arrow). Because this putative signal may gradually lose strength along its path, it may have a stronger effect on $HML-E$ (or any silencer ectopically placed there) than $HML-I$.

Fig. 2-5. Effect of inverting $HML$ and flanking sequences on the activity of $HML-I$. A sequence (large shaded arrow) encompassing $HML$ and ~1 kb left (zigzag line) and right (think line) flanking sequences, as well as $URA3$ inserted 600 bp from $HML-I$, in strains 1-01 and 1-02 was inverted to make 2-07 and 2-08. Growth phenotypes of strains 1-01, 1-02, 2-07 and 2-08 on SC, FOA and –Ura media were shown on the right. Possible means of interaction between the left telomere of chromosome III ($TEL \, III-L$) and silencers at $HML$ are shown at the bottom. Curved arrow, direct interaction. Large shaded arrow, propagation of a signal along the chromosome.
2.4.4 The directionality of a silencer can be altered by other silencing elements in its context

The fact that silencers exhibited distinct directionality in different chromosomal locations prompted us to investigate what in the context of a silencer regulated its function. I focused on possible elements at the HML locus that can influence the silencing activities of the resident or ectopic silencers. It has been shown previously that the HML-E and –I silencers could cooperate to promote stronger silencing in the HML sequence they bracketed, and the Rap1p-binding site within UASα (the shared regulatory region of the α1 and α2 genes) could serve as a protosilencer to enhance the silencing by HML-E (Boscheron et al., 1996; Cheng and Gartenberg, 2000; Feldman et al., 1984; Lebrun et al., 2003). I showed here that silencer-silencer and silencer-protosilencer interactions can affect the apparent directionality of a silencer.

As shown in Fig. 2-6, the basal expression of URA3 inserted between the α1 gene and HML-I was strongly silenced independent of the orientation of HML-I (strains 2-09 and 2-10 on FOA). The activated expression of URA3 was strongly silenced when HML-I was orientated toward URA3, but was only moderately silenced when HML-I was in the opposite direction (Fig. 2-6, 2-09 and 2-10 on –Ura). Therefore, URA3 experienced stronger silencing when located within HML than when located to the right of HML-I, but the directionality of HML-I was not changed (Fig. 2-6, compare 2-09 and 2-10 to 1-01 and 1-02, respectively, on -Ura). To test whether URA3 silencing in strains 2-09 and 2-10 resulted from combined actions of HML-E and –I, we examined the effect of deleting either silencer on silencing. Deletion of HML-I abolished URA3 silencing demonstrating that HML-E alone was not sufficient to silence URA3 (Fig. 2-6, 2-11). In the absence of HML-E, HML-I in its natural
direction (toward $\alpha 1$) silenced basal $URA3$ expression, but failed to do so in the opposite direction (Fig. 2-6, strains 2-12 and 2-13). $HML-I$ in either direction was not able to silence activated expression of $URA3$ in the absence of $HML-E$ (Fig. 2-6, strains 2-12 and 2-13 on –Ura). These results indicate that the inability of $HML-I$ to silence $URA3$ on its ORC side (Fig. 2-6, strain 2-13) could be overcome with the assistance of $HML-E$ (Fig. 2-6, strain 2-10). As $HML-E$ alone was not able to silence $URA3$ (Fig. 2-6, 2-11), silencing of $URA3$ in strain 2-10 reflected a synergistic interaction between $HML-E$ and $HML-I$. These results confirmed that $HML-I$ was a unidirectional silencer (Fig. 2-6, compare 2-12 and 2-13 on FOA), and also demonstrated that $HML-E$ could alter the apparent directionality of $HML-I$ (when the basal expression of $URA3$ was used as the silencing reporter) (Fig. 2-6, compare strains 2-09 and 2-10 to 2-12 and 2-13 on FOA, respectively).

**Fig. 2-6.** $HML-E$ affects the apparent directionality of $HML-I$. Left, strains used. Right, growth phenotypes of these strains on SC, FOA and –Ura media. [$\Delta$] denotes the deletion of a sequence.
I next examined whether the apparent directionality of HMR-E was affected by HMR-I at the HMR locus. The basal expression of URA3 inserted between HMR-E and the a2 gene was strongly silenced (Fig. 2-7, robust growth of 2-14 on FOA). This was independent of the orientation of HMR-E (Fig. 2-7, compare 2-15 and 2-14 on FOA). However, when HMR-I was deleted, HMR-E only silenced URA3 in one direction (Fig. 2-7, compare 1-10 and 1-11 on FOA). Therefore, HMR-I could alter the apparent directionality of HMR-E (when the basal expression of URA3 was used as the silencing reporter). On the other hand, HMR-E significantly silenced the activated expression of URA3 in one direction only when HMR-I was present (Fig. 2-7, compare 2-14 and 2-15 to 1-10 and 1-11 on –Ura, respectively).

Fig. 2-7. HMR-I affects the apparent directionality of HMR-E. Left, strains used. Right, growth phenotypes.

I also examined if the apparent directionality of HMR-E ectopically inserted at HML was affected by the resident silencing elements. Strains 2-16 and 2-17 were derived from 2-09 by replacing HML-I with HMR-E in opposite directions (Fig. 2-8, left). Robust silencing of both the basal and activated expression of URA3 was observed in these strains, which was independent of the orientation of HMR-E (Fig. 2-8, compare 2-16 and 2-17 on FOA and –Ura, respectively). Deletion of HML-E had no effect on the silencing of basal URA3.
expression regardless of the direction of \( HMR-E \) (Fig. 2-8, 2-18 and 2-19 on FOA). However, it greatly reduced the silencing of activated expression of \( URA3 \) when \( HMR-E \) was orientated away from it (Fig. 2-8, compare 2-18 and 2-16 on –Ura). As a consequence, \( HMR-E \) appeared to act unidirectionally (Fig. 2-8, compare 2-19 and 2-18 on –Ura). Further deletion of UAS\( \alpha \) containing a Rap1p binding site (designated R1) reduced the efficiency of silencing by \( HMR-E \) orientated away from \( URA3 \) but not that of \( HMR-E \) in the opposite orientation (Fig. 2-8, compare 2-20 and 2-21 to 2-18 and 2-19, respectively). Therefore, both the apparent potency and directionality of \( HMR-E \) was affected by the \( HML-E \) silencer and the protosilencer R1 at \( HML \). I conclude that the apparent potency and directionality of a silencer are regulated by other silencing elements in its context.

**Fig. 2-8.** Effect of UAS\( \alpha \) on the directionality of \( HMR-E \) in the context of \( HML-I \). The Rap1p-binding site (designated R1) in UAS\( \alpha \) (upstream activating sequence of the \( \alpha 1 \) and \( \alpha 2 \) genes) is indicated by a bar in strains 2-16 – 2-19. Note R1 was only highlighted in certain relevant strains in this report. UAS\( \alpha \) was deleted in strains 2-20 and 2-21. Growth phenotypes of strains 210-215 were shown on the right.
2.4.5 Dispersed silencing elements can act in synergy to promote silencing outside the region harboring them

Results from the above experiments revealed collaborative interactions among silencers and protosilencers at *HML* and *HMR*. These and previous examples of such interactions concerned the silencing of a reporter gene located between the two participating silencing elements (Fig. 2-6, 2-7 and 2-8; Feldman et al. 1984; Boscheron et al. 1996; Rivier et al. 1999; Cheng and Gartenberg 2000; Lebrun et al. 2001). Here we describe clear cases of two silencers separated by the 3.3 kb *HML* sequence functioning in synergy to silence a gene located outside *HML*. As shown in Fig. 2-5, in strain 1-02 bearing *HML-E* and the inverted *HML-I*, *URA3* inserted to the right of *HML* was silenced. However, neither *HML-I* nor *HML-E* alone was able to silence *URA3* (Fig. 2-9, 2-23 and 1-07 on FOA). Therefore, *URA3* silencing in strain 1-02 requires the synergistic interaction of *HML-E* and inverted *HML-I*. On the other hand, *HML-E* and *HML-I* in its native orientation failed to work together to silence *URA3* (Fig. 2-9, strain 1-01). Robust *URA3* silencing was retained when *HML-I* in strain 1-02 was replaced by *HMR-E* orientated toward *URA3* (Fig. 6, strain 1-04 on FOA). Deletion of *HML-E* decreased *URA3* silencing (Fig. 2-9, compare 2-25 to 1-04 on FOA) indicating that *HML-E* enhanced the silencing activity of *HMR-E* in strain 1-04. These results demonstrate that a silencer in the position of *HML-I* can collaborate with the *HML-E* silencer at a distance of 3.3 kb to silence a gene located to the right of the *HML* sequence. The *HMR-E* silencer placed in the context of *HML-E* did not require the assistance of *HML-I* to fully silence the basal expression of *URA3* located to the left of *HML* (Fig. 2-9, FOA panel, compare 2-26 and 2-27 to 2-03 and 2-04, respectively). However, *HML-I* helped *HMR-E* promote stronger silencing of the activated expression of *URA3* (Fig. 2-9, compare
In summary, these results clearly demonstrated that two silencers separated by the HML sequence can cooperate to silence genes outside of HML more efficiently.

Fig. 2-9. Two silencers separated by a sizable distance can synergize to promote silencing outside the region they bracket. Schematics of the modified HML loci in the strains were shown on the left. Symbols used were as described in Fig. 2-1. Growth phenotypes of these strains were shown on the right.

As the Rap1p site R1 in the middle of HML could cooperate with an ectopic HMR-E to silence URA3 within HML (Fig. 2-8, compare 2-20 to 2-18 on FOA), we wondered whether R1 could also collaborate with silencers to silence URA3 outside of HML. To address this question, we deleted UASα containing R1 from strain 1-02 resulting in strain 2-
The failure of strain 2-28 to grow on FOA (Fig. 2-10A) indicated that R1 was also required for *URA3* silencing in strain 1-02. On the other hand, R1 did not seem to be required for *URA3* silencing mediated by *HMR-E* (Fig. 2-10A, compare strains 2-29 to 1-04, as well as 2-30 to 2-25), which is consistent with the notion that *HMR-E* is stronger than *HML-I*.

The fact that R1 was required for the silencing of *URA3* outside of *HML* in strain 1-02 raised the question of whether the other known Rap1p site within *HML* was also required. This site (referred to as R2) resides near *HML-I* (Fig. 2-10B) and only weakly binds Rap1p *in vitro* (Boscheron et al. 1996). R2 is apparently not required for the silencing of *HMLα* by the resident *HML-I* (Feldman et al. 1984). However, we showed that deletion of R2 abolished the silencing of *URA3* inserted to the right of the inverted *HML-I* silencer (Fig. 2-10B, compare strains 1-02 and 2-33 on FOA). Therefore, R2, like R1, is also required for the silencing of *URA3* outside of *HML*. On the other hand, R2 was not required for the unidirectional silencing activity of the *HMR-E* silencer (Fig. 2-10B, compare 2-35 and 2-36 to 1-03 and 1-04, respectively). In fact, even when all of *HML-E*, R1 and R2 were simultaneously deleted, *HMR-E* in place of *HML-I* was still able to significantly silence *URA3* (Fig. 2-10A, 2-31 on FOA).

Taken together, the above data demonstrate that dispersed silencing elements can synergize to silence a gene located outside of the region harboring them. Remarkably, in strain 1-02, at least 4 silencing elements (*HML-E, HML-I, R1* and *R2*) are required for the silencing of *URA3* inserted outside *HML* (Fig. 2-9 and 2-10, compare strains 2-23, 1-07, 2-28 and 2-33 to 1-02; Fig. 2-10C).
Multiple dispersed silencing elements can cooperate to promote silencing outside the region harboring them. (A) The protosilencer R1 within the HML sequence is involved in silencing outside of HML. Left, strains used. Right, growth phenotypes. (B) The Rap1p-binding side near HML-I in HML (designated R2) is involved in silencing outside of HML. Left, strains used. Strains 2-37 and 2-38 were identical to 1-01 and 1-02, respectively, except that the HML sequence bracketed by the HML-E and –I silencers were inverted. Note R2 was only highlighted in certain relevant strains in this report. Right, growth phenotypes. (C) Four dispersed silencing elements at HML synergize to promote silencing outside HML. The HML-E and inverted HML-I silencers as well as the R1 and R2 protosilencers were all required for the silencing of URA3 located to the right of HML. This was a summary of results concerning strains 1-02, 2-23, 1-07, 2-28 and 2-33 described in panels A, B and Fig. 2-6 and 2-9.
2.4.6 The relative positions of the silencing elements are important for their functional interactions

As at least four silencing elements participated in URA3 silencing in strain 1-02, it was possible that these elements all contribute to the putative build up of the “strength” of silencing (perhaps in the form of the abundance of Sir proteins) over a threshold for silencing to occur. If this were the case, then the relative positions of the auxiliary protosilencers R1 and R2 might not be important for silencing. I tested this by precisely inverting the HML sequence in strain 1-02 making strain 2-38 (Fig. 2-10B). In strains 2-38, sequences within HML that were previously close to HML-I were now distant from it, and vice versa. It was clear that inversion of HML abolished URA3 silencing (Fig. 2-10B, compare 2-38 to 1-02 on FOA). One possibility was that R2 had to be physically close to HML-I for productive collaborations between the silencing elements. However, relocating R2 to a position near the right side of HML-I also abolished URA3 silencing (Fig. 2-10B, compare strain 2-34 to 1-02). Therefore, the mere proximity of R2 to HML-I was not sufficient for its role in promoting URA3 silencing. The above results demonstrated that the relative position of R2 is important for its role in facilitating silencing in strain 1-02, and suggested that R2 was part of an intricate silencing network consisting of HML-E, HML-I, R1 and R2 (Fig. 2-10C).
2.5 Discussion

The principal mechanism for how yeast silencers initiate the formation of transcriptionally silent chromatin has been elucidated (Grewal and Moazed, 2003; Moazed, 2001a). However, the issue regarding whether and how silencers function in an orientation-dependent or -independent manner has not been completely resolved (Bi et al., 1999; Brand et al., 1985; Shei and Broach, 1995). I have obtained evidence indicating that a silencer promotes asymmetric positioning of nucleosomes around it, leading to unequal silencing potentials on the two sides in chapter 1 (Zou et al., 2006a). In other words, silencers are by nature unidirectional. It is therefore puzzling that the same silencer (e.g., *HMR-E*) could act in an orientation-independent fashion as measured in one experiment, but appear unidirectional in another (Brand et al., 1985; Shei and Broach, 1995). Results presented in this work provided two explanations for this conundrum.

The first concerns the distinct sensitivities of silencing assays used to measure silencer activity in different studies. The efficiency of silencing of a particular reporter gene by a silencer depends on the potency of the silencer as well as the strength of the promoter of the reporter gene that is inversely correlated with its sensitivity to the silencing machinery (van Leeuwen and Gottschling, 2002). Using both the basal and activated expression of *URA3* as reporters, I found that a silencer may (i) silence both the basal and activated expressions; (ii) silence the basal but not the activated expression; or (iii) silence neither the basal nor activated expression. As a consequence, the directionality of a silencer examined in a specific experiment in this work depended on whether the basal or activated expression of *URA3* was used as the silencing reporter. For example, *HMR-E* inserted in the position of
$HML-E$ silenced the basal expression of $URA3$ on the left of $HML$ in an orientation-independent manner, but silenced activated $URA3$ expression in only one direction (Fig. 2-1B). Moreover, although $HMR-E$ was able to silence the $a1$ gene at $HMR$ independent of its orientation (Brand et al., 1985), I found it silenced basal $URA3$ expression in one direction when it is located at $HML-I$ locus, but failed to silence activated $URA3$ expression in either direction (Fig. 2-1B). In addition, despite the directional behavior of $HML-I$ detected by assaying $URA3$ silencing, the endogenous $CHA1$ gene located on the “disfavored” side of $HML-I$ is still subject to Sir-dependent repression in native strains (Moreira and Holmberg, 1998). In summary, the directional nature of the function of a silencer may only be revealed using a silencing assay with sufficient resolution, and regarding the silencing of native genes, the apparent directionality of a native silencer in its native context is not absolute.

The other explanation was based on my finding that the function of a silencer is influenced by its local genomic context. The directional nature of a silencer may be revealed in a “neutral” context, but may also be “masked” in a context that can increase silencing on the inherently disfavored side of the silencer to a level comparable to that of the preferred side.

I have demonstrated in this chapter that the activity of a silencer concerning both its potency and directionality can be affected by other silencing elements (silencers, protosilencers and telomeres) that are present in its context. For instance, the $HML-E$ silencer was able to increase silencing on the disfavored ORC side of $HMR-E$ (in the context of $HML-I$) to a level comparable to that on its Abf1p side (Fig. 2-8, compare strains 2-16 and 2-17). In other words, $HML-E$ served to transform $HMR-E$ into a bidirectional silencer.
This is one of many examples observed in this work regarding two silencing elements cooperating to promote stronger silencing in the region bracketed by them. Moreover, I also showed that two silencers separated by the 3.3 kb \textit{HML} sequence could synergize to silence a gene located outside of the region bordered by them (Fig. 2-9). In addition, I found evidence that being close to the left telomere of chromosome III enhances the efficiency of silencing by a silencer (Fig. 2-5).

The mechanism(s) underlying functional interactions among silencing elements have not been resolved, but several models have been proposed (Bi et al., 1999; Boscheron et al., 1996; Fourel et al., 2002). The fact that two silencers separated by up to several kb are able to cooperate to silence a reporter located between them can be explained by assuming that convergent spreading of Sir proteins emanating from the silencers is additive or synergistic so that silent chromatin established between the silencers is stronger than that formed by either silencer alone. In support of this model, it was shown that silent chromatin formed by two bracketing \textit{HMR-E} silencers had a higher density of positioned nucleosomes and thus a more compact chromatin structure than that formed by a single \textit{HMR-E} (Reimer and Buchman, 1997). However, this interpretation does not apply to silencer-protosilencer cooperation since a protosilencer is not able to autonomously initiate silencing. On the other hand, because a protosilencer is actually a binding site for Abf1p, Rap1p or ORC that can position nucleosomes (Bi et al., 2004; Lipford and Bell, 2001; Yarragudi et al., 2004; Yu and Morse, 1999; Zou et al., 2006a), it is conceivable that a protosilencer helps position nucleosomes in the region between it and the silencer in a configuration that is more favorable for the spread of Sir proteins from the silencer (Boscheron et al., 1996). Along this line, I think that the presence of a protosilencer on the “disfavored” side of a silencer may
alter the putatively inhibitory pattern of nucleosome positioning and allow silencing to occur more efficiently on this side, thereby masking the inherent directionality of the silencer.

The two models discussed above cannot readily explain how a silencer cooperates with another silencer or protosilencer to promote stronger silencing in areas outside the region containing the silencing elements. Ample evidence suggests that yeast telomeres cluster at the nuclear periphery creating discrete subnuclear compartments that are rich in Sir proteins (Maillet et al., 1996; Taddei and Gasser, 2004). Recent evidence indicates that HML silencers physically associate with the left telomere of chromosome III and therefore a Sir-rich compartment (Lebrun et al., 2003). Therefore, functional cooperation between two silencers could be the consequence of a mutual enhancement of their tethering to a Sir-rich compartment. I have presented a striking example of four silencing elements (silencers HML-E and HML-I, and protosilencers R1 and R2) working together to silence a distal gene (summarized in Fig. 2-10C). It is possible that all silencing elements act, at least in part, by contributing to the tethering of the locus to a Sir-rich subnuclear compartment.
Chapter 3

Positive roles of SAS2 in DNA replication and transcriptional silencing in yeast
3.1 Abstract

Sas2p is a histone acetyltransferase implicated in the regulation of transcriptional silencing, and ORC is the six-subunit origin recognition complex involved in the initiation of DNA replication and the establishment of transcriptionally silent chromatin by silencers in yeast. I show in this chapter that SAS2 deletion (sas2Δ) exacerbates the temperature sensitivity of the ORC mutants orc2-1 and orc5-1. Moreover, sas2Δ and orc2-1 have a synthetic effect on cell cycle progression through S phase and initiation of DNA replication. These results suggest that SAS2 plays a positive role in DNA replication and cell cycle progression. We also show that sas2Δ and orc5-1 have a synthetic effect on transcriptional silencing at the HMR locus. Moreover, I demonstrate that sas2Δ reduces the silencing activities of silencers regardless of their locations and contexts, indicating that SAS2 plays a positive role in silencer function. In addition, I show that SAS2 is required for maintaining the structure of transcriptionally silent chromatin.
3.2 Introduction

Eukaryotic DNA is packed into chromatin through the formation of nucleosomes. Chromatin can be roughly divided into condensed heterochromatin and decondensed euchromatin based on its cytological and molecular properties. Chromatin plays a pivotal role in the regulation of DNA-dependent processes including gene expression, DNA replication and DNA repair. Chromatin is subject to various modifications that differentially affect its compaction and accessibility to DNA/chromatin-interacting factors (Kouzarides, 2007). Acetylation of lysine residues of histones may alter nucleosomal conformation and/or chromatin compaction. Histone acetylation is usually associated with euchromatin, whereas histone hypoacetylation is a hallmark of heterochromatin. Histone acetylation is carried out by histone acetyltransferases (HATs) that fall into distinct families with specific substrate preferences (Carrozza et al., 2003; Roth et al., 2001). For example, *Saccharomyces cerevisiae* Sas2p belongs to the MYST (MOZ, Ybf2/Sas3, Sas2 and Tip60) HAT family that preferentially acetylates histones H4 and H2A. A native HAT usually functions as part of a multi-subunit complex that helps direct the HAT to proper targets (Roth et al., 2001). Sas2p is the catalytic subunit of the SAS complex consisting of Sas2p, Sas4p and Sas5p that specifically acetylates histone H4-K16 (Carrozza et al., 2003).

In *S. cerevisiae*, DNA replication is initiated at defined origins named autonomous replicating sequences (ARSs) across the genome (Bell, 2002). The origin recognition complex (ORC) that binds to ARS plays a key role in replication initiation. The timing and efficiency of the firing of replication origins appear to depend on their genomic locations and contexts. There is evidence that chromatin structure affects origin activity (Lipford and Bell,
Histone acetylation surrounding an origin directly influences its time of firing (Vogelauer et al., 2002). Removal of the histone deacetylase (HDAC) Rpd3p causes DNA replication to occur early in S-phase, and targeting the HAT Gcn5p to a late firing origin causes it to fire earlier (Aparicio et al., 2004; Vogelauer et al., 2002). There is also evidence that the HDAC Sir2p involved in transcriptional silencing negatively regulates initiation of DNA replication (Pappas et al., 2004). The HAT Hat1p was recently shown to physically interact with ORC, and deletion of HAT1 exacerbated the temperature sensitivity of *orc* mutants (Suter et al., 2007). These studies suggest that HATs play positive roles, and HDACs play negative roles in the initiation of DNA replication. However, interestingly, there is also evidence suggesting that the HAT Sas2p negatively regulate ORC function (Ehrenhofer-Murray et al., 1997; Weinberger et al., 1999).

ORC also plays a role in the establishment of yeast silent chromatin that is akin to metazoan heterochromatin (Rusche et al., 2003). Formation of silent chromatin at the HML and HMR loci is promoted by cis-acting E and I silencers flanking these loci (Rusche et al., 2003). Each silencer consists of recognition sites for ORC, Rap1p and/or Abf1p. ORC binding to the silencers recruits Sir proteins to initiate silent chromatin at *HM* loci. Silent chromatin is regulated by various factors including Sas2p. *SAS2* deletion (*sas2Δ*) was originally found to suppress the defects in *HMR* silencing caused by mutations in the *HMR-E* silencer, but decrease *HML* silencing in the *sir1Δ* background (Ehrenhofer-Murray et al., 1997; Reifsnyder et al., 1996). However, it was also found that *sas2Δ* decreased silencing of ectopic reporter genes inserted at an otherwise intact *HMR* locus (Meijsing and Ehrenhofer-Murray, 2001; Xu et al., 2006). Therefore, *SAS2* seems to have the ability to positively or negatively regulate *HM* silencing. What determines the mode of Sas2p action has not been
resolved. On the other hand, \textit{sas2} has been consistently found to abolish telomeric silencing (Reifsnyder et al., 1996), and it is proposed that Sas2p contributes to telomeric silencing by helping retain Sir complexes in telomeric silent chromatin (Kimura et al., 2002). This is based on the finding that acetylation of H4-K16 in subtelomeric euchromatic regions by Sas2p hinders ectopic spreading of Sir complex from telomeric silent chromatin (Kimura et al., 2002; Suka et al., 2002).

In this chapter, I examined functional relationships between Sas2p and ORC in DNA replication and transcriptional silencing in different genetic backgrounds. I found that \textit{sas2} exacerbated the temperature sensitivity of \textit{orc2-1} and \textit{orc5-1} mutants. I further showed that \textit{sas2} retarded cell cycle progression thorough S phase and reduced the origin activity of \textit{ARS1} in the \textit{orc2-1} mutant. These results suggest that Sas2p plays a positive role in DNA replication. I also obtained evidence suggesting that Sas2p positively regulates silencer function regardless of whether it is at \textit{HML} or \textit{HMR}. Moreover, I showed that Sas2p played a role in maintaining transcriptionally silent chromatin structure.
3.3 Materials and Methods

3.3.1 Yeast strains

Yeast strains are listed in Table 1. Strains carrying the $sas2\Delta::kanMX$ allele were made by transforming their corresponding parents to geneticin-resistant with a PCR-generated fragment composed of $kanMX$ bracketed by 5’ and 3’ flanking sequences of the SAS2 open reading frame (ORF). Strains carrying the $sas2\Delta1::TRP1$ allele were made by transforming their parents with plasmid pJR1642 (Ehrenhofer-Murray et al., 1997) digested with XhoI and BamHI. Strains 3-21, 3-23, 3-24, 3-26, 3-28, 3-30 and 3-32 are strains 2-05, 2-05s, 2-04, 2-02, 1-02, 1-04 and 1-09, respectively (see chapter 1 and chapter 2). Strains 3-36 and 3-37 were made by transforming strains 3-34 and 3-35, respectively, to $Ura^+$ with plasmid pES17 (Stone et al., 1991) digested with BamHI and Hind III. Strains 3-38 and 3-39 were made by transforming strains 3-34 and 3-35, respectively, to $Ura^+$ with EcoRI-digested plasmid pDM42 (Mahoney and Broach, 1989). Strains 3-45 and 3-47 were made by transforming strains 3-11 and 3-15, respectively, to $Ura^+$ with HindIII-digested plasmid pYZ1-10 (see 1.3.1). The relevant genotypes of all strains were confirmed by Southern blotting.

3.3.2 Fluorescence activated cell sorting

Cells for fluorescence activated cell sorting (FACS) analysis were grown at 23°C to log phase ($OD_{600} = ~0.5$) and divided into two aliquots that were grown for another 90 min at 23°C and 30°C respectively. Samples were prepared as described (Haase and Reed, 2002) and analyzed on a FACSCalibur (Becton, Dickinson and Company). Data acquisition was performed with the CellQuest software. Quantification of the percentages of cells in G1
phase (with C1 DNA content), S phase, and G2-M (with C2 DNA content) were performed using the MODFIT software program (Verity Software House).

3.3.3 Two-dimensional gel electrophoresis

Cells used for isolating replication intermediates (RIs) were grown at 23°C to log phase and divided into two aliquots that were grown for another 90 min at 23°C and 30°C respectively. For each sample, 1.5 x 10⁹ cells were collected and used for genomic DNA isolation as described (Wu and Gilbert, 1995). Genomic DNA was digested with NcoI and subjected to 2-D gel analysis as described (Brewer and Fangman, 1987). The conditions for the first dimension were: 0.35% agarose, 1 x TBE buffer, 0.6 V/cm for 45 hr, room temperature; and for the second dimension: 0.95% agarose, 0.3 µg/ml ethidium bromide, 2.8 V/cm for 20 hr, 4°C. The Southern blot was first hybridized with an ARS1 probe to reveal RIs associated with ARS1. The blot was then stripped off the ARS1 probe and hybridized with an ARS305 probe to reveal RIs associated with ARS305.

3.3.4 Chromatin mapping

Chromatin mapping was carried out as described in Chapter 1. About 2 x 10⁸ permeabilized spheroplasts were treated with MNase at 120 or 160 units/ml at 37°C for 5 minutes. Purified genomic (naked) DNA from untreated cells was digested with MNase at 7.5 units/ml.

3.3.5 Analysis of DNA topology

Cells were grown in YPR medium (1% yeast extract, 2% bacto-peptone and 2% raffinose) to log phase. Galactose (2%) was added to the culture which was further incubated for 2.5 hr to induce the expression of P_{GAL10-FLP1}. Nucleic acids were isolated using the
glass bead method and fractionated on an agarose gel supplemented with chloroquine. DNA circles were detected by Southern blotting.
3.4 Results

3.4.1 Synthetic genetic interactions between SAS2 and ORC genes

All six subunits of ORC, Orc1p-Orcl6p, are essential for cell viability, but temperature sensitive mutations of the ORC genes such as orc2-1 and orc5-1 have been described (Bell, 2002; Foss et al., 1993; Loo et al., 1995). It was reported previously that sas2Δ partially suppressed the temperature-sensitivity of the orc2-1 mutant (Ehrenhofer-Murray et al., 1997). In an attempt to further examine functional interactions between the SAS2 and ORC genes, I made orc2-1 sas2Δ::kanMX and orc5-1 sas2Δ::kanMX double mutants in the W303-1A (MATa) background. I was surprised to find that our orc2-1 sas2Δ double mutant grew less robustly than the orc2-1 and sas2Δ single mutants at 30°C, a semi-permissive temperature for orc2-1 (Fig. 3-1, compare 3-04 to 3-03). In line with this finding, the orc5-1 sas2Δ double mutant also showed reduced growth compare to the orc5-1 single mutant at the semi-permissive temperature of 34°C (Fig. 3-1, compare 3-07 to 3-06). These results suggest that sas2Δ has a synthetic effect on cell growth with orc2-1 or orc5-1. Since Sas2p is a subunit of the SAS complex that also contains Sas4p and Sas5p, I asked whether SAS4 also genetically interacts with the ORC genes. I found that similar to sas2Δ, sas4Δ exacerbated the growth defect of orc2-1 at semi-permissive temperatures (Fig. 3-1, compare 3-10 to 3-03 at 26°C and 30°C). These results suggest that the SAS complex performs an overlapping role with ORC in cell growth.
Considering the cause of the discrepancy between our results and previously published results regarding genetic interactions between orc mutants and sas2Δ, I noted that the genetic background of our strains was different from that of the previous strains. My sas2Δ strains differed from published ones in the way of SAS2 disruption. In my strains, the
kanMX cassette was used to precisely replace the entire open reading frame (ORF) of SAS2, whereas in the previous strains TRP1 was used to replace most of the SAS2 ORF resulting in sas2Δ1::TRP1 (Ehrenhofer-Murray et al., 1997; Weinberger et al., 1999). I analyzed the sequence of sas2Δ1::TRP1 and found it contained a new ORF encoding a short peptide composed of the N-terminal 4 amino acids of Sas2p followed by 20 other residues. To test whether sas2Δ1::TRP1 differed from sas2Δ::kanMX in its interaction with orc2-1 or orc5-1, I made orc2-1 sas2Δ1::TRP1 and orc5-1 sas2Δ1::TRP1 double mutants in the W303-1A background. The growth phenotypes of these double mutants at various temperatures were similar to orc2-1 sas2Δ::kanMX and orc5-1 sas2Δ::kanMX strains, respectively (Fig. 3-1, compare 3-05 to 3-04 and 3-03, and 3-08 to 3-07 and 3-06). Therefore, the sas2Δ::kanMX and sas2Δ1::TRP1 alleles interact similarly with orc2-1 or cor5-1, ruling out the method of SAS2 disruption as the cause of the discrepancy between our data and previously published results.

The orc mutants used in previous studies were derivatives of W303-1B (MATα) instead of W303-1A (MATα). I therefore examined if the cell type affected genetic interactions between orc mutants and sas2Δ. I remade orc2-1 sas2Δ1::TRP1 and orc5-1 sas2Δ1::TRP1 double mutants in the W303-1B background and compared their growth phenotypes with orc single mutants. The double mutants exhibited more severe temperature sensitivity than their corresponding orc single mutants (Fig. 3-2B, compare 3-14 to 3-13, and 3-16 to 3-15), which was similar to our results concerning W303-1A derivatives (Fig. 3-1). I noticed that the temperature sensitivity of orc mutants was more severe in the MATα background than in the MATα background as the MATα orc2-1 strain was not able to grow at
30°C, whereas the MATα orc2-1 strain was (albeit poorly) (Fig. 3-1 and 3-2, compare 3-13 to 3-03). Possible causes of this phenomenon are noted later in the Discussion.

Another difference between our strains and previous strains was that the latter contained a modified HMR locus with a synthetic HMR-E silencer and deletion of the HMR-I silencer (HMR-SSΔI) (Ehrenhofer-Murray et al., 1997), whereas all our strains were wild type for HMR. I remade the double mutant orc2-1 sas2Δ1::TRP1 in the MATα HMR-SSΔI background and found that it still exhibited growth defects compared to the orc2-1 single mutant (Fig. 3-2, compare 3-20 to 3-19).

Fig. 3-2. Synthetic interactions between sas2Δ and orc mutations in the MATα background. Cells of strains 11-20 were grown to log phase and serial 10-fold dilutions were spotted on SC plates and incubated for 3 days at 23°C, 26°C, 30°C and 34°C, respectively. Two independent clones of each strain were examined.
The above data demonstrate that *sas2Δ* exacerbates the temperature sensitivity of *orc* mutants. In addition to its temperature sensitivity, the *orc2-1* mutant is sensitive to hydroxyurea (HU) which induces replicative DNA damage at permissive temperature (Fig. 3-1, compare 3-03 to 3-01 on HU-containing medium) (Shimada et al., 2002). *sas2Δ* rendered *orc2-1* cells moderately more sensitive to HU (Fig. 3-1, compare 3-04 and 3-05 to 3-03 on HU-containing medium). Taken together, these results suggest that *SAS2* plays a positive role in ORC-mediated functions.

### 3.4.2 Synthetic effect of *sas2Δ* and *orc2-1* on cell cycle progression through S phase

ORC is involved in the initiation of DNA replication, and defects in ORC function interrupt cell cycle progression (Bell et al., 1993; Gibson et al., 2006; Loo et al., 1995). To further examine the role of Sas2p in ORC-mediated functions, I tested whether *sas2Δ* affected cell cycle progression of *ORC2* and *orc2-1* cells using FACS (fluorescence-activated cell sorting) analysis. Exponentially growing asynchronous cultures of two independent clones of each strain were subjected to FACS analysis and the results are shown in Fig. 3-3A. The percentages of cells in G1 phase (with C1 DNA content), S phase, and G2-M (with C2 DNA content) were measured for each culture and shown in Fig. 3-3B.

At 23°C, the wild type culture has about 21% G1, 38% S and 41% G2-M cells (Fig. 3-3, a), which was not significantly affected by *sas2Δ* (Fig. 3-3, compare b to a). *orc2-1* reduced the proportion of cells in G1 (Fig. 3-3, compare c to a), which is consistent with previous results showing that temperature-sensitive mutations in *ORC1, ORC2* and *ORC5* all reduce the proportion of G1 cells (Gibson et al., 2006). *sas2Δ* had no effect on the proportions of G1, S and G2-M cells in the *orc2-1* culture (Fig. 3-3, compare d to c). After
being incubated at the semi-permissive temperature of 30°C for 90 min, SAS2 and sas2Δ cells were roughly equally divided among the G1, S and G2-M phases (Fig. 3-3B, e and f). As for the orc2-1 mutant, the proportion of G1 cells decreased whereas that of S or G2-M cells increased at 30°C (Fig. 3-3, compare g to c), which suggests that compromising Orc2p function induces a delay of progression through S and/or G2-M phases. Compared to orc2-1 cells, a strikingly larger percentage of orc2-1 sas2Δ cells were in S phase at 30°C than at 23°C (Fig. 3-3, compare h to g), indicating that sas2Δ delayed or arrested progression of orc2-1 cells through S phase.

Fig. 3-3. Synthetic effect of sas2Δ and orc2-1 on cell cycle progression. (A) FACS analysis. Cells were grown to log phase and subjected to FACS analysis. Two independent clones of each strain grown at 23°C and 30°C, respectively, were examined. The x-axis represents DNA content and the y-axis the number of cells in the culture. Data for strains 3-01 – 3-04 at 23°C are shown in panels a-d, respectively, and data for strains 3-01 – 3-04 at 30°C are shown in panels e-h, respectively. (B) Quantification of the proportions of G1, S and G2-M cells. The percentages of G1, S and G2-M cells for each strain are the averages of data for the two independent clones examined by FACS in (A).
3.4.3 SAS2 plays a positive role in ORC-dependent initiation of DNA replication

The above results suggest that SAS2 is important for the passage of the orc2-1 mutant through S phase. Sas2p may regulate replication initiation that is ORC-dependent, and/or replication elongation that is ORC-independent. I directly examined whether sas2Δ affected initiation of DNA replication. A neutral-neutral two dimensional (2-D) gel electrophoresis technique was used to examine the abundance of replication intermediates (RIs) associated with ARS1, an early-firing origin of replication, in wild type as well as orc2-1 and sas2Δ single and double mutants. This type of 2-D gel separates replication bubbles, forks and linear DNA based on the size and shape of the DNA fragment containing a replication origin (Brewer and Fangman, 1987). A fragment containing an active replication origin in the middle would yield a bubble arc, whereas a fragment being replicated by passing replication forks would yield a fork arc (Fig. 3-4A).

ARS1 activity in wild type, sas2Δ, orc2-1, and sas2Δ orc2-1 strains was examined. For each strain, cells were grown to log phase at 23°C and divided into two halves that were grown for another 90 minutes, one at 23°C and the other at 30°C, before being harvested. Genomic DNA was isolated from each sample and digested with NcoI, and subjected to 2-D gel analysis followed by Southern blotting. ARS1 is located in a 4.7 kb NcoI fragment of chromosome IV (coordinates 460989-464386) (Fig. 3-4B). Because the position of ARS1 is asymmetric, replication initiated from ARS1 will initially yield bubbles and will then produce large forks when one of the two replication forks reaches one end of the fragment before the other (Fig. 3-4A).
Fig. 3-4. Synthetic effect of \textit{sas2}\textsuperscript{Δ} and \textit{orc2-1} on the initiation of DNA replication. (A) Scheme of replication intermediates fractionated by 2-D gel electrophoresis. The bubble arc, small-fork and large-fork arcs are indicated. (B) Schematics of the genomic NcoI fragment of chromosome IV containing \textit{ARS1} and the NcoI fragment of chromosome III containing \textit{ARS305}. The ACS (ARS consensus sequence), B1-B4 and DUE sequence in the two ARSs are shown. Note that ACS is the recognition site of ORC, and B3 is an Abf1p-binding site. (C) 2-D gel analysis of replication intermediates. Data for \textit{ARS1} in strains 3-01 – 3-04 at 23°C are shown in panels a-d, respectively, and data for \textit{ARS1} in strains 3-01 – 3-04 at 30°C are shown in panels e-h, respectively. Data for \textit{ARS305} in strains 3-03 and 3-04 at 23°C are shown in panels c’ and d’, respectively. Data for \textit{ARS305} in strains 3-03 and 3-04 at 30°C are shown in g’ and h’, respectively. See text for descriptions.
In the wild type strain at either 23°C or 30°C, the majority of the RIs were bubbles and large forks, which is consistent with the notion that ARS1 is an active origin (Fig. 3C, a and e). The presence of some small forks indicated that the ARS1 did not fire in a small fraction of the cells in the culture (Fig. 3-4C, a and e) (Liang et al., 1995). sas2Δ alone had little or no effect on ARS1’s origin activity at either 23°C or 30°C (Fig. 3-4C, compare b to a, and f to e). The orc2-1 mutation reduced ARS1 activity at 23°C as evidenced by a decrease in the relative intensity of the bubble arc compared to the fork arc (Fig. 3-4C, compare c to a). sas2Δ further reduced the relative intensity of the bubble arc in the orc2-1 mutant (Fig. 3-4C, compare d to c), suggesting that SAS2 is required for efficient ARS1 function in an orc2-1 mutant. At 30°C, the bubble arc in the orc2-1 mutant was barely detectable, which is consistent with the temperature sensitivity of orc2-1 (Fig. 3-4C, compare g to c). The bubble arc could not be detected in the orc2-1 sas2Δ double mutant at 30°C (Fig. 3-4C, h). These results suggest that Sas2p plays a positive role in the initiation of DNA replication at ARS1 in an orc2-1 mutant.

I next tested whether sas2Δ also affected the activities of other origins in an orc2-1 mutant. I examined ARS305, which is also an early firing origin but differs from ARS1 in sequence/structure (Fig. 3-4B). ARS305 is asymmetrically located in a 5.1 kb NcoI fragment of chromosome III (coordinates 36563-41669). In the orc2-1 mutant, bubble and large-fork arcs were readily detectable, but little or no small-fork arc was observed at 23°C (Fig. 3-4C, c’), suggesting that ARS305 was a very active origin. Raising the temperature from 23°C to 30°C increased the intensity of small fork arc (Fig. 3-4C, compare g’ to c’). However, the bubble arc was still readily detectable (Fig. 3-4C, g’), indicating that ARS305 was less sensitive to orc2-1 mutation than ARS1 (Fig. 3-4C, compare g’ and g). sas2Δ does not
significantly affect *ARS305* activity in the *orc2-1* background at either 23°C or 30°C (Fig. 3-4C, compare d’ to c’, and h’ to g’). Therefore, compared to *ARS1*, replication initiation at *ARS305* seems less sensitive to *orc2-1* and *sas2Δ*.

3.4.4 **SAS2 does not affect chromatin structure at replication origins**

ORC bound to a replication origin serves as a platform for the formation of the pre-replication complex. Meanwhile, it also directs the establishment of proper chromatin structure around the origin that is important for its function (Lipford and Bell, 2001). Because Sas2p is a HAT that can acetylate histone H4 in chromatin, and plays a positive role in replication initiation at *ARS1*, we wondered whether it contributes to chromatin structure at *ARS1*. I examined chromatin around *ARS1* in *sas2Δ* and *orc2-1* single and double mutants using micrococcol nuclease (MNase) digestion followed by indirect end labeling. The *ARS1* region is associated with three major MNase cleavage sites (designated α, β and γ) corresponding to a nucleosome-free region defined by ORC and Abf1p bound to the ACS and B3 elements of *ARS1*, respectively (Fig. 3-5) (Lipford and Bell, 2001). The pattern of MNase sensitive sites around *ARS1* was not significantly altered by *orc2-1* and *sas2Δ* single mutations or the *orc2-1 sas2Δ* double mutation at 23°C or 30°C (Fig. 3-5, compare b, c and d to a, and f, g, h to e). Therefore, Sas2p does not seem to contribute to chromatin structure at *ARS1*. 
3.4.5 A positive role of SAS2 in silencer function

SAS2 was originally identified as a gene involved in transcriptional silencing (Ehrenhofer-Murray et al., 1997; Reifsnyder et al., 1996). However, conflicting effects of sas2Δ on silencing have been reported. sas2Δ was found to eliminate HML silencing in a
sir1Δ mutant, and abolish telomeric silencing ( Ehrenhofer-Murray et al., 1997; Reifsnyder et al., 1996). These results indicate that SAS2 plays a positive role in silencing at HML and telomeres. On the other hand, it was shown that sas2Δ suppressed the silencing defect of a HMR-E silencer with mutated Rap1p- and Abf1p-binding sites (HMRa-e**) (Ehrenhofer-Murray et al., 1997; Meijsing and Ehrenhofer-Murray, 2001; Reifsnyder et al., 1996). It also suppressed the silencing defect of a synthetic HMR-E silencer at the HMR locus deleted for the HMR-I silencer (HMR-SSΔI) (Ehrenhofer-Murray et al., 1997). These results suggest that SAS2 plays a negative role in silencing at HMR. Other investigations into SAS2 function in silencing have only added more conflicting evidence to this apparent paradox. For instance, it was shown that sas2Δ decreased silencing of an ADE2 reporter inserted at HMR (Meijsing and Ehrenhofer-Murray, 2001). A recent study found that sas2Δ reduced silencing at both HML and HMR, and sas2Δ and sir1Δ had a synthetic effect on both HML and HMR silencing (Xu et al., 2006). In that work, the reporters for HML and HMR silencing were YFP and CFP genes respectively, under the control of the URA3 promoter, and silencing in individual cells was examined (Xu et al., 2006).

Reconciling the conflicting results concerning the effect of SAS2 on silencing has been complicated by the fact that they were obtained from various silencing assays that involved different loci (e.g., HML vs. HMR), silencers (e.g., intact HMR-E vs. HMRa-e** or HMR-SS), reporters (e.g., HMRa vs. ADE2) and/or genetic backgrounds (SIR1 vs. sir1Δ). Since distinct silencers have different properties in silencing, and the function of a silencer is affected by its genomic context (Rusche et al., 2003; Zou et al., 2006b), is the mode of Sas2p action on a silencer also dependent on the intrinsic features and/or the context of the silencer? To avoid potential complications that might result from comparing data from different
silencing assays, I examined the effect of $sas2\Delta$ on the silencing of the $URA3$ reporter by the $HML-E$, $HMR-E$ and $HML-I$ silencers in the same context of the $HML-E$ silencer (Fig. 3-6B, strains 3-21 – 3-27). Cells expressing $URA3$ are sensitive to the drug 5-fluoroorotic acid (FOA) so cell growth on medium containing FOA is a measure of $URA3$ silencing (van Leeuwen and Gottschling, 2002).

$URA3$ inserted to the left (telomere-proximal) side of $HML$ was strongly silenced by $HML-E$ (Fig. 3-6B, robust growth of strain 3-21 on FOA). $HML-I$ and $HMR-E$ placed in the context of $HML-E$ also efficiently silenced $URA3$ (Fig. 3-6B, 3-24 and 3-26 on FOA). $sas2\Delta$ decreased the growth of strain 3-22 on FOA medium (Fig. 3-6B, compare 3-22 to 3-21). Since $sas2\Delta$ does not affect the function of the $URA3$ promoter per se (Pillus and Rine, 1989), decreased growth of strain 3-22 on FOA medium demonstrates that $URA3$ silencing by $HML-E$ is reduced by $sas2\Delta$. It is noteworthy that in the $sas2\Delta$ mutant, silencing was not completely abolished as it was in the $sir3\Delta$ strain (Fig. 3-6B, compare 3-22 to 3-23). The sizes of colonies of the $sas2\Delta$ strain 3-22 on FOA were uniformly smaller than those of the $SAS2$ strain 3-21 (Fig. 3-6B), suggesting that $URA3$ silencing was reduced to an intermediate level by $sas2\Delta$. $sas2\Delta$ also decreased $URA3$ silencing by $HMR-E$ and $HML-I$ (Fig. 3-6B, compare 3-25 to 3-24, and 3-27 to 3-26). Taken together, these results demonstrate that $SAS2$ plays a positive role in silencing by all three silencers in the context of $HML-E$. 
Fig. 3-6. A positive role of $SAS2$ in silencer function. (A) Schematics of the $HML$ and $HMR$ loci on chromosome III in $S. cerevisiae$ (not drawn to scale). $HML$-E and -I silencers are shown as filled arrows with white letters, and $HMR$-E and -I are shown as open arrows with black letters. The directions of silencers are drawn pointing toward the inside of $HML$ or $HMR$. The sites for Abf1p, Rap1p, and ORC binding in silencers are indicated by stippled, filled, and open rectangles, respectively. $HML\alpha$ genes at $HML$ and $HMR\alpha$ genes at $HMR$ are also indicated. Tandem arrowheads indicate telomeric repeats. (B) Growth phenotypes of strains 3-21 – 3-33. Left, modified $HML$ and $HMR$ loci in strains 3-21 – 3-33. Cells of each strain were grown to log phase, and serial 10-fold dilutions were spotted on test plates and allowed to grow for 3 days. Two independent clones of each strain were examined. FOA, SC plus 1 mg/ml of 5-fluoroorotic acid (FOA).
I next examined whether the location of a silencer affects its response to $sas2\Delta$. I deleted $SAS2$ from strains in which $URA3$ inserted to the right of $HML$ is subject to silencing by inverted $HML-I$ or $HMR-E$ (Fig. 3-6B, strains 3-28 and 3-30). Growth of these $sas2\Delta$ strains on FOA was reduced compared to their $SAS2$ counterparts (Fig. 3-6B, compare 3-29 to 3-28, and 3-31 to 3-30). Therefore, the activities of $HML$-$I$ and $HMR$-$E$ in the context of $HML$-$I$ were also reduced by $sas2\Delta$. I next deleted $SAS2$ from strains 3-32 in which $URA3$ was inserted to the left of an inverted $HMR$-$E$ silencer at $HMR$ (Fig. 3-6B). I found again that $sas2\Delta$ reduced $URA3$ silencing by $HMR$-$E$ (Fig. 3-6B, compare 3-33 to 3-32). The above results suggest that $SAS2$ is required for efficient silencing by all tested silencers in all tested contexts.

### 3.4.6 A role of $SAS2$ in maintaining transcriptionally silent chromatin structure

Transcriptional silencing in yeast is mediated by a special silent chromatin (Rusche et al., 2003). One of the characteristics of silent chromatin is a distinct topology of its DNA. DNA at $HML$ and $HMR$ is more negatively supercoiled when these loci are silenced than when they are derepressed (Bi and Broach, 1997; Cheng et al., 1998). As the topology of eukaryotic DNA reflects the density and conformation of nucleosomes, the degree of negative supercoiling of $HM$ DNA can be used as a measure of the state of silent chromatin (Bi and Broach, 1997; Cheng et al., 1998).

To investigate whether $sas2\Delta$ affects the structure of silent chromatin, I compared the supercoiling of $HML$ DNA in wild type and $sas2\Delta$ strains. In strain 3-34, the modified $HML$ locus was bracketed by two copies of FRT (Flp1p recombination target), the recognition site for the site-specific recombinase Flp1p (Fig. 3-7A, top). Induction by galactose of a $P_{GAL}$-
*FLP1* fusion gene resident elsewhere in the genome would lead to the expression of Flp1p and recombination between the FRT sites resulting in the excision of *HML* as a closed minichromosome circle. After being deproteinized, the supercoiling of the circle can be examined by gel electrophoresis in the presence of the DNA intercalator chloroquine (Fig. 3-7A, strain 3-34). Deletion of *SIR3* reduced the negative supercoiling of *HML* DNA by a linking number change (ΔLk) of 7 (Fig. 3-7, compare the centers of topoisomer distributions in strains 3-38 and 3-34; note more negatively supercoiled circles migrate more slowly under the condition used). *sas2Δ* induced a reduction in negative supercoiling of *HML* DNA of a ΔLk of 1 (Fig. 3-7, compare 3-35 and 3-34). This effect was specific to silent *HML*, as *sas2Δ* did not reduce the negative supercoiling of derepressed *HML* in a *sir* background (Fig. 3-7, compare 3-39 and 3-38). Therefore, the supercoiling of *HML* (hence the configuration of silent chromatin) in a *sas2Δ* strain is in an intermediate state between fully silenced and derepressed states (Fig. 3-7, compare 3-35 to 3-34 and 3-38). This is consistent with the result that *sas2Δ* reduced transcriptional silencing to an intermediate level (Fig. 3-6).

As *sas2Δ* and *sir1Δ* have a synthetic effect on *HML* silencing ( Ehrenhofer-Murray et al., 1997; Reifsnyder et al., 1996; Yu and Morse, 1999), I tested whether they also have a synthetic effect on the topology of *HML* DNA. Sir1p is believed to be involved in the establishment but not the maintenance of silent chromatin at the *HM* loci (Rusche et al., 2003). Single-cell assays revealed that a culture of *SIR1* null cells consists of two populations of mitotically stable cells that differ in the state of *HML* silencing (Pillus and Rine, 1989; Xu et al., 2006). *HML* is silenced in one population of cells, but derepressed in the other. Consistently, the topoisomers of *HML* circles isolated from a *sir1Δ* culture appear to be a mixture of topoisomers from *SIR*+ cells where *HML* is silenced and those from *sir*−
cells where \( HML \) is derepressed (Fig. 3-7, compare 3-36 to 3-34 and 3-38). However, in a \( sas2\Delta \, sir1\Delta \) double mutant, there was only one population of \( HML \) circles, which is similar to that in \( sir^{-} \) cells (Fig. 3-7, compare 3-37 and 3-38). Therefore, \( sas2\Delta \) and \( sir1\Delta \) have a synthetic effect on the topology of \( HML \) DNA, which is consistent with the finding that \( sas2\Delta \) abolished \( HML \) silencing in a \( sir1\Delta \) background (Ehrenhofer-Murray et al., 1997; Reifsnyder et al., 1996; Xu et al., 2006).

**Fig. 3-7.** \( sas2\Delta \) reduces the negative supercoiling of silent \( HML \) DNA. (A) Top, diagram of the modified \( HML \) locus in strains 3-34 – 3-39. The \( HML\)-E and –I silencers and a pair of FRTs (Flp1p recombination targets) were shown. Each strain bears a \( P_{GAL-FLP1} \) gene in the genome. Bottom, the topology of \( HML \) DNA in strains 3-34 – 3-39. DNA isolated from each strain was subjected to agarose gel electrophoresis in the presence of 13 \( \mu \)g/ml chloroquine. Under this condition more negatively supercoiled circles migrate more slowly. After Southern blotting, topoisomers of the \( HML \) circle were detected by an \( HML \)-specific probe. The center of distribution of topoisomers in each lane was marked by a dot. The nicked/relaxed form of the \( HML \) circle was indicated as N. (B) Profiles of topoisomers in lanes 3-34 – 3-39 shown in (A) as determined by using NIH Image.
3.4.7 Synthetic effect of \(sas2\Delta\) and \(orc5-1\) on transcriptional silencing

Given the aforementioned functional interactions between \(sas2\Delta\) and \(orc\) mutants regarding cell growth and DNA replication, I asked whether they also interact to affect transcriptional silencing. I showed that either \(orc5-1\) or \(sas2\Delta\) alone reduced silencing of a \(URA3\) reporter integrated at \(HMR\) in W303-1B (Fig. 3-8, compare 42 and 41 to 40 on FOA), and \(sas2\Delta\ orc5-1\) double mutation abolished \(URA3\) silencing (Fig. 3-8, compare 43 to 40). Therefore, \(SAS2\) is required for \(HMR\) silencing in an \(orc5-1\) mutant.

![Fig. 3-8. Synthetic effect of \(sas2\Delta\) and \(orc2-1\) on transcriptional silencing. Growth phenotypes of strains 3-40 – 3-43 on SC and FOA media at 30°C are shown.](image-url)
3.5 Discussion

The initiation of DNA replication in eukaryotes depends on the assembly of the pre-RC via the sequential binding to the origin of ORC, Cdc6p, Cdt1p and the MCM complex (Bell, 2002). I have presented evidence to suggest that the HAT Sas2p plays a positive role in replication initiation in yeast. This is in line with the notion that chromatin structure regulates origin function. It has been shown that increasing histone acetylation around a replication origin in yeast enables it to fire earlier in S phase (Vogelauer et al., 2002). Recently, it was found that the yeast HAT Hat1p physically interacts with ORC, and HAT1 deletion exacerbates the temperature-sensitivity of orc mutants (Suter et al., 2007). Hat1p acetylates histone H4-K5, K12 and, to a lesser extent, histone H2A (Parthun, 2007), and it may contribute to replication initiation by modifying nucleosomes near the origins. There is also evidence suggesting that Hat1p contributes to chromatin assembly during replication elongation (Parthun, 2007; Suter et al., 2007). In vertebrates, Hbo1p, a member of the MYST family of HATs, has been shown to interact with ORC and MCM and positively regulate pre-RC formation (Iizuka et al., 2006; Iizuka and Stillman, 1999). Depletion of Hbo1p does not affect chromatin binding of ORC and Cdc6p but prevents the recruitment of the MCM complex. It is proposed that Hbo1p facilitates MCM loading by acetylating chromatin at the origin and/or components of the pre-RC complex such as Orc2p (Iizuka et al., 2006; Iizuka and Stillman, 1999).

I showed that SAS2 deletion (sas2Δ) does not affect DNA replication in the presence of an intact ORC, but reduces origin firing when ORC function is compromised by orc2-1 mutation (Fig. 3-4). Sas2p’s role in DNA replication may overlap with that of other factors involved in ORC function such as Hat1p. It is not known whether Sas2p directly interacts
with ORC like Hat1p and Hbo1p. Sas2p may help ORC function by acetylating histones near origins. I have tested the possibility that Sas2p contributes to the formation of proper chromatin structure at origins such as ARS1 that is required for origin firing. However, I found that sas2Δ caused no significant change in chromatin structure at ARS1 in wild type or orc2-I cells at nucleosome positioning level (Fig. 3-5). It is also possible that H4-K16 acetylation by Sas2p helps an origin fire by disrupting higher order chromatin structure. This is based on the finding that H4-K16 acetylation is inhibitory to 30 nm fiber structure formation from a nucleosome array (Shogren-Knaak et al., 2006). In addition, Sas2p may also contribute to chromatin assembly during DNA replication. Consistent with this notion is the finding that Sas2p physically interacts with CAF-I and Asf1p, two factors that mediate chromatin assembly during DNA synthesis (Meijsing and Ehrenhofer-Murray, 2001; Osada et al., 2001; Shogren-Knaak et al., 2006).

I found, interestingly, that compared to ARS1, ARS305 firing was significantly less sensitive to orc2-I and sas2Δ despite the fact that ARS305 is also an early-firing origin (Fig. 3-4). ARS305 is distinct from ARS1 in sequence and structure. Whereas the ARS1 contains ACS, B1, B2 and B3 elements, ARS305 contains ACS, B1 and B4 but lacks B2 and B3 (Fig. 3-4B) (Huang and Kowalski, 1996; Shogren-Knaak et al., 2006). Moreover, ARS305 contains a DUE (DNA unwinding element) that is essential for its function (Fig. 3-4B) (Huang and Kowalski, 1993; Huang and Kowalski, 1996). The unique presence of this DUE sequence may be the reason why ARS305 firing is less dependent on the functions of ORC and Sas2p than ARS1.

The results in this chapter demonstrating a synthetic genetic-functional interaction between SAS2 and ORC genes are apparently contrary to the previous finding that sas2Δ
suppressed *orc2-1* phenotypes (Ehrenhofer-Murray et al., 1997; Weinberger et al., 1999). The temperature-sensitive *orc2-1* and, to a lesser extent, *orc5-1* mutants exhibit slow growth phenotypes even at permissive temperatures. It is possible that a spontaneous secondary mutation that suppresses the growth defect exists in one or more of the *orc2-1* and *orc5-1* mutants used in this and/or other studies. In fact, Shimada et al. demonstrated that their batch of a widely used *orc2-1* mutant JRY125 had an extragenic suppressor mutation that mitigated the temperature sensitivity of *orc2-1* so that cells could grow at 30°C (but not higher temperatures) (Shimada et al., 2002). The JRY125 derivatives we made were not viable at 30°C (Fig. 3-2), and therefore did not seem to contain the additional mutation. On the other hand, my *MATa orc2-1* strain was able to grow, albeit slowly, at 30°C, and therefore might possess a secondary mutation that partially suppresses the growth defect of *orc2-1* (Fig. 3-1). There has been no report regarding whether *orc5-1* mutants contain extragenic suppressor mutations. Nevertheless, the fact that *sas2Δ* showed synthetic growth defect with both *orc2-1* and *orc5-1* in three genetic backgrounds in this study (Fig. 3-1 and 3-2) strongly supports the notion that Sas2p plays a positive role in ORC-mediated functions. As for previous results of *sas2Δ* suppressing *orc2-1* phenotypes (Ehrenhofer-Murray et al., 1997; Weinberger et al., 1999), it is possible that an extragenic suppressor mutation alone, or together with *sas2Δ*, was responsible for the suppression.

Previous studies on the role of *SAS2* in transcriptional silencing yielded conflicting results, with some suggesting a positive role for *SAS2* in silencing, while others concluding that Sas2p was a negative regulator (Ehrenhofer-Murray et al., 1997; Meijsing and Ehrenhofer-Murray, 2001; Reifsnyder et al., 1996; Xu et al., 2006). However, the silencing assays used in these studies were distinct from each other concerning the silencing reporter,
the silent locus, and/or the genetic background, making it difficult to compare the results directly. In this work, I examined whether the intrinsic properties and/or the context of a silencer (HML vs. HMR) affected its response to sas2Δ. I showed that the function of each of silencers HMR-E, HML-E and HML-I was reduced to an intermediate level when put in the same context of HML-E (Fig. 3-6). The activity of HMR-E in its native context is also reduced to an intermediate level by sas2Δ (Fig. 3-6 and 3-8). The same silencing reporter URA3 was used in all our experiments so the results can be readily compared directly. These results demonstrate that SAS2 plays a positive role in the function of any of the tested silencers regardless of their genomic contexts, which are consistent with the finding that SAS2 is required for telomeric silencing. Acetylation of histone H4-K16 by Sas2p in euchromatin was proposed to prevent ectopic spreading Sir complex from telomeric silent chromatin (Kimura et al., 2002; Suka et al., 2002). This model may also apply to silent chromatin at HM loci. According to this model, sas2Δ allows a portion of Sir proteins to leave silent HML and HMR and spread into euchromatin regions, thereby reducing Sir abundance at HM loci. As a result, silent chromatin adopts an intermediate state between fully silent and fully derepressed structures.

Given the functional interactions between the SAS2 and ORC genes in DNA replication, I envision that Sas2p may also regulate silencing by affecting ORC function at the silencer. This is supported by our finding that sas2Δ and orc5-1 have a synthetic effect on HMR silencing (Fig. 3-10). ORC bound to a silencer contributes to the establishment of silent chromatin by recruiting Sir1p which interacts with the Sir complex (Rusche et al., 2003). In addition, ORC also directs nucleosome positioning at the silencer that affects the efficiency and directionality of silencing (Zou et al., 2006a). It is possible that Sas2p-
mediated histone acetylation near the silencer facilitates one or both of these ORC-mediated processes.

It is noteworthy that my experiments demonstrating a positive role of \textit{SAS2} in silencing involved wild type silencers (Fig. 3-6 – 3-8), while previous studies reporting a negative role of \textit{SAS2} in silencing employed a mutated or synthetic \textit{HMR-E} silencer that is weaker than the wild type one (Ehrenhofer-Murray et al., 1997; Reifsnyder et al., 1996). It is not clear why wild type and mutated \textit{HMR-E} silencers respond to \textit{SAS2} in opposite ways.

I found that \textit{sas2}\textsuperscript{$\Delta$} reduced the negative supercoiling of \textit{HML} DNA circles bearing the silencers by a \textit{\Delta}\textsubscript{Lk} of 1 (Fig. 3-7). This reduction is less than the \textit{\Delta}\textsubscript{Lk} of 7 caused by \textit{sir3}\textsuperscript{$\Delta$} (Fig. 3-7), but is consistent with the finding that \textit{sas2}\textsuperscript{$\Delta$} decreases but does not completely abolish transcriptional silencing at the \textit{HM} loci (Fig. 3-6 and 3-8), and suggests that \textit{HML} chromatin assumes an intermediate state that is distinct from both the fully silenced state (in \textit{SIR}\textsuperscript{+} cells) and fully derepressed state (in \textit{sir\textsuperscript{-}} cells).

The topology of eukaryotic DNA is determined by multiple factors including the density of nucleosomes along the DNA and the conformation of individual nucleosomes. The major contribution to the negative supercoiling of DNA is its wrapping into nucleosomes with an average \textit{\Delta}\textsubscript{Lk} of about –1 per nucleosome formed \textit{in vitro} (Simpson et al., 1985). Changing the conformation of a nucleosome may alter the number of supercoils constrained on it. For example, acetylation of the core histones reduces the negative supercoiling of nucleosomal DNA (Norton et al., 1989). The higher negative supercoiling of DNA in silent versus derepressed \textit{HML} locus is a reflection of increased regularity and stability of nucleosomes as well as reduced histone acetylation in silent chromatin (Braunstein et al., 1993; Weiss and Simpson, 1998). The reduction of negative supercoiling induced by \textit{sas2}\textsuperscript{$\Delta$}
might be the result of a decrease in nucleosome density/stability and/or conformational changes of nucleosomes, but our preliminary chromatin mapping results suggest that the former is unlikely the case (Y. Zou and X. Bi, unpublished results).
References


