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Studies on the Establishment of Transcriptional Silencing in Yeast

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Abstract of the Dissertation

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Epigenetic silencing refers to a transcriptionally inactive state, which involves the formation, maintenance and heritable transmission of heterochromatin, in concert with cell-cycle progression. It plays an important role in growth and development in eukaryotes ranging from yeast to human. My dissertation research concentrated on this process with budding yeast as the model system.

I have studied how silencing is established in concert with cell-cycle progression. In *S. cerevisiae*, the two cryptic mating type loci, *HML* and *HMR*, are transcriptionally silent. Previous studies on one locus (*HMR*) identified an S-phase requirement for establishment of silencing, but couldn't explain the underlying mechanism. Although the other locus (*HML*) was assumed to be the same, I found it didn't impose such a requirement. That is, silencing could be partially established at *HML* without passage through S phase. Further

analysis identified the promoters at these loci as the cause of this difference. Experiments with modified *HM* loci containing transcription units of different promoter strength demonstrated the competition between transcription and silencing: the stronger the promoter, the more resistant it is to silencing. This competition can be overcome by passage through S phase.

Another part of my study focused on how the silent information regulator 1 (Sir1) facilitates the establishment of silencing. Current understanding suggests that the origin recognition complex (ORC) recruits Sir1, and Sir1, in turn, recruits other Sir proteins to the silencers, the cis-regulatory sites flanking the silent region. I have analyzed the distribution of Sir1 on silent chromatin and its interaction with other proteins both *in vitro* and *in vivo*. Sir1 contains an N-terminal domain which is homologous to its well-characterized C-terminal domain. My studies on the Sir1 N-terminal domain showed that, although it is not necessary for complementation of *sir1* null mutants, it is important for protein stability and suppression of certain silencing defects. Using the known structure of the Sir1 C-terminal domain, I predicted the structure of the Sir1 N-terminal domain and tested its interaction with the Sir3 BAH domain.

As an independent project of my dissertation research, I have investigated the cyclin-dependent kinase Bur1 and its interacting partners. A two-hybrid screen for Bur1 interacting proteins was carried out. Various assays were used to characterize the function of such interactions in the regulation of transcription, mRNA maturation and export.

Dedication

This work is dedicated to my parents, father Li Ren and mother Song Yue.

All I have and will accomplish are only possible due to their love, support, sacrifices, guidance and everything.

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Acknowledgements

Faculty, friends and family members have gave me tremendous help to complete this dissertation. This is a great opportunity to express my gratitude to those people who have made this dissertation possible and because of whom my graduate experience has been one that I will cherish forever.

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My sincere appreciations also go to the members of my dissertation committee, Prof. Janet Leatherwood, Prof. Peter Gergen and Prof. Neta Dean. They have generously given their time and expert advices to make sure my research went into the right direction. I would also like to thank people from the Yeast floor for generously sharing reagents and facilities.

I am very grateful to all the past and present members of the Sternglanz lab: Ann Sutton, who gave me tremendous help and guidance when I first joined the lab; Vinaya Sampath, who is the first person I will turn to for my experimental problems and has always been patient, caring and insightful; Xiaorong Wang, who is like a big sister to me and has been a wonderful source of advice and encouragement; Yao Yu and Madhusudhan Srinivasan, who not only gave amazing suggestions on science, but also have a wicked sense of humor to cheer me up; Chia-lin Wang and Peihua Yuan, who are great colleagues to work with; and Evelyn Prugar, who gave critical technical support.

Finally and most importantly, none of this could be possible without the love, understanding and patience of my family. My mom and dad, Song Yue and Li Ren, to whom this thesis is dedicated to, has been a constant source of love, concern, support, guidance and strength for all these years. It was under their watchful eyes that I gained the courage and ability to tackle the challenges head on.

Chapter One: Background and Significance

I. Epigenetics: establishment and inheritance of epigenetic state

The concept of epigenetics was first proposed by Waddington in 1942 to describe the causal mechanism by which a genotype brings about a phenotype (134). Since the discovery of DNA methylation and post-translational modifications of histones, which can change gene activity without changing the sequence, the term "epigenetics" has been applied to such heritable changes in genome function that occur without alterations to the DNA sequence (106). Currently, the candidates of "epigenetic marks" include DNA and histone modifications, histone variants, non-histone chromatin proteins, nuclear RNA, as well as the resulting higher-order chromatin organization (99).

Epigenetic information provides a form of memory for chromatin regulation, which operates at both local (single genes) and global levels (chromosome domains or even entire chromosomes) (33). Epigenetic regulation plays an important role in establishing and maintaining differentiated patterns of gene expression in cells which are genetically identical (63, 99). Examples for epigenetic silencing include the maintenance of a cell identity after differentiation, dosage compensation and imprinting in mammals, position-effect variegation in *Drosophila melanogaster*, and the cryptic mating-type loci

in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* (99, 111, 119, 142). Epigenetic regulation also functions in genome structure and stability, as demonstrated at rDNA repeats, telomeres, centromeres, etc. (99).

The status of epigenetic marks can be divided into three phases: establishment, maintenance and inheritance. The establishment phase refers to the initial setting up of these epigenetic marks, which is to be sustained during one cell cycle in the maintenance phase, and to be transmitted from generation to generation for inheritance. During DNA replication, chromatin undergoes disruption and subsequent restoration of epigenetic marks onto daughter strands according to the parental information (99). However, such parental guidance is not available for the establishment phase, indicating that a different mechanism may be utilized for the initial step.

Moreover, both establishment and inheritance are concerted with cell cycle progression, but it is not clear if the underlining mechanisms are the same or different. For inheritance, the cell-cycle dependence is conceivable because the process of inheritance is coupled to DNA replication in several aspects; e.g., DNA methylation is inherited at the replication fork (148). Another example is that part of the inheritance of histones and their modification is also associated with DNA replication, as suggested by the evidence that parental and newly synthesized histones are distributed to the daughter strands in a random fashion, and the maintenance of the epigenetic marks on newly synthesized histones are guided by neighboring parental nucleosomes (37). The other part

of the inheritance of the epigenetic marks, such as the incorporation of centromere-specific histone variants in humans (79), operates outside S phase . Therefore, epigenetic inheritance should be in concert with cell-cycle progression. On the other hand, several studies have also shown that the establishment phase is cell-cycle dependent (63-65, 69, 72, 85), which does not necessarily have a direct link to DNA replication. Part of my study tried to understand the underlining mechanism for this dependence. The difference between epigenetic establishment and inheritance will be compared again later in this chapter using transcriptional silencing at *HM* loci of *S. cerevisiae* as a paradigm.

II. Heterochromatin and its assembly

Epigenetic silencing, or transcriptional silencing, refers to a transcriptionally inactive state and its heritable transmission (14, 99). It relies on the establishment and inheritance of a specialized, constitutively compact chromatin structure, termed heterochromatin (63, 69, 111). At a heterochromatic region, there is no accessibility of DNA methyltransferases and restriction endonucleases (31, 78). In contrast, euchromatin assumes more open chromatin structures, and is actively transcribed. Heterochromatin and euchromatin constitute the organization of the eukaryotic DNA.

The assembly of heterochromatin utilizes various mechanisms and involves different enzymes, structural proteins, or small RNAs, depending on the species and the region to be silenced. Nevertheless, the assembly process still possesses some conserved themes. It

usually occurs in a stepwise manner; silencing complexes are recruited to the nucleation site, followed by sequential rounds of histone modification and spreading of the silencing complex, resulting in a compact higher-order chromatin structure (33) (FIG 1-1). Many varieties exist within this common theme, such as the recruitment of a silencing complex, which can occur either via interaction with DNA binding proteins (e.g., at the mating type loci and telomeres of *S. cerevisiae*), or is targeted by small RNAs (e.g., at centromeres of *S. pombe* or X inactivation in mammals). Also, the silencing complex can be composed of a variety of chromatin-modifying enzymes and structural proteins.

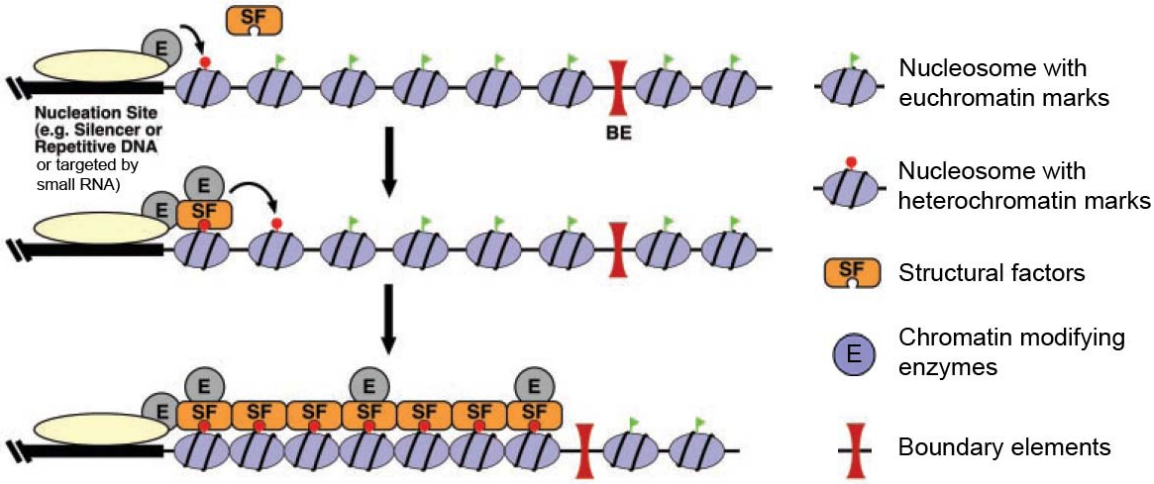

FIG 1-1 Common theme of heterochromatin assembly (adapted from FIG 4 of ref.(33)). The nucleation sites recruit chromatin-modifying enzymes (E) through protein-protein interaction or protein-RNA interaction. Once targeted, the chromatin-modifying enzymes exchange euchromatin marks for heterochromatin marks, and create binding sites for structural factors on neighboring nucleosomes. After sequential rounds of chromatin modification and spreading of the silencing complex, the nucleosomes are packed into a tight higher-order structure. The boundary elements prevent heterochromatin from spreading further.

III. Transcriptional silencing in *S. cerevisiae*

In *S. cerevisiae*, the budding yeast, there are heterochromatin-like regions at the two cryptic mating type (*HM*) loci, telomeres and rDNA repeats. Silent chromatin at the *HM* loci and telomeres share many mechanistic features, while rDNA silencing is achieved through a distinct mechanism (111). Although the mechanisms and players of epigenetic silencing are not well conserved from *S. cerevisiae* to other species, they share common features as mentioned above. Therefore, transcriptional silencing in *S. cerevisiae* serves as a paradigm. Especially, silencing at *HM* loci has been studied extensively to dissect the steps of establishment of silencing (54).

HM loci, *HML* and *HMR*, harbor cryptic copies of the mating type information genes, α and **a**, respectively. The mating type information at each of the *HM* loci is transcriptionally silenced, while the same cassette of either mating type at a different genomic locus (*MAT*) is actively transcribed and determines the cell type. Transcriptional silencing at *HM* loci relies on *cis*-regulatory DNA elements called silencers, and on a number of *trans*-acting gene products (FIG 1-2). The assembly of heterochromatin also follows the stepwise manner as described above. Silencers flank the silent region and recruit the DNA binding proteins Rap1, Abf1, and the origin replication complex (ORC), which in turn recruit the silent information regulator (Sir) proteins, Sir1, Sir2, Sir3 and Sir4. Therefore, silencers serve as the nucleation site for the binding of Sir proteins. The spreading of the Sir2-Sir3-Sir4 complex requires the self-association of Sir proteins and

the deacetylase activity of Sir2, which removes the euchromatic marks on histone H4 K16, thereby creating better binding sites on nearby nucleosomes for Sir3 and hence the complex. The spreading of the SIR complex brings Sir2 in vicinity to euchromatin marks on neighboring nucleosomes for deacetylation, and in turn, creates binding sites for the complex. After multiple rounds of deacetylation and spreading, the modified chromatin with interacting silencing complexes form a higher-order structure characteristic of heterochromatin (FIG 1-3) (44, 86, 110, 112).

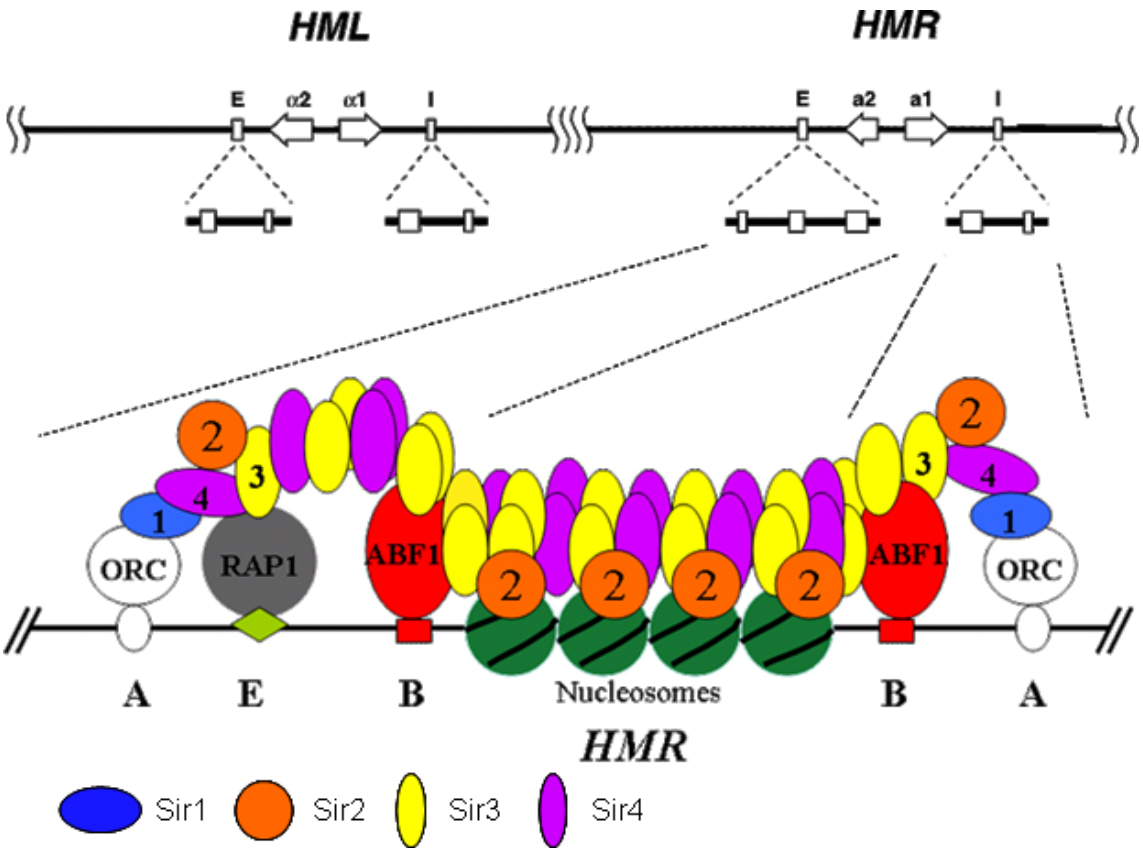

FIG 1-2 Diagrams of the *HM* loci (adapted from FIG 1 of ref(111) and FIG 2 of ref(13)). The upper panel shows the composition of *HML* and *HMR* loci. They harbor silent transcription units of the mating type information, α1/α2 and **a**1/**a**2, respectively. The transcription units are flanked by E and I silencers, which are composed of binding sites for two or three DNA-binding proteins, as illustrated in the lower panel. The schematic representation of the *HMR* locus (the lower panel) is not in proportion to the actual genomic distance, but shows in detail the protein-DNA and protein-protein interactions which establish the heterochromatin at this locus. The *HMR-E* silencer contains binding sites for ORC, Abf1 and Rap1, while the *HMR-I* silencer only binds the former two. Among these DNA-binding proteins, ORC has high affinity for Sir1, which interacts directly with Sir4; Abf1 interacts with Sir3; and Rap1 recruits Sir4 and hence the Sir4/Sir2 complex. Once targeted to the silencer, the SIR complex carried out iterative cycles of deacetylation and spreading to establish heterochromatin at the locus. The spreading of the SIR complex mainly occurs in the region between the two silencers, and the spreading outside is restricted by boundary elements.

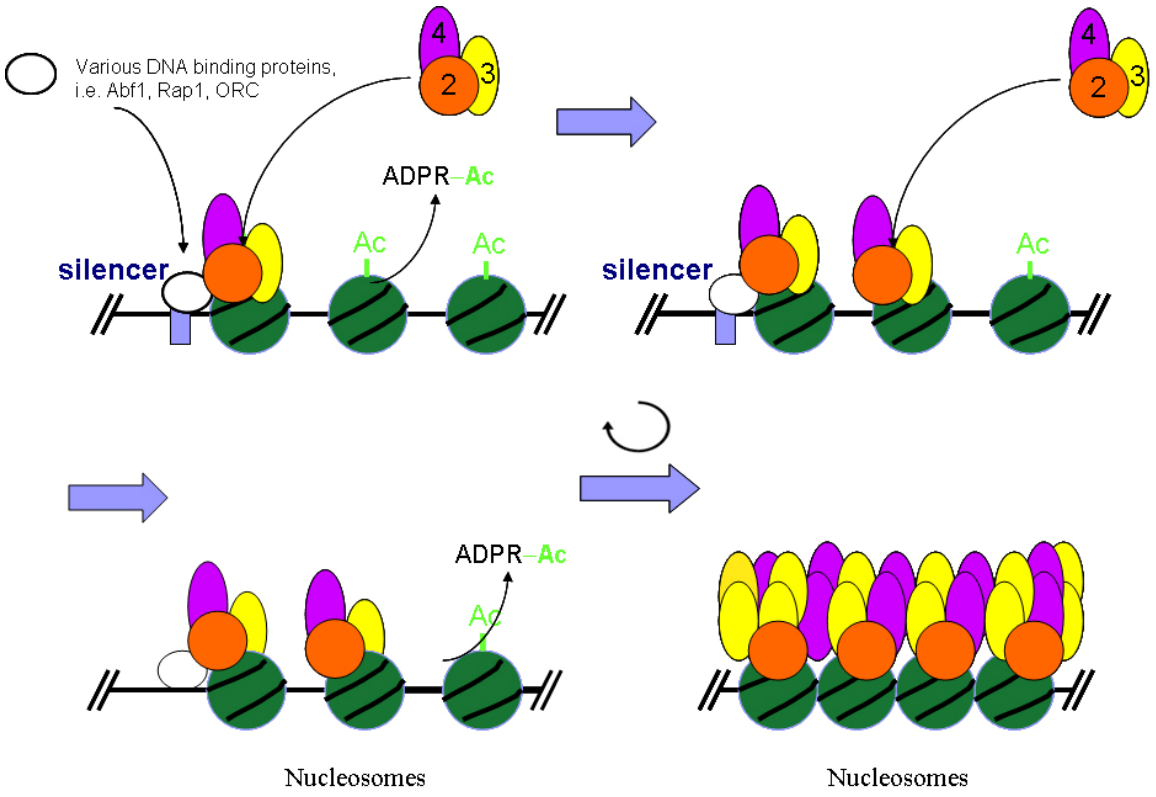

FIG 1-3 A simplified model of stepwise assembly of heterochromatin at the *HM* loci (adapted from ref(54, 86)). The silencer recruits DNA-binding proteins to direct the SIR complex to target chromosomal regions. At this region, an iterative cycle of NAD-dependent histone deacetylation and direct chromatin association leads to spreading of the SIR complex and produces a compact SIR-coated chromatin fiber that silences transcription. In this cycle, the deacetylation of histone H4 K16 creates a binding site for Sir3, which can then promote the loose association between Sir4-Sir2 complex and the acetylated chromatin to a tighter SIR-chromatin interaction; also, one product of the reaction, O-acetyl-ADP-ribose (labeled as ADPR-Ac in the diagram), may be incorporated into the SIR complex and contribute to the SIR-chromatin association. The spreading of the SIR complex brings Sir2 to euchromatin marks on neighboring nucleosomes for a new round of deacetylation and association.

IV. Establishment of silencing and cell-cycle requirements in *S. cerevisiae*

The *S. cerevisiae* cell cycle has been well studied, as depicted in FIG 1-4 labeled with major landmark events.

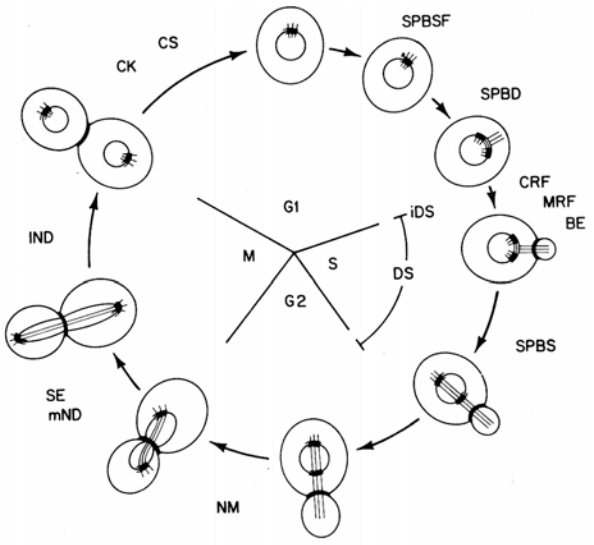

FIG 1-4 *S. cerevisiae* cell cycle labeled with major landmark events (98). Abbreviations: SPBSF, spindle-pole-body satellite formation; SPBD, spindle-pole-body duplication; CRF, formation of the chitin ring (shown in the diagram as a heavy line at the mother-bud junction); MRF, formation of the microfilament ring (not shown in the diagram, but found adjacent to the cell membrane in the region of mother-bud junction); BE, bud emergence; iDS, initiation of chromosomal DNA synthesis; DS, chromosomal DNA synthesis; SPBS, spindle-pole-body separation (and formation of a complete spindle); NM, nuclear migration; mND, medial stage of nuclear division; SE, spindle elongation; lND, late stage of nuclear division; CK, cytokinesis; CS, cell separation. The intervals from SPBSF to BE and from IND to CK may be exaggerated in the diagram.

Earlier in this chapter, I have compared the establishment and inheritance of epigenetic marks in general. In FIG 1-5, they are put side by side again to illustrate their specific processes in transcriptional silencing. For inheritance, the parental nucleosomes are distributed randomly onto two daughter strands, serving as the template for modification of newly synthesized histones and also as the recruiter of the SIR complex for the assembly of heterochromatin. However, such parental guidance is not available for the establishment phase, which starts from a derepressed locus. In addition, there are

euchromatin marks at the derepressed locus that have to be removed during the establishment of silent chromatin.

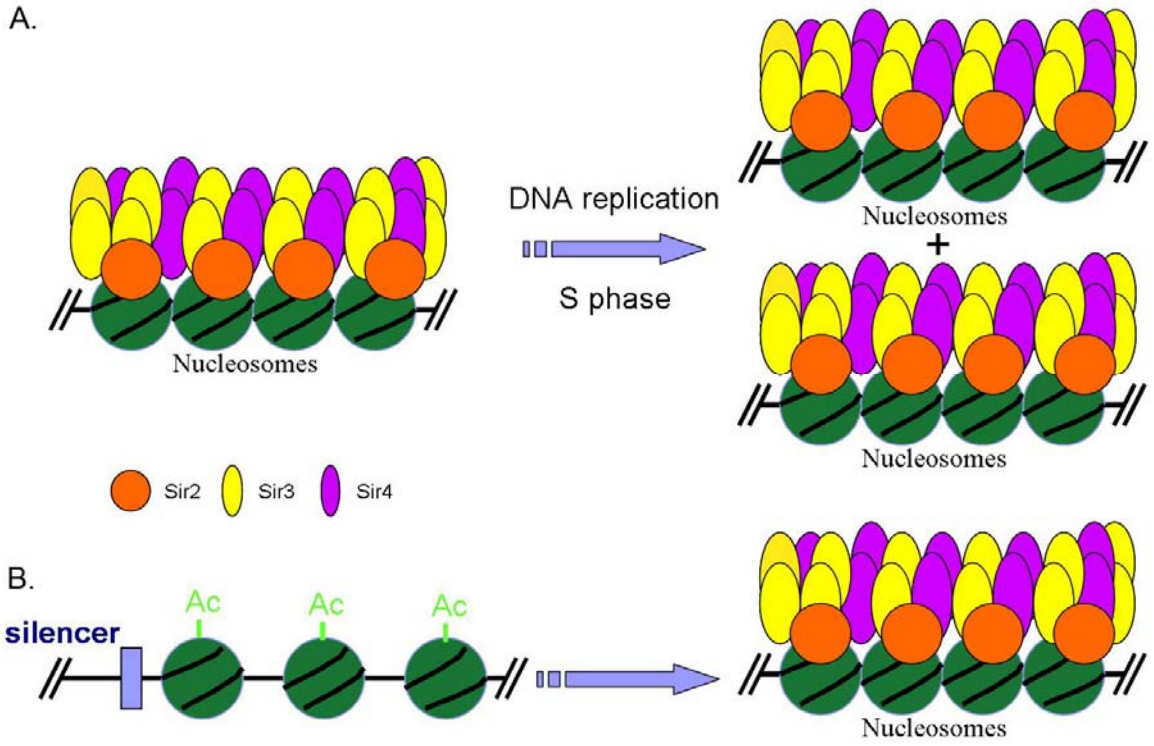

FIG 1-5 Inheritance and establishment of transcriptional silencing at the *HM* loci of *S. cerevisiae*. A. Inheritance of a silent *HM* locus. After DNA replication, parental nucleosomes with heterochromatin marks may be distributed randomly onto two daughter strands. To avoid the dilution of epigenetic marks and to inherit the heterochromatic structure, active maintenance is carried out by modification of newly synthesized nucleosomes with neighboring parental nucleosomes as the template for modification, or even as the recruiter of the SIR complex to interact with the chromatin and assemble it into a heterochromatic structure. B. Establishment of silencing at a derepressed *HM* locus. For the assembly of heterochromatin, euchromatin marks, such as histone H4 K16 acetyl group and histone H3 K79 methyl groups, need to be removed. The silencer recruits the SIR complex for subsequent cycles of deacetylation and spreading onto the nucleosomes to pack them into a higher-order structure.

Despite all the differences, both the establishment and inheritance of transcriptional silencing depends on cell cycle progression (63-64, 69, 72, 85). As discussed previously and in FIG 1-5A, the process of inheritance is coupled to DNA replication and other cell cycle events in many aspects, such as the propagation of DNA methylation and histone modifications. However, the relationship between establishment and cell cycle events are not so obvious. And the underlying mechanism is still not clear yet. Part of my thesis (Chapter Two) is devoted to this topic.

In order to study the establishment of silencing and its relationship with cell cycle, certain conditional or inducible mutants have been used to create a transition from Sir^- to Sir^+ to recapitulate the establishment of silencing (22, 63-64, 69, 72, 83, 92, 135, 142). For example, with a temperature-sensitive allele of *SIR3* (*sir3-8*^{ts}), the mutant sir3 protein is thermolabile (13, 122); it is not detectable at the non-permissive temperature (122). As a critical subunit of the silencing complex, the loss of Sir3 disrupts heterochromatin. Such a ts mutant can be shifted to the permissive temperature to follow the establishment of silencing as functional Sir3 protein becomes available (69, 83, 85, 142). Another approach utilized synthetic silencers in which Sir1 is essential for silencing (22, 63-64, 72). Expression of Sir1 was controlled by an inducible promoter. Therefore, researchers could observe the establishment of silencing by turning on the expression of Sir1. Using these systems, previous work identified certain cell cycle requirements for establishment of silencing.

It was first discovered by Miller and Nasmyth that establishment of silencing requires passage through S phase (85). They used a *sir3-8*^{ts} mutant in a strain which contained **a**-mating type information at both *HML* and *HMR* while *MAT* was deleted. Thus, whether the *HM* loci were silenced or not, this strain was able to respond to alpha factor (αF), a pheromone which arrests the cells in G1 phase. However, the establishment of silencing at *HML* and *HMR* could not be distinguished in this strain, because both loci expressed the same transcripts while derepressed. After shifting the culture from the non-permissive to permissive temperature, the cells were examined for the establishment of silencing under two conditions, arresting in G1 phase or released for cell-cycle progression. They found that silencing could not be established without cell-cycle progression. Furthermore, they used different reagents to block cell-cycle progression at different stages after releasing from G1 phase and found that passage through S phase was required for establishment of silencing.

This S phase requirement was confirmed in a later study by Lau and colleagues in a more quantified manner. They used similar conditions and demonstrated a substantial decrease in the amount of transcription from the derepressed *HMR* locus after passage through S phase, indicating the establishment of silencing at that stage. Moreover, they identified an additional cell-cycle requirement at G2/M phase by showing silencing was established more completely at telophase, when sister-chromatid cohesion was resolved. They suggested that this event constituted the G2/M-phase requirement because

expression of a non-cleavable cohesin subunit inhibited the establishment of silencing. They also showed that the two cell-cycle requirements were independent of each other, because loss of sister chromatid cohesion could not bypass the S-phase requirement, although it did bypass the G2/M phase requirement.

In spite of these studies, the exact nature of the S-phase requirement remained unknown. It was generally assumed to be DNA replication. However, a subsequent study in which ORC binding sites were deleted from the *HMR* silencers found that silencing of the locus still required passage through S phase, suggesting that S phase requirement was independent of the initiation of replication (22). Two subsequent groups extended this conclusion by showing that heterochromatin could be formed without passage of a replication fork (64, 72). They used an inducible promoter to control expression of a chimeric DNA-binding-Sir1, which could be targeted to a modified *HMR* locus through its DNA-binding domain, instead of the usual recruitment by ORC. In order to prevent the passage of a replication fork through this locus, the replication origins were deleted, and the modified *HMR* locus was excised from the chromosome by site-specific recombination. They demonstrated that silencing on this nonreplicating extrachromosomal *HMR* circle could still occur and thus was independent of DNA replication, but, surprisingly, still required passage through S phase. Therefore, the underlying mechanism for the S phase requirement remains unknown.

Notably, there is evidence showing that full silencing cannot be achieved within one

cell cycle (58). Although certain euchromatin marks, i.e. histone acetylation, are removed rapidly during establishment, the loss of others, such as euchromatic histone methylation, occurs gradually through several cell generations (58). It was suggested that the initial establishment of silencing was followed by a slow maturation phase, in which gradual loss of histone methylation enhances SIR complex association and full silencing.

V. The role of Sir1 in transcriptional silencing

1. Sir1 and epigenetics

SIR1 was identified in genetic screens as its mutants allowed expression from the *HM* loci (50, 107). Unlike the tight mutant alleles of *SIR2, SIR3* and *SIR4* which exhibited complete silencing defects, *sir1* mutants including the null, caused only partial derepression of *HM* loci in a population, leading to a weak mating-defective phenotype (51, 107). An epigenetic phenotype for *sir1* mutants was demonstrated by single-cell assays (96), in which individual cells (*MAT***a** *sir1*Δ) were exposed to mating pheromone αF. Wild-type **a** cells normally arrest in G1 phase and undergo morphological changes, forming shmoos in the presence of αF. In contrast, cells with a derepressed *HML* locus exhibit properties of **a**/α diploid cells, thus not responding to αF and continuing to divide. Using the shmoo assay, the isogenic population of *MAT***a** *sir1*Δ cells showed two distinct transcription states: ~20% of the population was silenced like wild-type, while the remaining 80% had silencing defects (96). Both transcription states were stably inherited

with a low frequency of switching to the opposite phenotype (on the order of once in every 250 cell divisions) (96). This stable transmission of the silent chromatin in the 20% of the population can be explained by the model of epigenetic inheritance, in that the parental heterochromatic marks helped to recruit Sir2-Sir3-Sir4 complex for the packing of daughter strands after DNA replication. On the other hand, in the other 80% of the population, the parental euchromatic marks disrupted the association of the SIR complex, especially Sir3, to the nucleosome, thus preventing the establishment of silent chromatin on the progeny. Since both epigenetic statuses were stably inherited, Sir1 was not needed at this stage, but functioned only for establishment. However, the reason why *sir1* null mutants demonstrate two different transcription states initially is still unknown.

2. Sir1 and *eso* mutants

Since in 20% of the population of *sir1* null mutants, silencing was executed as in wild-type cells, it suggested that there is some overlapping pathway(s) that can carry out Sir1's function. Therefore, although deletion of *SIR1* only causes partial silencing defects, it can abolish silencing completely in combination with mutations in parallel pathways. Researchers have tried to find out such pathways with a screen for enhancers of the *sir one* (*eso*) mutant mating-defective phenotype. Such screens, along with targeted mutagenesis studies, have identified *eso* mutations in several genes including *SIR3*, *SIR4*, *SIR2*, *SAS2*, *SAS4, SAS5, NAT1* and *ARD1* (24, 104, 122, 141). A single *eso* mutation usually does not cause a significant silencing defect, but it has a strong effect in

combination with a *SIR1* deletion. Notably, most *eso* mutations were found to cause derepression at telomeres even in a $Sir1^+$ background, usually more severe than the phenotype at *HM* loci. Because Sir1 is not found at telomeres, telomeric silencing may be considered as a system already defective by lacking Sir1, and in combination with *eso* mutations it is logical to show more severe phenotype than the *HM* loci where Sir1 is present.

The original expectation from screening for *eso* mutants was to identify gene products which can take the place of Sir1 and carry out the same function. However, as will be discussed in detail later, genes identified with *eso* mutations mediate a diverse range of pathways which contributes to silencing from different aspects (FIG 1-7). It is unlikely that Sir1 can be involved with all of them at the same time. Instead, Sir1 probably has a major effect on one of these pathways. The synergistic effect of deletion of *SIR1* and *eso* mutations in this pathway abolished silencing. As with *eso* mutations involved in other pathways, the double mutant weakens two independent pathways that affect silencing, thus having an additive effect.

A large fraction of the *eso* mutations were identified in *SIR3*. Moreover, as described in FIG 1-3, Sir3 plays a critical role in SIR-complex spreading through its association with nucleosomes. Therefore, I propose the pathway that leads to Sir3-nucleosome association as the one Sir1 is involved in. The interaction between Sir3 and nucleosome will be described in relation to *sir3 eso* mutants in the following part, and experiments

testing this hypothesis and further discussion are included in Chapter Three. Other *eso* mutants will also be briefly introduced in this chapter.

1) *sir3 eso* mutants

Sir3 interacts with the nucleosome through multiple regions *in vitro*, including two C-terminal patches (aa 623-762 and aa 799-910) (38) and the conserved N-terminal bromo-adjacent-homology (BAH) domain (90-91, 113, 147). In addition, the Sir3 extreme N-terminus is a N^{α}-acetyltransferase (NatA) substrate, and its acetylation is also important for silencing (136).

Correspondingly, nucleosomes also utilize multiple domains to interact with Sir proteins. For example, the N-terminal tails of histone H3 and H4 are important for silencing. Mutations in these tail regions, e.g., histone H4 K16, cause silencing defects due to compromised association with Sir proteins (55, 60, 128). In addition, several residues in the core domain of histone H3 and H4, e.g., histone H3 K79 and the surrounding residues, form a patch on the surface of nucleosomes which affects Sir3-interaction, hence being important for silencing (94, 129). The hypoacetylation and hypomethylation of these regions are characteristics of yeast heterochromatin. Specifically, the Sir3-nucleosome interaction is sensitive to the acetylation of histone H4 K16, which should be removed via the histone deacetylase activity of Sir2, as well as the methylation of histone H3 K79 (74, 91).

Through a targeted screening for dominant negative *sir3* mutants that disrupt

telomeric silencing, Buchberger and colleagues identified a group of mutants and demonstrated their defects in interacting with nucleosomes. Interestingly, the majority of the mutants clustered at the Sir3 BAH domain and even localized on the same side of the structure. Based on these mutants, they predicted that the nucleosome-interacting surface on Sir3 should be composed of an acidic patch and a "lysine-knob" (5) (FIG 1-6 B). The surface of the BAH domain is highly negatively charged (14), while histones are rich in positively-charged residues. Within the dominantly negative surface of the BAH domain lies an acidic patch, which may interact directly with the histone H4 tail and with histone H3 K79 in the core domain. In addition, the "lysine-knob", the positively charged lysine residues on two C-terminal helixes of the BAH domain, may interact with DNA or the negatively charged region around histone H3 K79 on the nucleosome (5, 91).

Not surprisingly, most of the *SIR3 eso* mutations (113, 122), except S813F, also clustered at the BAH domain. All the *SIR3 eso* mutations were mapped to the Sir3 BAH domain (PDB ID: 2FVU) (FIG 1-6A). It was remarkable that they overlap with the predicted Sir3-nucleosome interacting region extensively. They can be divided into three groups, labeled as group A, B, and C in FIG 1-6A. Group A was comprised of mutants of Ala2 residue. Previous work from our lab and other groups has shown that the N-terminal Ala2 and its acetylation play an important role in silencing (113, 136). There is also *in vitro* evidence of compromised Sir3-nucleosome association caused by lack of N-terminal acetylation (113). Mutations in Group B were encompassed by the acidic patch (FIG

1-6B). Several residues (F123, R92, F94, and E140) were exposed on the surface and may directly mediate the interaction, while the remaining (L96, A181, T135) located beneath the surface and may affect the surface indirectly. Group C located within the lysine knob, including mutations S204P, Y207C, L208S, and K209R. Therefore, it is conceivable for these *eso* mutants to have compromised the Sir3-nucleosome interaction. Moreover, the absence of Sir1 further disrupts this interaction and causes severe silencing defects.

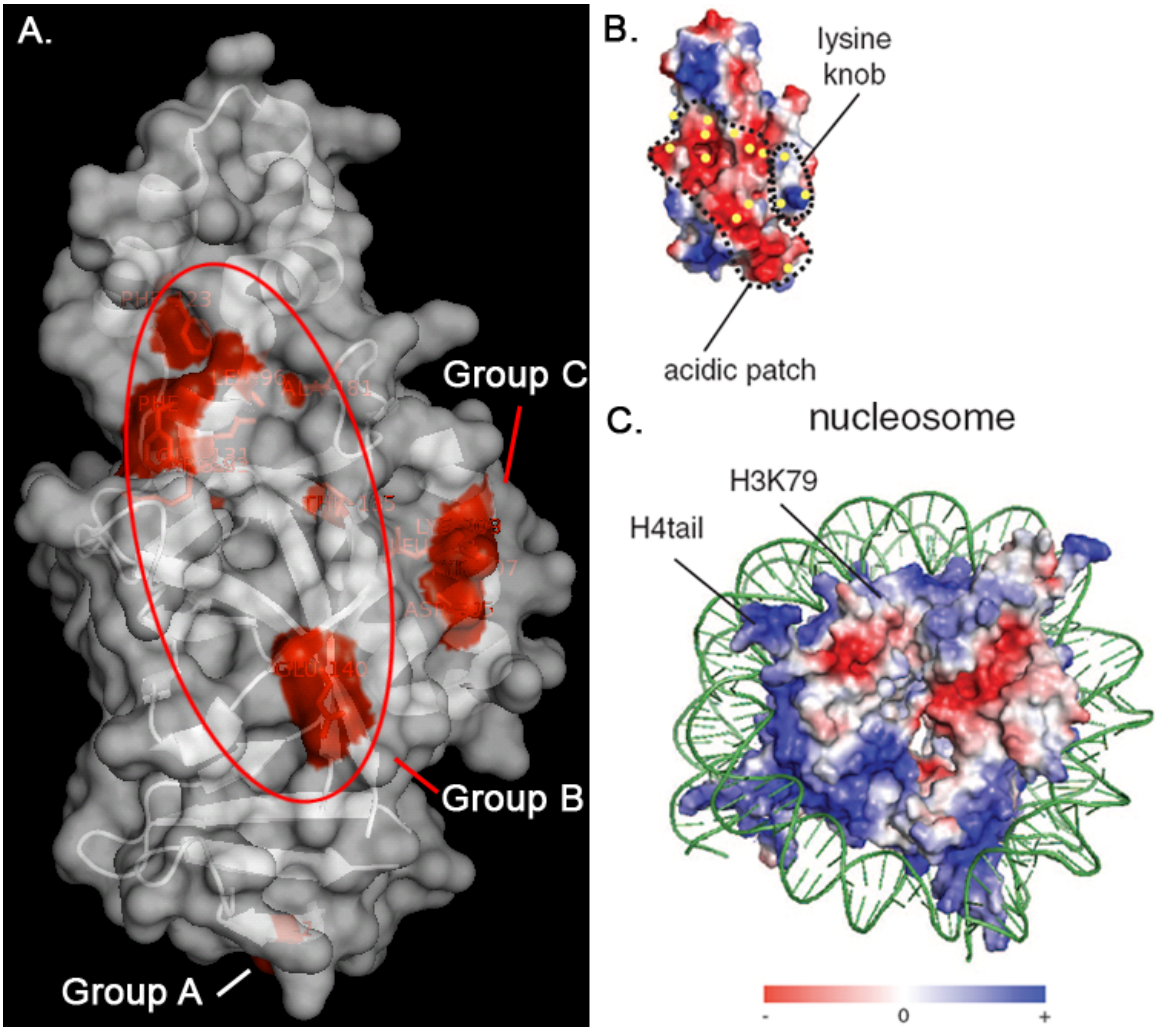

FIG 1-6 *SIR3* eso mutations and predicted Sir3-nucleosome interacting regions. A) *SIR3* eso mutations (labeled in red) mapped to the BAH domain structure (PDB ID: 2FVU). Mutations of group B are encompassed by a circle. Group A: A2T, A2V, A2G; Group B: R92K, F94L, L96F, F123P, T135I, E140K, and A181V; Group C: S204P, Y207C, L208S, and K209R. The remaining *eso* mutations: R30K and N80D are not available in the structure, and S813F does not locate in BAH domain. B) Electrostatic map of the BAH domain (PDB ID: 2FL7) (FIG 10 from ref(5)). The locations of dominant negative mutations causing telomeric silencing defects are indicated by yellow dots. The acidic patch and lysine knob are marked by dashed circles. The size of the BAH domain structure is in proportion to the yeast nucleosome in C. C) Electrostatic map of the surface of yeast nucleosome (PDB ID: 1ID3) (FIG 10 from ref (5)). The histone H4 tail and H3 K79 regions on the surface of the nucleosome may constitute an interface for interaction with the acidic patch of Sir3 BAH domain. And the lysine knob may interact with DNA and the acidic region around histone H3 K79 on the surface of the nucleosome.

2) Other *eso* mutants

Besides the *sir3* mutants, *eso* mutations have also been identified on other gene loci, such as *SIR2 R139K*, *G270E*, and *F296L*, impairing Sir2's deacetylase activity (24) which is needed to remove the acetyl group form histone H4 K16 for the Sir3-nucleosome association. Interestingly, *eso* mutants were also found in the SAS complex which antagonizes Sir2's function by acetylating histone H4 K16 (120). It is possible that acetylated histone H4 K16 in flanking euchromatic region acts as a barrier to keep Sir3 within the region to be silenced, thus increasing its local concentration.

Since a group of *sir3 eso* mutants were found at the Ala2 residue, whose acetylation by N^{α}-acetyltransferase (NatA) is important for transcriptional silencing, it was not surprising to also identify *eso* mutations on *NAT1* and *ARD1*, genes coding subunits of NatA. In addition, *eso* phenotype can be caused by certain mutations on histone chaperones, i.e. Chromatin assembly factor I (CAF-I), HIR complex, and Rtt106 (19, 48, 59), which are involved in the nucleosome assembly in replication-dependent and/or replication-independent manner. However, the function of histone chaperones in heterochromatin assembly is not clear. There is yet another type of *eso* mutants which contain weakened silencers with one or two binding sites eliminated, resulting in reduced association of ORC, Abf1 or Rap1, and consequently less Sir protein recruitment.

3. Over-expression of Sir1 can suppress certain silencing defects

In some cases of severely compromised silencing, the physiological level of Sir1

cannot suppress the defect. Instead, over-expression of Sir1 is needed to restore silencing. For example, only higher dosage of Sir1 can suppress a combination of *eso* mutations, such as in a *nat1*Δ *ard1*Δ strain with a weakened *HMR-E* silencer (123, 136). Another example is the *sir3-8*^{ts} allele, which is an *eso* mutation (E131K) at the permissive temperature, because it has no silencing defect with the physiological amount of Sir1, but is a non-mater in the absence of *SIR1* (122). However, since the sir3-8 mutant protein is thermolabile (13, 122), it can cause silencing defects at the non-permissive temperature even when physiological amounts of Sir1 are available. Surprisingly, such silencing defects can be suppressed by Sir1 overexpressed from a 2 micron plasmid (123). The bases for suppression by high amounts of Sir1 will be further investigated in Chapter Three.

Histone tails are critical for SIR complex-nucleosome interaction. In an *hhf2*Δ*(4-23)* mutant, silencing is completely abolished at the *HML* locus and severely disrupted at the *HMR* locus. Overexpressing Sir1 can improve silencing by more than 20-fold, as judged by a quantitative mating assay (55). In another extreme case, when *SIR3* is deleted, overexpressing the BAH domain of Sir3, or the closely related Orc1 BAH domain, can lead to some silencing at *HM* loci, but only when excess Sir1 is available (14).

To summarize, the *eso* mutations which can be suppressed by the physiological level of Sir1, as well as mutations that require over-expressed Sir1 to suppress, can be mapped to a broad spectrum of pathways in the stepwise model of heterochromatin assembly

(FIG 1-7). According to these genetic interactions, Sir1 may have three possible roles: 1) since most of the *eso* mutants affects the Sir3-nucleosome association, Sir1 may function in a pathway that facilitate this interaction directly; 2) Sir1 may function indirectly by increasing local Sir3 or Sir4 concentration; 3) Sir1 may play a role in forming a higher-order structure that favors the heterochromatin assembly. These hypotheses, especially the first one, will be tested and discussed in Chapter Three.

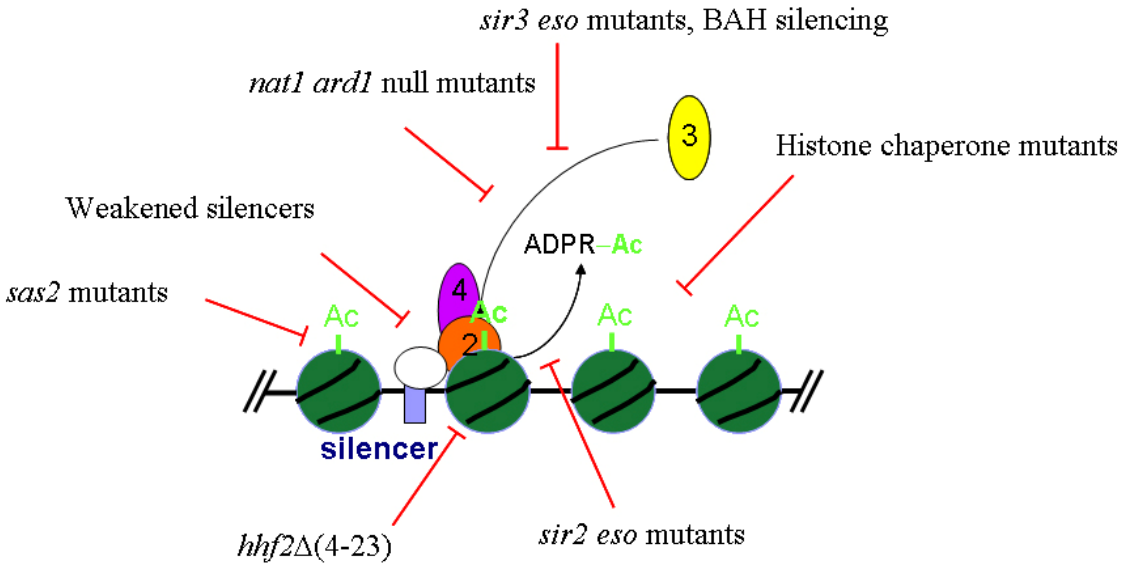

FIG 1-7 Summary of the silencing defects that Sir1 can suppress in relation to the assembly of heterochromatin (See text for detail). Different types of *eso* mutants are depicted with its possible function as indicated by the arrows. Silencing defects that can only be suppressed by over-expressing Sir1 are also included.

4. The N- and C-terminal domains of Sir1

There is a great deal of evidence that the Sir1 C-terminal domain interacts with the N-terminal BAH domain of Orc1, the largest subunit of ORC (3, 26, 45, 47, 130, 147). Interestingly, although the BAH domains of Orc1 and Sir3 are well conserved, with 50%

sequence identity within the first 214 amino acids (14), the Sir1 C-terminal domain binds to Orc1^{BAH} but not to Sir3^{BAH} (46). This can be explained by the difference at some surface areas of the two BAH domains. In addition, the Sir1 C-terminal domain not only binds to Orc1^{BAH}, but also interacts with Sir4 (3). The interaction with ORC targets Sir1 to the silencers, and then Sir1 recruits Sir4 through the other interaction. In FIG 1-8, residues important for the interaction with Orc1 are labeled with red triangles, those for the interaction with Sir4 are labeled with green diamonds, and those labeled with yellow stars are important for both interactions. These residues are identified by mutagenesis and confirmed for function by two-hybrid interaction (3, 26).

Sir1 contains an N-terminal region which is homologous to its well-studied C-terminal domain. The two regions share 27% identity and 46% similarity (14, 46). The sequence alignment is shown in FIG 1-8 and plotted to the secondary structure known for the C-terminal domain. Despite the high similarity between the two domains, there is no clear evidence of the Sir1 N-terminal domain interacting with Orc1 or Sir4. Therefore, the remaining candidate to bind to the Sir1 N-terminal domain is the Sir3 BAH domain. This hypothesis will be examined in Chapter Three.

The N-terminal domain of Sir1 is not needed for complementation of a *sir1*Δ phenotype (14). However, it is needed for suppression of some severe silencing defects, especially in cases where overexpression of Sir1 is needed. For example, *SIR1 L15P* and *D17N* mutations compromise the BAH silencing (14). It is of interest to investigate why

Sir1 has an N-terminal duplication. A large section of Chapter Three is devoted to this purpose.

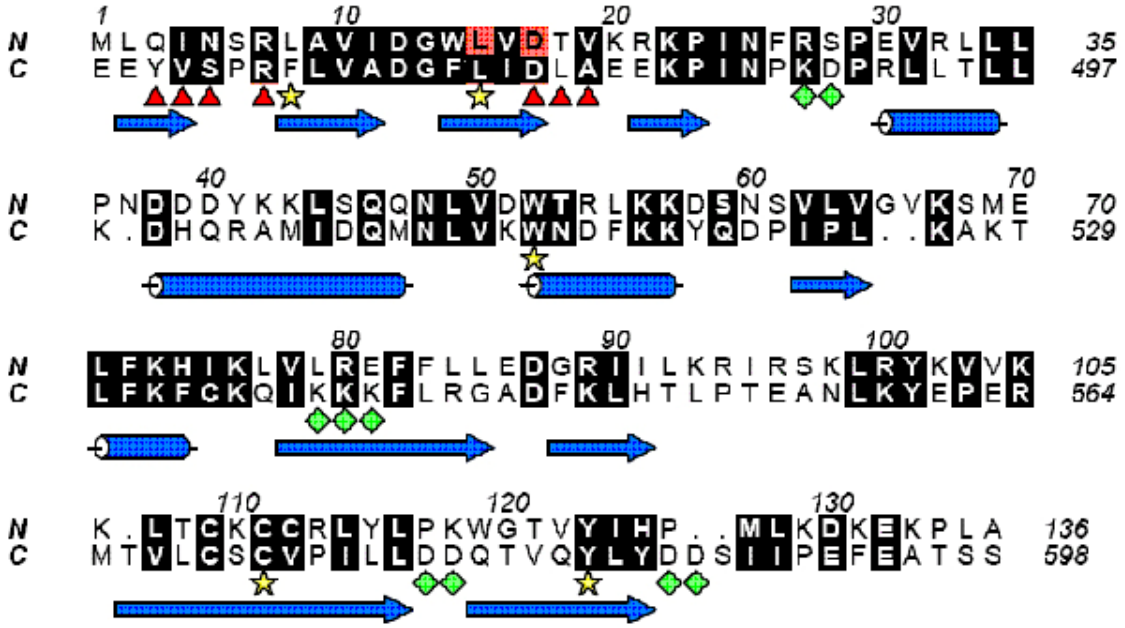

FIG 1-8 Structure-guided sequence alignment of the N- and C-terminal domains of Sir1. The identical and similar residues are shown with white letters over dark background. C-terminal residues important for interaction with Orc1 are labeled with red triangles, those for interaction with Sir4 are labeled with green diamonds, and those labeled with yellow stars are important for both interactions (3, 26). N-terminal residues which can disrupt BAH silencing are highlighted in pink (14).

5. Sir1 family

SIR1 homologs have not been found outside of the *Saccharomyces* genera. However, in several *Saccharomyces* species, Gallagher and colleagues discovered several paralogs of *SIR1*, named *KOS1-KOS4* (*K*in *o*f *SIR1*) (23). Like *S. cerevisiae* Sir1, all Sir1 family members have a duplication of the C-terminal domain at the N-terminus, except for Kos3

which only has the C-terminal domain. As tested in *S. bayanus*, all paralogs have more or less similar function as Sir1 does for silencing at *HM* loci.

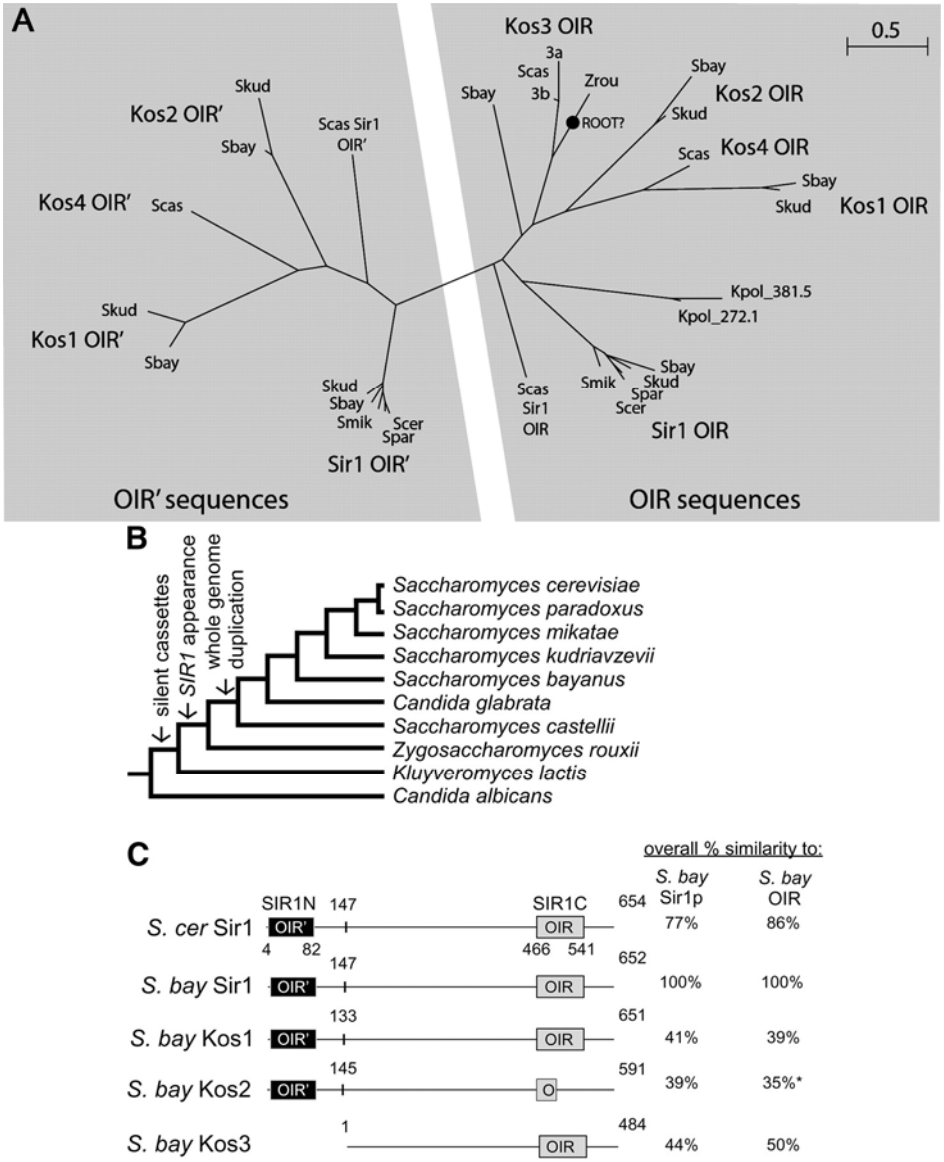

FIG 1-9 *SIR1* family is composed of *SIR1* and its paralogs in the *Saccharomyces* genera (adapted from figures of ref(23)). A. Phylogenetic tree of Sir1 C-terminal Orc1 Interacting Region (OIR) and the N-terminal duplication, OIR' (23). B. Evolutionary tree of several *Saccharomyces* species and other yeast (139). C. Representation of *S. cerevisiae* Sir1 protein and paralogs from *S. bayanus* (23).

VI. Overview of Thesis

I have undertaken a study of different aspects of the establishment of silencing at *HM* loci. First in Chapter Two, I have studied how silencing is established in concert with cell-cycle progression. And I demonstrated the different cell cycle requirement for silencing at *HM* loci. That is, silencing could be partially established at *HML* without passage through S phase, while not at *HMR*. Further analysis identified the promoters at these loci as the cause of this difference. Experiments with modified *HM* loci containing transcription units of different promoter strength demonstrated the competition between transcription and silencing: the stronger the promoter, the more resistant it is to silencing. This competition can be overcome by passage through S phase. This work has been submitted for publication. Another student from our lab, Chia-Lin Wang, contributed some preliminary data and is an author on the paper. However, none of his data are in the paper nor in this thesis.

In Chapter Three, I focused on how Sir1 facilitates the establishment of silencing. I have analyzed the distribution of Sir1 on silent chromatin and its interaction with other proteins both in vitro and in vivo. In addition, my studies on the N-terminal domain showed that, although it is not necessary for complementation of null mutants, it is important for protein stability and suppression of certain silencing defects. Using the known structure of the Sir1 C-terminal domain, I predicted the structure of the N-terminal domain and constructed mutants accordingly to explore their effects.

Finally in Chapter Four, the cyclin-dependent kinase Bur1 and its interacting partners are described. Since this is a separate topic, the background information will be introduced in that chapter. I carried out a two-hybrid screen for Bur1 interacting partners and used various assays to characterize the function of such interactions in the regulation of transcription, mRNA maturation and export.

Chapter Two: Promoter Strength Influences the S Phase Requirement for Establishment of Silencing at the *Saccharomyces cerevisiae* Silent Mating Type Loci

I. Introduction

In *S. cerevisiae*, the two cryptic mating type loci, *HML* and *HMR*, are transcriptionally silent. Previous studies on the establishment of silencing at *HMR* identified a requirement for passage through S phase. However, the underlying mechanism for this requirement is still unknown. In contrast to *HMR*, we found that substantial silencing of *HML* could be established without passage through S-phase. In order to understand this difference, we analyzed several chimeric *HM* loci and found that promoter strength determined the S phase requirement. To silence a locus with a strong promoter such as the **a***1*/**a***2* promoter required passage through S phase while *HM* loci with weaker promoters such as the α*1*/α*2* or *TRP1* promoter did not show this requirement. Thus, transcriptional activity counteracts the establishment of silencing but can be overcome by passage through S phase.

II. Results

1. Silencing at *HMR* requires passage through S phase; however, it could be

partially established at *HML* during early S phase arrest

Previous work from our laboratory identified a point mutation in the *SIR2* gene which caused mating defects in haploid strains of either mating type at 37°C but not at 23°C (135). RNA measurements demonstrated that the lack of mating at 37°C was due to loss of silencing. We also noted that when cultures were shifted from 37°C to 23°C, it took more than 8 hours for silencing to be re-established at the *HMR* locus (135). On the other hand, it took less than 4 hours to achieve a similar extent of silencing at *HML* (data not shown). These observations prompted us to consider the possibility that establishment of silencing at *HML* might not have the same cell-cycle requirement as had been described previously for *HMR*. In order to characterize the difference in cell-cycle requirement we monitored the establishment of silencing at the *HMR* locus in a *MAT*α strain and at the *HML* locus in a *MAT***a** strain with the same *sir3-8* temperature-sensitive allele that had been used in several previous studies on this topic. Cells were grown to log-phase at 37°C (the nonpermissive temperature which disrupts silencing), synchronized in early S phase with HU, then shifted to 23°C (the permissive temperature) either in the presence of HU to prevent passage through S phase, or released into fresh medium without HU to allow cell-cycle progression (FIG 2-1A). HU rather than α factor (αF) was used because only the *MAT***a** strain is sensitive to αF while HU allowed us to compare strains of either mating type under the same condition. Samples were withdrawn at the times indicated, and their DNA content monitored by flow cytometry. The cells

held in HU maintained a 1n peak of DNA content during the course of the experiment, demonstrating an early S phase arrest by HU (FIG 2-1B), whereas cells incubated without HU progressed through the cell cycle (data not shown). RNA was extracted from the above samples, subjected to RT-PCR, and quantified by real-time PCR. The amount of HMLα1 and HMR**a**1 RNA level was normalized to the ACT1 RNA control, respectively. At the 0 h time point, just after cells have been shifted to 23°C, when they were still fully derepressed, the ratio of HMR**a**1/ACT1 RNA (FIG 2-1C) or HMLα1/ACT1 RNA (FIG 2-1D) for each strain was set to 1.0. As shown in FIG 2-1C, the expression level of HMR**a**1 RNA remained high in cells held in HU, consistent with previous studies (22, 63-64, 69, 72, 85, 92). Although there was a slight decrease during incubation at 23°C in HU (to 0.85 at the 4 h time point), this extent of silencing may have been due to a small portion of the cells that escaped the HU block and entered the cell cycle. In contrast, as shown in FIG 2-1D, the HMLα1 RNA level showed a significant decrease under the same condition (to 0.28 at the 4 h time point). This demonstrated that substantial silencing of *HML* locus could occur without passage through S phase, and thus contrasted with the well-documented cell-cycle requirement for establishment of silencing at *HMR*. Cells released from the S phase block had an even greater drop in the HMLα1 RNA level, to 0.028 at the 4 h time point. This is to be compared with cells grown at 23°C for many generations in which the HMLα1 RNA level was even lower, 0.004 (FIG 2-1D). Thus, it took more than one generation for silencing to be fully established, consistent

with previous observations and our results on the *HMR* locus, which also required several cell division cycles for the locus to be fully silenced (58) (FIG 2-1C).

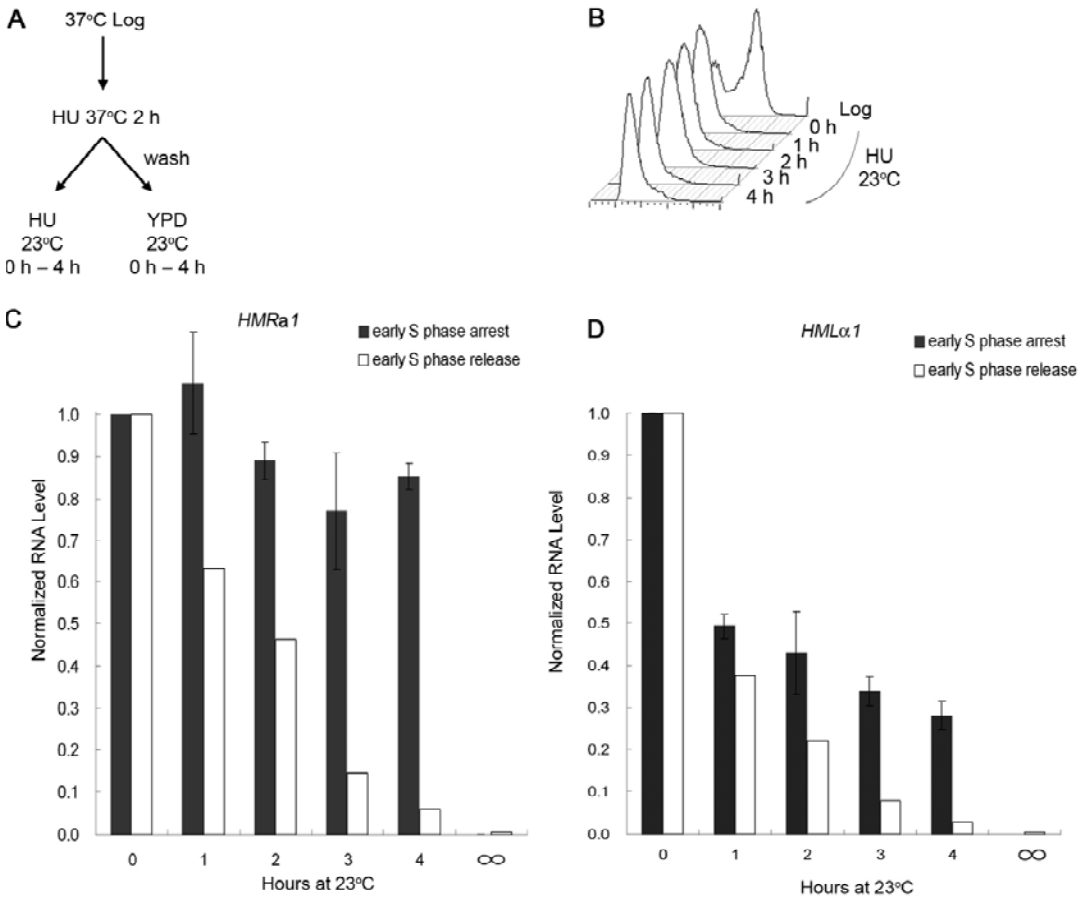

FIG 2-1 Silencing can be partially established at *HML* without passage through S phase, while silencing at *HMR* cannot. (A) Experimental outline. *MAT*α *sir3-8* cells (RS1230) and *MAT***a** *sir3-8* cells (RS1231) were used to analyze silencing at *HMR* and *HML*, respectively. Cells were grown to log-phase at 37°C, synchronized in early S phase with HU, then shifted to 23°C either with HU to prevent passage through S phase, or released into fresh YPD to allow for cell-cycle progression. (B) DNA content. Samples at 23°C were withdrawn at the times indicated, and their DNA content monitored by flow cytometry. A representative result of samples held in HU is shown. (C) *HMR***a***1* expression. RNA was extracted at the indicated time points from both S phase-arrested and released samples, subjected to RT-PCR, and quantified by real-time PCR. The HMR**a**1 RNA level at the 0 h time point, normalized to the *ACT1* control, was set to 1.0. The average of two independent experiments is shown for the S phase arrested samples (shaded bars) and one representative experiment is shown for S phase-released samples (open bars). Also shown is the RNA level for fully silenced cells grown at 23°C overnight (labeled ∞). (D) *HMLα1* expression. A similar procedure and analysis was done as described in (C), except that the HMLα1 RNA level was measured.

2. The difference in cell-cycle requirement for establishment of silencing at *HML* versus *HMR* was due to the transcription units of these loci rather than the flanking silencers

Despite some similarities between the *HML* and *HMR* loci, they are comprised of different transcription units and somewhat different flanking silencers. Therefore, there were two possible explanations for the difference in cell-cycle requirement, the transcription units or the flanking silencers. In order to distinguish these possibilities, we constructed a strain carrying an *HML***a** locus, which contained the **a***1*/**a***2* transcription unit from the *HMR* locus instead of the usual *HML* α*1*/α*2* transcription unit, but flanked by the usual *HML* silencers, as diagrammed in FIG 2-2A. Thus, if the silencers caused the different cell-cycle requirement of the *HML* locus, they should be able to convey the difference to the *HML***a** locus, allowing silencing to be partially reestablished without passage through S phase. On the other hand, if the difference was linked to the transcription units, this substitution should prevent the establishment of silencing before S phase.

To test it, we used a similar experimental strategy (FIG 2-2B) as we did for WT *HM* loci, except that α-factor was used instead of HU to achieve better synchrony. A strain with the *HML***a** locus and mutations at MAT and HMR so that there was no other source of a1 mRNA was synchronized in G1 phase with α-factor at the non-permissive temperature, then shifted to the permissive temperature, either with α-factor for G1 phase

arrest, or released into fresh medium to allow for cell-cycle progression (FIG 2-2B and C). Cells arrested in G1 phase kept expressing **a**1 RNA from the *HML***a** locus at a high level, while cells released from G1 phase established silencing as cells progressed through the cell cycle (FIG 2-2D). The normalized HML**a**1 RNA level was 0.94 after arresting in G1 phase for 4 hours. In contrast, the HML**a**1 level decreased to 0.15 at the 4 h time point in G1 released samples (FIG 2-2D). Moreover, allowing these cells to progress through G1 but blocking them again in early S phase with HU still did not allow silencing of this hybrid locus (data not shown), indicating arresting with α-factor or HU did not change the conclusion. Therefore, silencing was not established at the *HML***a** locus without passage through S phase. These results indicated that the difference in the cell-cycle requirement for establishment of silencing at *HML* versus *HMR* was linked to the transcription units.

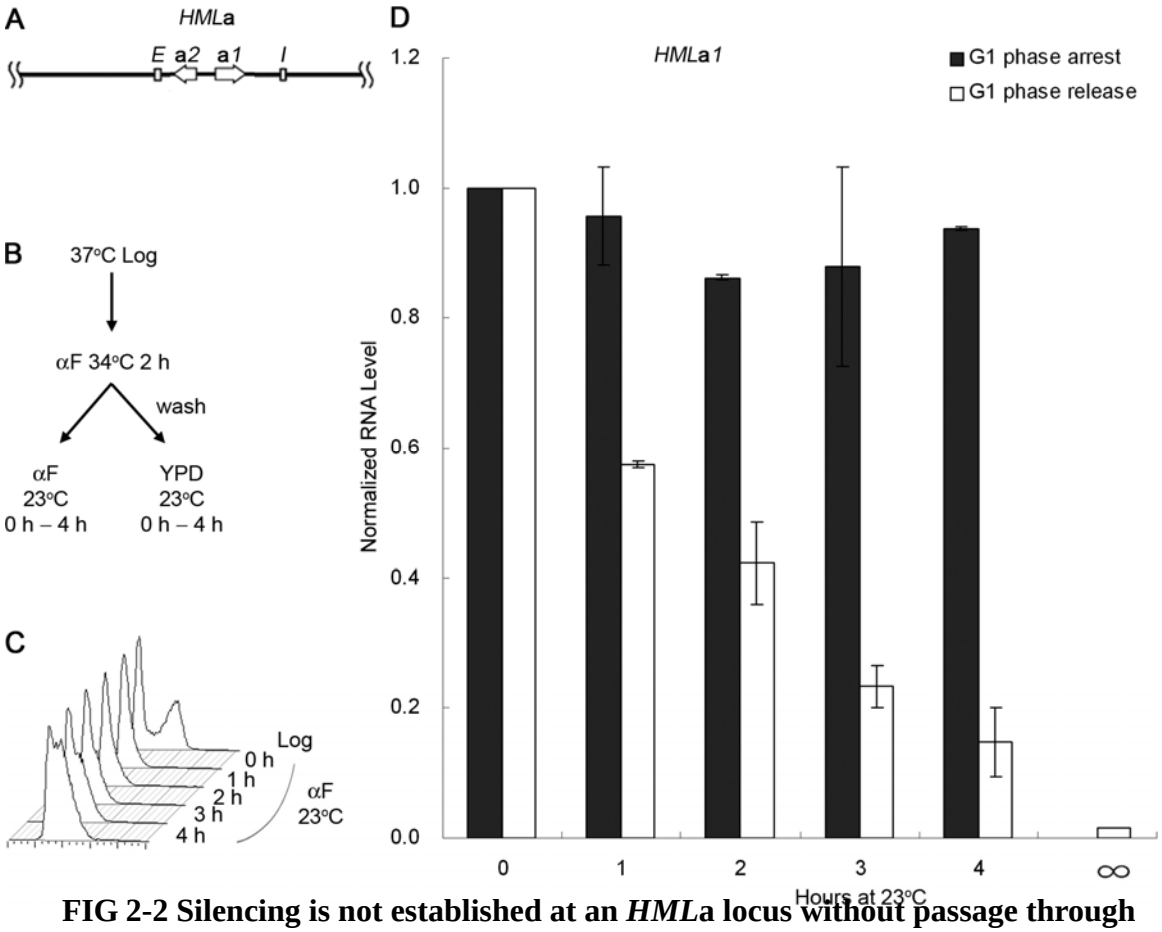

FIG 2-2 Silencing is not established at an *HML*a locus without passage through S phase. (A) A diagram of the modified *HML* locus, *HML***a** is shown. It contains the **a***1*/**a***2* transcription unit from *HMR* instead of the usual *HMLα1/α2* transcription unit, but flanked by the usual *HML* silencers. (B) Experimental outline. The scheme for this experiment is similar to that described in FIGURE 1, except that *HML***a** *sir3-8* strains (JRY19 or JRY25) were synchronized in G1 phase by αF at 34°C, then shifted to 23°C, either with αF for G1 phase arrest, or released into fresh YPD to allow for cell-cycle progression. (C) DNA content. Samples for G1 phase arrest were withdrawn at the time points indicated, and their DNA content monitored by flow cytometry. (D) *HML***a***1* expression. RNA was extracted at the indicated time points from both G1 phase arrested and released samples, subjected to RT-PCR, and quantified by real-time PCR. The HML**a**1 RNA level at the 0 h time point, normalized to either an 18S rRNA or the *ACT1* internal control, was set to 1.0. The average of two independent experiments is shown. Also shown is the RNA level for cells grown at 23°C for 11 h after release from α factor (labeled ∞).

3. The difference in cell-cycle requirement for establishment of silencing

between *HML* and *HMR* was due to transcription, rather than the gene product

In order to further delineate which part of the transcription units, i.e., the promoter or the open reading frame (ORF), caused this difference, we constructed a strain (JRY27) with a hybrid *HML-P***a** locus by substituting the usual α*1*/α*2* divergent promoter with the **a***1*/**a***2* promoter. This construct expressed the α1 protein from the **a***1* promoter instead of the usual α*1* promoter (FIG 2-3A). Because the C-terminal sequence of the **a***2* ORF is identical to that of α*2* ORF, and part of the N terminus of the α*2* ORF was removed in this promoter swap, this construct expresses **a**2 protein instead of α2 protein. The lack of functional α2 protein results in a strain expressing both **a-** and α-specific genes at the non-permissive temperature, thus escaping from the α-factor arrest. Therefore HU was used to synchronize cells at 37^{o}C as in FIG 2-1. After the HU block cells were shifted back to 23^{o}C, either with HU for continued S phase arrest, or released into fresh medium to allow cell-cycle progression. Similar to the result with an *HML***a** locus (FIG 2-2), the α1 RNA level expressed from the **a***1* promoter at the hybrid *HML-P***a** locus showed no significant decrease without passage through S phase (FIG 2-3D, 0.77 for the 4 h time point). On the other hand, in cells allowed to pass through the cell cycle, silencing was reestablished and transcription dropped to 0.11 after 4 hours. Since silencing was not established at the hybrid *HML-P***a** locus without passage through S phase, the difference in cell-cycle requirement between *HML* and *HMR* was due to the promoter-based transcription activity, rather than to the gene product from the ORF.

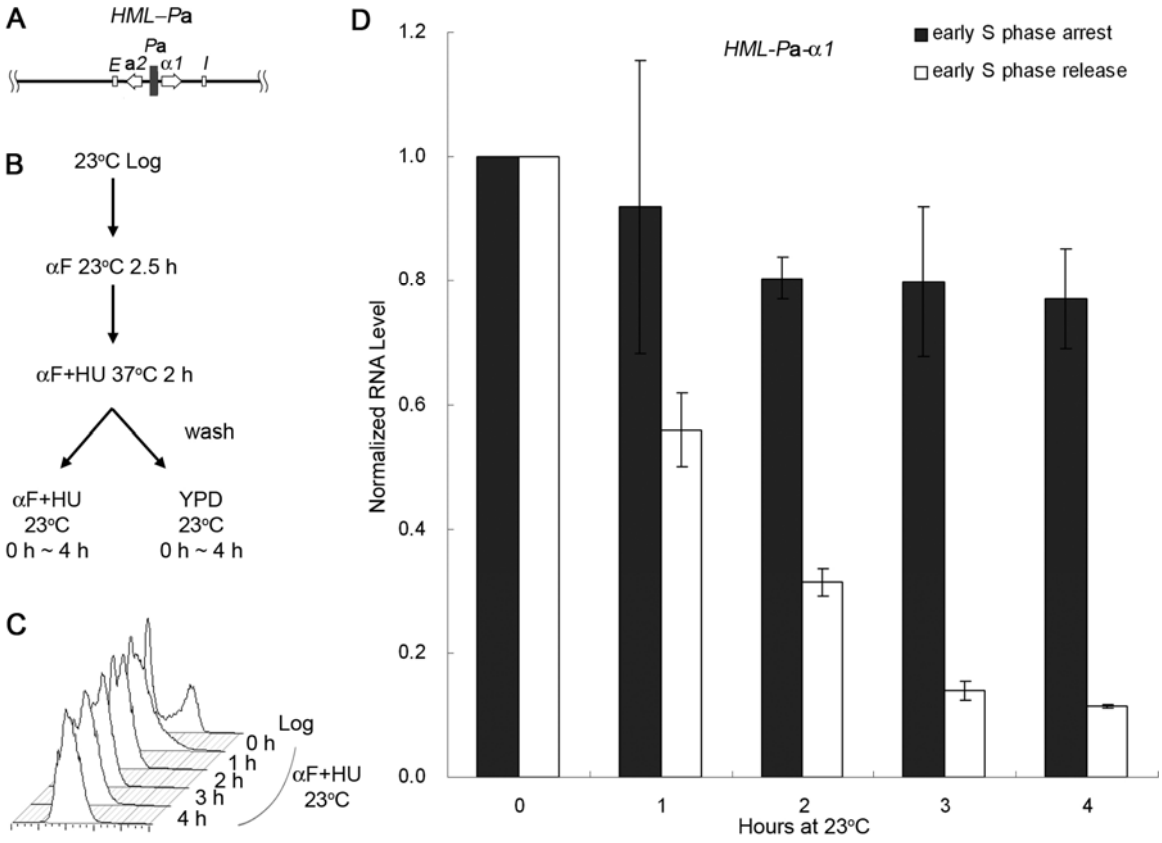

FIG 2-3 Silencing is not established at a hybrid *HML-P*a locus without passage through S phase. (A) A diagram of the hybrid *HML-P***a** locus is shown. It expresses the α1 protein from the **a**1 promoter instead of the usual α*1* promoter. (B) Experimental outline. An *HML-P***a** *sir3-8* strain (JRY27) was synchronized in G1 phase by αF at 23°C, then shifted to 37 °C in the presence of αF and HU for 2.5 h. The culture was then shifted back to 23°C, either with HU for S phase arrest, or released into fresh YPD to allow for cell-cycle progression. (C) DNA content. Samples for S phase arrest were withdrawn at the times indicated, and their DNA content monitored by flow cytometry. (D) *HML*α*1* expression at the *HML-P***a** locus. RNA was extracted at the indicated time points from both S phase arrested and released samples, subjected to RT-PCR, and quantified by real-time PCR. For either cell cycle condition, the HMLα1 RNA level at the 0 h time point, normalized to 18S rRNA, was set to 1.0. The average of two independent experiments is shown.

4. The a*1* promoter was significantly stronger than the α*1* promoter

In order to understand the linkage between the cell-cycle requirement and the corresponding promoter, we measured the relative strength of the **a***1* and α*1* promoters. First, the RNA level from the derepressed *HML*α*1* and *HMR***a***1* loci was measured as an indicator of their promoter strength. We found that the HMLα1 RNA level was 0.13, relative to 1.0 for HMR**a**1 (FIG 2-4A). In order to confirm that the measurement of these RNA levels reflected the promoter strength rather than half-life of the RNAs, the **a***1* promoter and α*1* promoter were fused to a *yEmRFP* reporter gene (62) and the amount of this transcript from each promoter was measured. When the normalized yEmRFP RNA level from the **a***1* promoter was set to 1.0, the level from the α*1* promoter was 0.18 (FIG 2-4B). Therefore, using two different methods, we found that the **a***1* promoter was significantly stronger than the α*1* promoter.

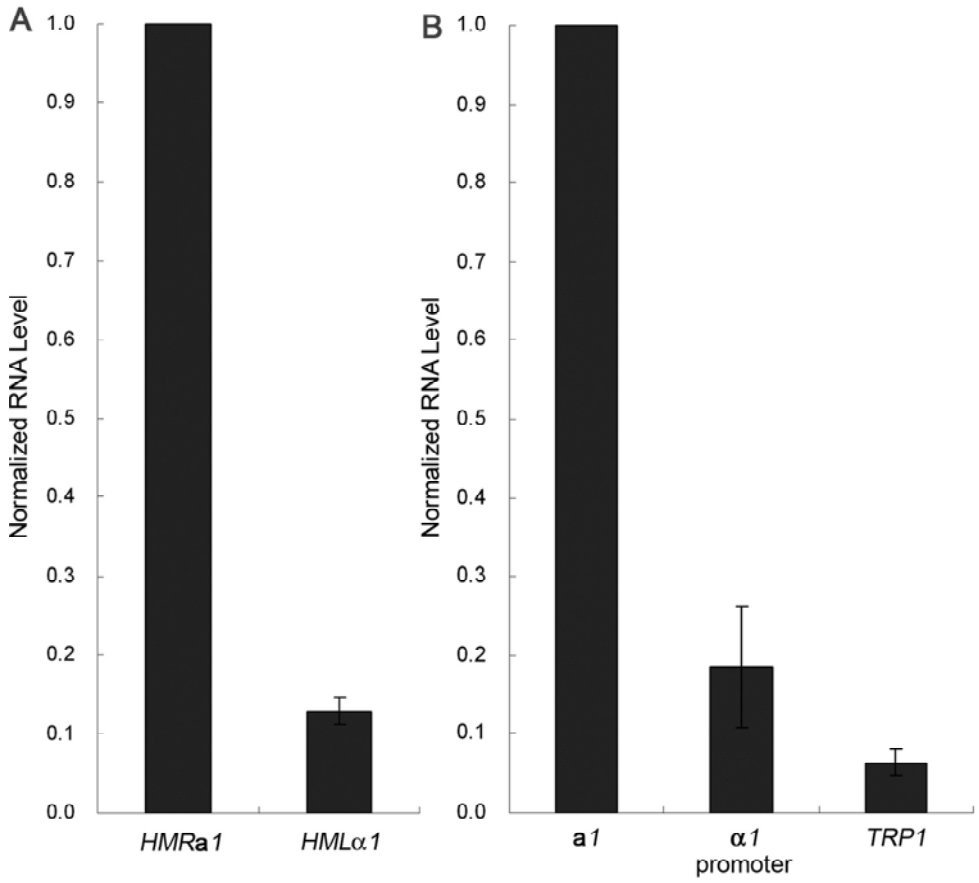

FIG 2-4 The a*1* promoter is significantly stronger than the α*1* promoter and a weakened *TRP1* promoter. (A) RNA levels from derepressed *HMLα1* and *HMR***a***1*. An *HMLα matΔ::kanMX6 HMR***a** *sir3-8* strain (JRY30) was grown at the non-permissive temperature and used to extract RNA for RT-PCR. RNA was quantified as described in Materials and Methods. (B) Measurement of promoter strength. The **a***1* promoter, α*1* promoter and *TRP1* promoter present at *hmr::TRP1* were fused to a *yEmRFP* reporter gene, and expressed from 2μ plasmids. RNA was extracted, subjected to RT-PCR, and quantified by real-time PCR. The yEmRFP RNA level from the **a***1* promoter, normalized to the *ACT1* internal control, was set to 1.0. The average of two independent experiments is shown.

5. Silencing was partially reestablished without passage through S phase at a chimeric *HMR* locus containing a weaker promoter, but not at the wild type *HMR* locus

The results presented above indicated that the strength of the promoter and hence the amount of transcription through the locus determined the cell-cycle requirement or lack thereof. To test this in another way, a strain (JRY27) with an *hmr::TRP1* locus harboring a weakened *TRP1* promoter, flanked by the usual *HMR* silencers, was used (FIG 2-5A). Measurement of promoter strength with the *yEmRFP* reporter gene showed that this *TRP1* promoter was much weaker than the **a***1* promoter (FIG 2-4B). This strain also contained the hybrid *HML-P***a** locus. As we showed in FIG 2-3, silencing was not established at that locus without passage through S phase. In contrast, the *TRP1* transcript from the *hmr::TRP1* locus measured from the same samples decreased significantly during S phase arrest (FIG 2-5B). When the TRP1 RNA level at the 0 h time point was set to 1.0, after 4 hours of arrest in early S phase, the RNA level from *hmr::TRP1* dropped to 0.31, a much greater drop than that seen from the *HML-P***a** promoter driving the α1 transcript in the same strain (compare FIG 2-5B and 3D). Therefore, in contrast to the WT *HMR* locus, silencing could be partially established at the hybrid *HMR* locus containing a weaker promoter.

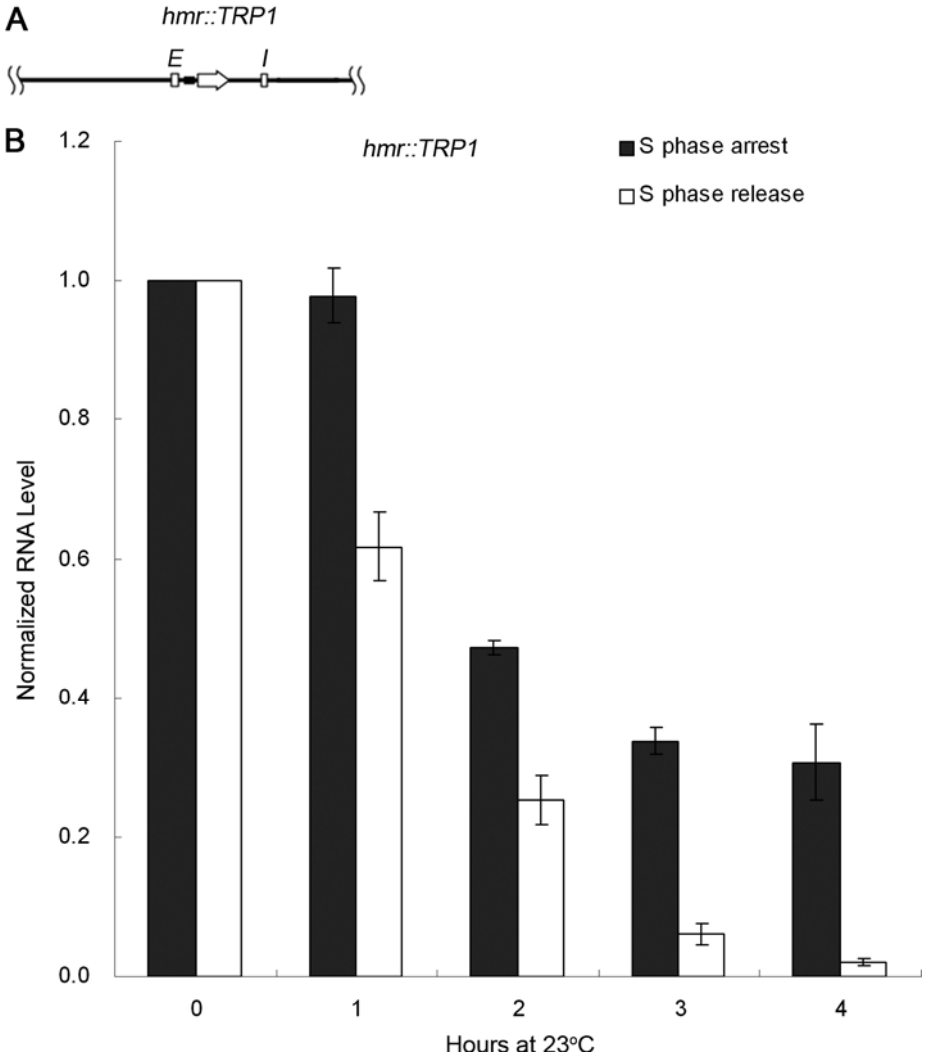

FIG 2-5 Substantial silencing can occur at an *hmr::TRP1* locus without passage through S phase. (A) A diagram of the *hmr::TRP1* locus (JRY27), containing the *TRP1* transcription unit driven by a weakened *TRP1* promoter, flanked by the usual *HMR* silencers. (B) *TRP1* expression at the *hmr::TRP1* locus. The strain and the samples used are the same ones as were used for the experiment shown in FIG 2-3 although TRP1 RNA quantification is shown here. For both S phase arrest and release, the TRP1 RNA level at time point 0 h, normalized to an 18S rRNA internal control, was set to 1.0. The average of two independent experiments is shown.

III. DISCUSSION

Our results demonstrate a difference in the S phase requirement for establishment of

silencing at *HML* and *HMR*. While silencing cannot occur at the *HMR* locus without passage through S phase (22, 63-64, 69, 72, 85, 92) (FIG 2-1C), it can be established to a significant extent at the *HML* locus under the same conditions (FIG 2-1D). This difference explains our previous result that silencing was established at *HML* much more rapidly than at *HMR* after shifting a *sir2* temperature-sensitive strain from a non-permissive to a permissive temperature (135).

Using various chimeric constructs we determined that the different S-phase requirement for silencing *HML* and *HMR* was due to the transcription units of these loci rather than to the flanking silencers. For example, an *HML* locus with the **a***1*/**a***2* transcription unit instead of the usual α*1*/α*2* transcription unit, but flanked by the usual *HML* silencer elements, could not be silenced without passage through S phase (FIG 2-2). We narrowed down this difference by showing that a substitution of the α*1*/α*2* promoter at *HML* with the **a***1*/**a***2* promoter also prevented the establishment of silencing before passage through S phase (FIG 2-3). Therefore, the different S phase requirement for silencing *HML*α and *HMR***a** was due to the different promoters present at those loci.

To test if the two promoters had different strengths we measured transcription activity from each promoter and found that the **a***1* promoter was significantly stronger than the α*1* promoter (FIG 2-4). We did this in two ways. First we compared the amount of RNA from derepressed *HMR***a***1* with the amount from *HML*α*1* (FIG 2-4A). To correct for the possibility that **a**1 mRNA might have a greater half life than α1 mRNA, we also

fused each of these promoters to a reporter gene and measured the amount of RNA from this gene (FIG 2-4B). Both experiments showed that the **a***1* promoter was significantly stronger than the α*1* promoter. Furthermore, by substituting the **a***1*/**a***2* promoter and gene at *HMR* with the much weaker *TRP1* promoter and its gene, we observed that silencing could be established at the *HMR* locus without passage through S phase (FIG 2-5B). Therefore, we propose that the amount of transcription through a gene counteracts establishment of silencing, and that influences the cell-cycle requirement. That is, the stronger the promoter, the more resistance there is to establishment of silencing, and the more stringent is the S-phase requirement. It seems reasonable that the frequent passage of RNA polymerase II from a relatively strong promoter inhibits the spreading of the Sir complex from the silencers. The euchromatin marks that result from active transcription may also hinder the establishment of heterochromatin.

Previous studies have also observed a competition between transcription and silencing. For instance a *URA3* reporter gene could be silenced at a greater distance from the telomere when *PPR1*, the trans-activator of *URA3*, was deleted (105). In addition, it was found that a silent telomeric *URA3* gene could become expressed if cells were arrested in G2/M and that depended on the Ppr1 activator (2).

Additional support for the competition between transcription and silencing came from studying silencing in mutants lacking the chromatin-modifying enzymes Dot1 or Set1, responsible for euchromatic methyl marks on histone H3K79 and H3K4,

respectively. In *dot1*Δ and *set1*Δ mutants, establishment of silencing was more rapid than in wild-type cells, probably because active transcription was compromised by the hypomethylated chromatin, and hence was less resistant to silencing (92). However, it may also have been caused by the better binding of Sir proteins to hypomethylated histones (91, 113).

Interestingly, the reason why S phase passage is necessary for establishing silencing at *HMR* is still not understood. Studies with non-replicating *HMR* circles provided strong evidence that it is not DNA replication itself that is needed for establishing silent chromatin (64, 72). Based on our findings that promoter strength influences the S phase requirement, we propose two different S phase events which may facilitate the spreading of the Sir complex and allow it to overcome the competition from transcription (FIG 2-6). One is an S-phase dependent post-transcriptional modification of a Sir protein or a histone that would strengthen the association between the Sir complex and nucleosomes. A recent study by Holt *et al.* identified Sir2, Sir3 and Sir4 among 308 substrates of the cyclin-dependent kinase Cdc28/Cdk1 in cells synchronized at M phase (43). Conceivably, similar modifications of Sir proteins or histones could explain the S phase requirement.

Another explanation could be that histone synthesis and deposition occur during S phase and that facilitates silencing. It is well established that transcription tends to reduce histone occupancy on chromosomal DNA. For example, the histone occupancy on the *GAL10* coding region is inversely correlated with transcription activity (117). Using anti-

histone H3 chromatin immunoprecipitation (ChIP), we obtained a similar result. We observed a bigger decrease in histone occupancy at the *HMR***a***1* transcription unit than at the *HML*α*1* transcription unit when shifting an exponentially growing *sir3-8* ts strain from 23^{o}C to 37^{o}C (data not shown), agreeing with our result that the **a***1* promoter is stronger than the α*1* promoter. The frequent passage of RNA polymerase II from the relatively strong **a**1 promoter may cause reduced nucleosome occupancy, which in turn, provides less binding surface for the Sir complex, thus counteracting silencing. During passage through S phase, when histone synthesis and deposition are robust, more nucleosomes may be incorporated into the silent regions, providing a better binding surface for the Sir complex. This process is not necessarily coupled to DNA replication since it can take place on a non-replicating *HMR* circle (64, 72).

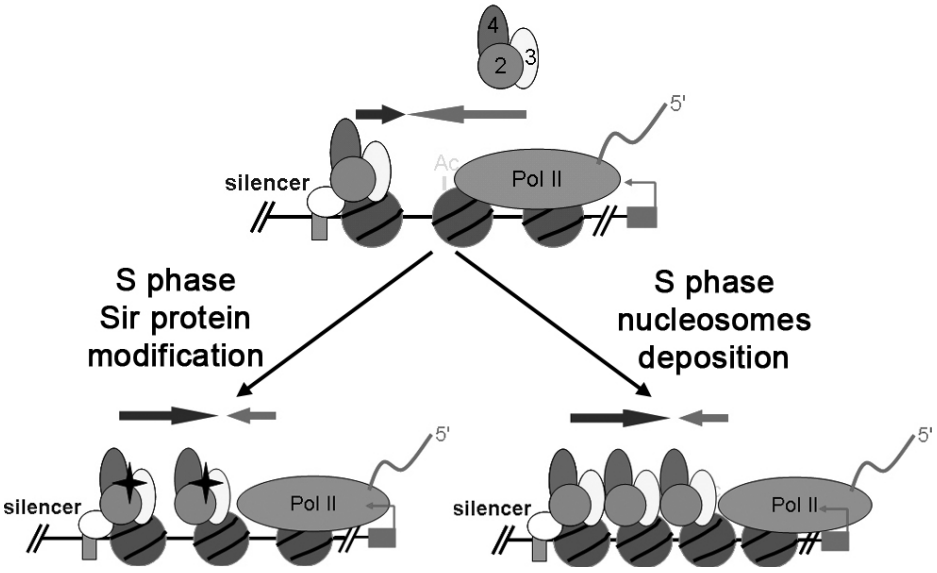

FIG 2-6 Model for competition between silencing and transcription. The grey box at the left end of the chromatin template represents one of the silencers that flank the chromatin region to be silenced. The silencer recruits various DNA binding proteins, generalized as an open circle associated with the silencer, which, in turn, recruit Sir proteins onto nearby nucleosomes. The Sir2-3-4 complex is shown as three associated grey ovals. Active transcription from a strong promoter counteracts the spreading of the Sir2-3-4 complex through the frequent passage of RNA polymerase II. Strong transcription activity may also reduce the nucleosome occupancy on the gene, thus reducing the binding surface for the Sir2-3-4 complex. Two possible S phase events may facilitate silencing to overcome this competition. On one hand, Sir proteins may be modified at this stage to strengthen their association with the nucleosomes. (The modification is represented with the star symbol in the lower left part of the figure.) On the other hand, during S phase, robust histone modification and deposition may increase the nucleosome occupancy and provide more binding surface for Sir2-3-4 complex. Either one or both of these S phase events may explain the S phase requirement for silencing.

Martins-Taylor and colleagues previously observed that establishment of silencing at *HML* did not require passage through S phase, but did require passage through G2/M

(83). However, their protocol was very different than ours and did not compare *HML* and *HMR*. They synchronized *sir3-8* ts cells in G2/M at 23º C and then released them into αF at 37ºC. They measured the fraction of cells blocked in G1 by αF as a measure of silencing at *HML*. Our results agree with their conclusion and extend it by showing that it is the strength of the promoter that influences the S phase requirement.

One interesting question not answered by our results is how the amount of silencing observed for the population relates to that of the individual cell. For example, in the experiment shown in FIG 2-1D, when the amount of HMLα1 RNA during S phase arrest decreased to 30% of its original level after 4 hr at a permissive temperature, was that because 70% of the cells were fully silenced or because the entire population was partially silenced? The two possibilities correspond to two different views for the establishment of silencing. One is that intermediate states of silencing exist and complete silencing is achieved gradually as cells continue to divide. The other assumes an all-or-none model, that a locus is either completely silenced or derepressed (32). Two recent studies showed that complete silencing required several generations and thus favor the former model (58, 92). Therefore, the decrease in RNA level we detected at *HML* in the first few hours at the permissive temperature (FIG 2-1) is likely to reflect a reduced RNA level in the population of cells, few or none of which are completely silenced.

Even though substantial silencing was established without passage through S phase at the *HM* loci with a weak promoter, e.g., *HMLα* and *hmr::TRP1*, it didn't reach the

same extent as that seen for cells allowed to pass through the cell-cycle. For example, as shown in FIG 2-1D, the HMLα1 RNA level decreased substantially to 0.28 after 4 h in early S phase arrest, while it showed an even greater drop to 0.028 at the corresponding time point when released from the S phase block. A similar difference was observed at the *hmr*::*TRP1* locus (FIG 2-5B). One possible cause is the previously described G2/M phase requirement, which is independent of the S-phase requirement (69). That study concluded that it was the dissolution of sister-chromatid cohesion at anaphase that accounted for the G2/M-phase requirement (69).

In summary, the results presented have clarified the different cell-cycle requirement for establishment of silencing at *HML* and *HMR*. That is, silencing can be partially established at *HML* without passage through S phase, but not at *HMR*. We have analyzed the difference and attributed it to the transcriptional activity of these loci. We found that the greater the transcriptional activity, the more resistance there is to silencing, and the more stringent the S-phase requirement. The competition between transcription and silencing may allow for a certain amount of plasticity for switching to the opposite phenotype, and this may be particularly important in metazoans.

Chapter Three: the Role of Sir1 in Transcriptional Silencing

I. Introduction

As mentioned in Chapter One, Sir1 contains an N-terminal domain ($Sir1^{N}$: aa 1-136) and a C-terminal domain ($Sir1^{C}$: aa 463-598) which are homologous to each other (14, 46) (see FIG 1-8). Although the known $Sir1^{C}$ – $Orc1^{BAH}$ and $Sir1^{C}$ – Sir4 interactions support the facilitating role of Sir1 during the establishment of silencing, it is possible that Sir1 has additional roles in silencing. Although the deletion of *SIR1* alone only causes minor silencing defects at the *HM* loci, it can disrupt silencing drastically in combination with *eso* mutations, which suggests that Sir1 functions in some unknown pathway(s) that overlap with *eso* mutants (see Chapter One for details). Moreover, when over-expressed, Sir1 can suppress some severe silencing defects. The Sir1 N-terminal domain was shown to be important for some of the above functions, but little is known about this domain. Therefore, I investigated the N-terminal domain and how it contributes to Sir1's function in silencing.

II. Results

1. The Sir1 N-terminal domain was not necessary for complementation of a

***sir1*Δ mutant, but was required for suppressing certain severe silencing defects and establishing BAH silencing**

Previous students from our lab have tried to identify the function of the Sir1 N-terminal domain by comparing the full-length protein and an N-terminal truncated one in various silencing assays. And they came to the same conclusion as stated in the preceding title (13-14, 123). However, their investigation was flawed by using a plasmid-borne $sir1^{\Delta N}$ allele without its own promoter. The mutant protein was probably expressed from some cryptic promoter in the plasmid backbone instead of its natural promoter. There was no information about the protein level or the fragment size of this additionally compromised version of $sir1^{\Delta N}$ mutant protein. The only indication of the existence of a functional fragment came from its ability to compliment *sir1* null mutants (14, 123). Therefore, it was necessary to construct a $sir1^{\Delta N}$ allele from its own promoter to test its expression and to examine if the silencing defects were due to the N-terminal truncation or were artifacts caused by the deletion of the natural promoter. Since the Sir1 N-terminal domain (aa 1-136) contains a Met127 residue near its C-terminus, the new construct used Met127 as the start codon and expressed $sir1^{127\text{-}654}$ as the N-terminal truncated protein from its own promoter on a 2-micron plasmid. Both Sir1 full-length (FL) and $sir1^{127\text{-}654}$ (ΔN) were tagged with a FLAG-epitope at the C-terminus for the following complementation and suppression tests (FIG 3-1), as well as for assessment of the protein level (FIG 3-2) to be discussed later.

First, I tested if the N-terminal domain is dispensable for complementation of *sir1*Δ mutants as previously found (14, 123). Because a complete deletion of *SIR1* alone only causes a minor silencing defect, to check on the role of the Sir1 N-terminal domain, I used a sensitive assay with a *TRP1* reporter inserted into the *HMR* locus (i.e., in strain JCY36) and tested for growth on SC-Trp medium. In this assay, less growth on SC-Trp medium indicated better silencing. As shown in FIG 3-1A, strains expressing either full-length Sir1 or Sir1$^{\Delta N}$ couldn't grow on SC-Trp medium, showing that the remaining part of Sir1$^{\Delta N}$ can function as well as the full-length version. Therefore, the N terminus is not critical for the physiological role of Sir1 in otherwise wild-type strains.

Next, I checked the necessity of the Sir1 N-terminal domain for silencing in some defective backgrounds as suggested by previous studies with the flawed *sir1*$^{\Delta N}$ allele (123), e.g., the *sir3-8*^{ts} mutant at the non-permissive temperature. Sir1$^{\Delta N}$ expressed from the new construct could not suppress the silencing defects (FIG 3-1B). It was previously suggested that a large amount of the full-length Sir1 protein can stabilize the thermolabile sir3-8^{ts} mutant protein, because sir3-8 protein can only be detected by immunoblotting when over-expressing full-length Sir1 protein at the same time (13, 122). However, the mechanism of this stabilization is still unknown.

Another test of Sir1 function involves so-called BAH silencing, in which the Sir3 BAH domain or the closely related Orc1 BAH domain are over-expressed in the absence of full-length Sir3 (14). Overexpression of full-length Sir1 was required for silencing in

this situation, while overexpressing Sir1$^{\Delta N}$ couldn't establish BAH silencing (FIG 3-1C). Therefore, I confirmed that the Sir1 N-terminal domain is indispensible for suppressing certain silencing defects. The proposed mechanism for this suppression will be discussed later in this chapter.

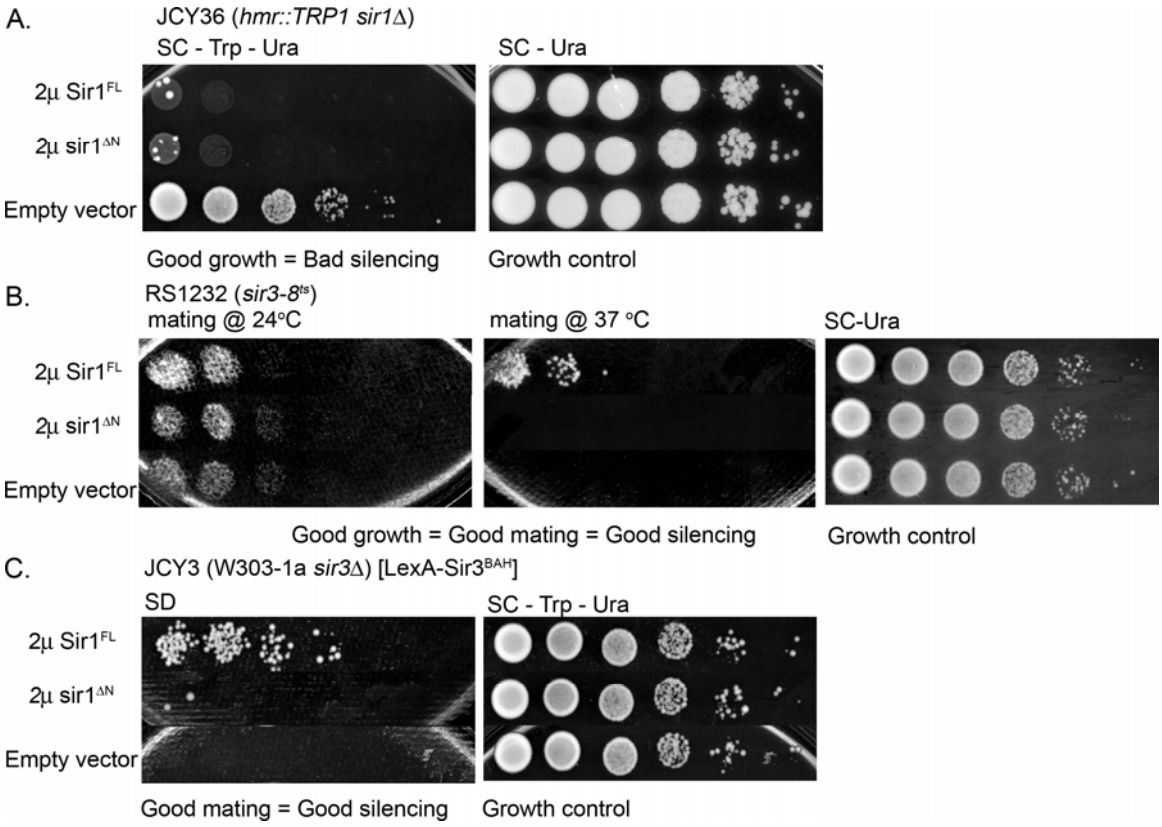

FIG 3-1 The Sir1 N-terminal domain is not required for complementation, but is critical for suppressing certain silencing defect and for establishing BAH silencing. Serial ten-fold dilutions were plated on supplemented synthetic medium for plasmid selection to monitor growth (SC-Ura, A and B right; SC-Ura-Trp, C right) or the same medium without tryptophan (SC-Trp-Ura, A left) to monitor silencing at *hmr::TRP1* locus. Serial ten-fold dilutions were also spotted onto a lawn of tester strain (DC17 *MAT*α) for mating at the permissive (24°C, B left) and non-permissive temperature (37°C, B middle), or for mating at 30°C (C left), and followed by replica to SD plates to measure the silencing at *HML* locus. Transformants of a *sir1* null mutant strain with *hmr::TRP1* reporter (JCY36) for complementation test (A), or of *sir3-8*^{ts} mutant strain to test suppression of silencing defect (B), or of *sir3* null mutant strain but overexpressing the Sir3 BAH domain for BAH silencing assay (C), contained the following plasmids: full-length Sir1 with C-FLAG$_3$ tag (2μ Sir1^{FL}, pJR52, *SIR1-G$_8$-FLAG$_3$-T$_{SIR1}$*), sir1$^{127\text{-}654}$ with C-FLAG$_3$ tag (2μ sir1$^{\Delta N}$, pJR55, *sir1*$^{127\text{-}654}$*-G$_8$-FLAG$_3$ -T$_{SIR1}$*), and vector control (pRS316).

2. The Sir1 N-terminal domain was important for protein stability

Although with the new construct, Sir1$^{\Delta N}$ was expressed from its own promoter, it

was still in question if it was expressed at the same level as the full-length protein. The relative amount of Sir1 was measured semi-quantitatively by immunoblotting 3-fold serial dilutions of whole-cell extracts of strains expressing FLAG-tagged full-length Sir1 or N-terminal deleted fragment with anti-FLAG antibody (FIG 3-2). The level of $Sir1^{\Delta N}$ fragment is about 7-fold lower than that of the full-length protein (compare lane 2 and 5, FIG 3-2). Notably, in the strain over-expressing full-length protein from a two-micron plasmid, besides the full-length protein, there is also another fragment detected with the same mobility as the $Sir1^{\Delta N}$ (aa 127-654) fragment, indicating a putative product from the internal start codon (Met 127) or from a specific protease digestion.

Interestingly, direct tagging to the C-terminus and consequent changing to the *ADH1* terminator compromised the silencing ability of the full-length Sir1 protein in BAH silencing. As shown in FIG 3-3A, *SIR1-FLAG*$_3$*-T*$_{ADH1}$ (pJR50) transformants cannot establish BAH silencing. Another group also found similar effect with Sir1-HA_3 which exhibited a partial silencing defect when assayed by a sensitive colony color assay for silencing of an *HMR::ADE2* reporter (147). I found that inserting an 8-Gly linker between the Sir1 C-terminus and the $FLAG_3$-epitope, while keeping Sir1's own terminator, preserved the suppressing ability (FIG 3-3A). Moreover, the protein levels expressed from *SIR1-FLAG*$_3$*-T*$_{ADH1}$ (pJR50) and *SIR1-G*$_8$*-FLAG*$_3$*-T*$_{SIR1}$ (pJR52) were similar (FIG. 3-3B). Thus, the adverse effect of the former tagging strategy was not due to a change in the expression level. These adverse effects were only visible in some very

sensitive assays, while not affecting Sir1's function in most cases (data not shown). For the assays in FIG. 3-1 and 3-2, the latter tagging strategy which did not cause a silencing defect was used.

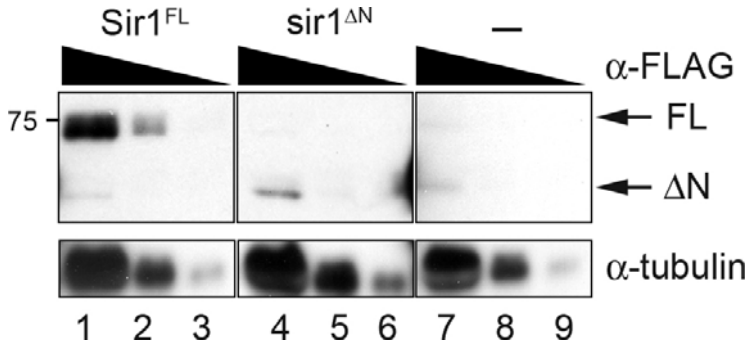

FIG 3-2 The Sir1 N-terminal domain is required for protein stability. Three-fold serial dilutions of total proteins extracted from strains expressing the FLAG-tagged $Sir1^{FL}$ protein (pJR52) (lane 1-3) or the $Sir1^{\Delta N}$ fragment (pJR55) (lane 4-6), or the no-tag control (lane 7-9) were resolved by SDS-PAGE, transferred to the membrane and detected with anti-FLAG monoclonal antibodies (M2, Sigma^{TM}).

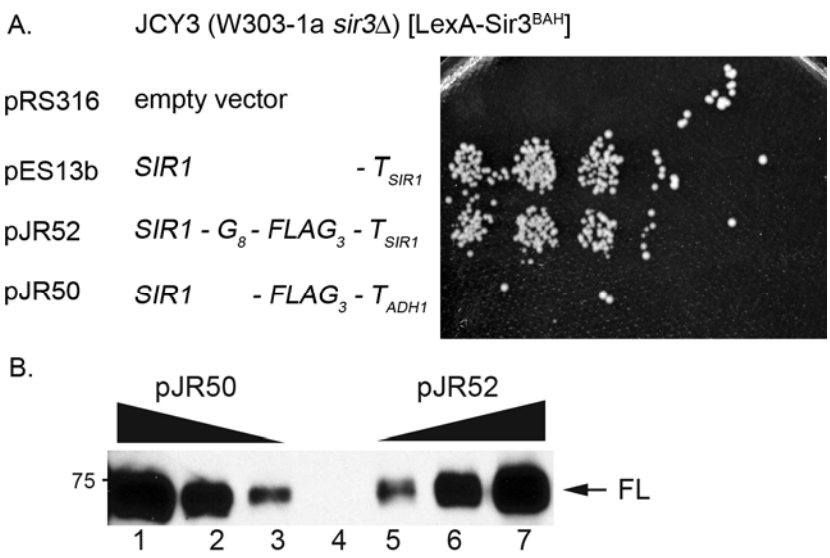

FIG 3-3 Different tagging strategies caused different silencing abilities in BAH silencing, but do not affect the protein level. (A) The BAH silencing assay as described in FIG 3-1C. Transformants of *sir3* null mutants but overexpressing $Sir3^{BAH}$ containing either of the following plasmids: full-length Sir1 without tag (pES13b), or with 8-Gly linker to C-FLAG tag and its own terminator (pJR52), or direct C-FLAG tag and *ADH1* terminator (pJR50), or vector control (pRS316). (B) The protein level assay as described in FIG 3-2. Three-fold serial dilutions of total proteins extracted from strains expressing FLAG-tagged $Sir1^{FL}$ protein from either strategy pJR52 (lane 1-3) or pJR55 (lane 5-7) or empty vector (lane 4) were analyzed here.

3. Prediction of the structure of the Sir1 N-terminal domain

The N- and C-terminal domains of Sir1 share 27% identity and 46% similarity (14, 46). Because of their conservation at the level of both primary sequence and secondary structure prediction (FIG. 1-8), the three-dimensional structure of the N-terminal domain can be predicted by template-based homology modeling utilizing the known C-terminal structure (PDB: 1ZBX). Shown in FIG 3-4 is the predicted N-terminal structure by the Phyre (protein-homology/analogy recognition engine) server (http://www.sbg.bio.ic.ac.uk

/phyre/html/index.html) (61) using a profile-profile alignment algorithm, with an E-value of 0.032 and an estimated precision score of 95%. That is, 95% of the predictions with an E-value of 0.032 or lower were correct homologs and 5% were false positive.

Based on the prediction and alignment, the N-terminal domain has a very similar tertiary structure as the C-terminal domain, especially for the first 76 aa whose corresponding part at the C-terminal domain (aa 463-535) is involved in the interaction with the Orc1 BAH domain. However, it is not so conserved at the region responsible for interacting with Sir4. Based on the co-crystal structure of $Sir1^{C}$-$Orc1^{BAH}$, most of the Sir1 residues important for this interaction (3, 26) are on the interface (residues labeled in red, FIG 3-4D), and some of them are conserved between the Sir1 N- and C-terminal domains (FIG 1-8). On the other hand, although there is not a structure available for the $Sir1^{C}$-Sir4 complex, Sir1 residues which are important for this interaction (45) also appear to be on the surface (residues labeled in green, FIG 3-4D). These residues are all charged. However, most of the corresponding residues in the N-terminal domain (7 out of 9) are neutral or even have the opposite charge, indicating much less conservation in the Sir4 interacting domain compared to that for the $Orc1^{BAH}$ interacting domain (FIG 1-8). Overall, the features for the Sir1 C-terminal interaction with the Orc1 BAH domain are more conserved on the Sir1 N-terminal domain than those for the Sir4 association. Therefore, the putative interacting partner of the Sir1 N-terminal domain is more likely to be similar to the Orc1 BAH domain rather than Sir4.

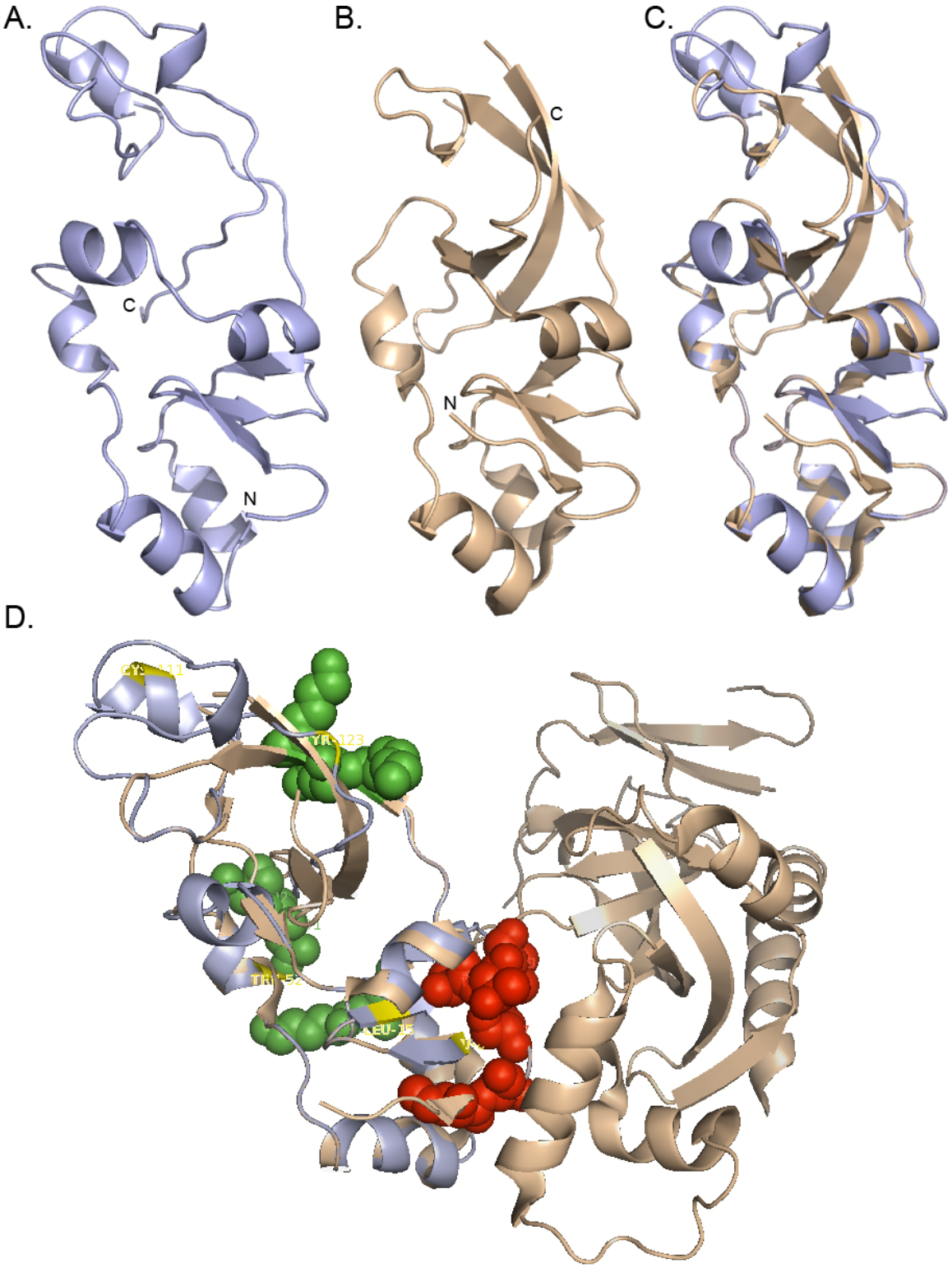
A.
B.
C.
C
N
C
N
D.

FIG 3-4 The structural prediction of the Sir1 N-terminal domain. (A) The predicted N-terminal structure (aa 4-132) by the Phyre (protein-homology/analogy recognition engine) server (http:// www.sbg.bio.ic.ac.uk/phyre/html/index.html) shown in a ribbon presentation. The N- and C-ends of the N-terminal domain are labeled. (B) The Sir1 C-terminal domain structure (aa 462-585) (PDB ID: 1ZBX) viewed from the same direction as in A. The N- and C-ends of the C-terminal domain are labeled. (C) Superposition of the Sir1 N- and C-terminal domain structures, which are colored the same as in A and B, respectively. (D) Superposition of the Sir1 N-terminal structure to the C-terminus, which is in complex with the Orc1 BAH domain, shown on the right. The C-terminal residues important for the interaction with the Orc1 BAH domain (Y465, V466, S467, R469, D479, L480, A491) are labeled in sphere-side-chain form in red, those for the interaction with Sir4 (K489, D490, K538, K539, K540, D579, D580, D587, D588) are labeled in green, and those labeled in yellow (F470, L477, C573, Y584) are important for both interactions (3, 26).

4. There was weak association between the Sir1 N-terminal domain and the Sir3 BAH domain *in vitro*

Despite the sequence and structural similarity between the Sir1 N- and C-terminal domains, a previous two-hybrid interaction screen failed to identify any interacting partner for the N-terminal domain (13) as was found for the C-terminal domain (130). As mentioned before, the putative $Sir1^{N}$-interacting partner is more likely to be similar to the Orc1 BAH domain. As described previously, the BAH domains of Sir3 and Orc1 are very similar in their backbones structures (with a root-mean-squared deviation of 1.1 Å using the Cα positions for alignment (14)), but have some differences on the surface area that may exert different specificities for binding. This may explain why the Sir1 C terminal domain interacts with the Orc1 BAH domain rather than with the Sir3 BAH domain (13, 47). Therefore, we wanted to test if it is the Sir1 N-terminal domain that binds to the Sir3

BAH domain.

In order to test this putative interaction, we carried out GST pull-down assays with two fragments of the Sir3 N-terminus, $Sir3^{253}$-GST and $Sir3^{219}$-GST. Both fragments contained the BAH domain (aa 1-214), and could pull down similar amounts of $Sir1^{136}$-His_6 (FIG 3-5). However, only a small fraction of $Sir1^{136}$-His_6 was pulled down out of the total input (less than 5%, data not shown). Also, there is some background binding due to the GST tag, although it is weaker than the BAH domain results. Notably, the molar ratio for the GST pull-down assay was ten $Sir3^{BAH}$ molecules to one $Sir1^{N}$ molecule. Therefore, the Sir1 N-terminal domain interacted with the Sir3 BAH domain weakly *in vitro*.

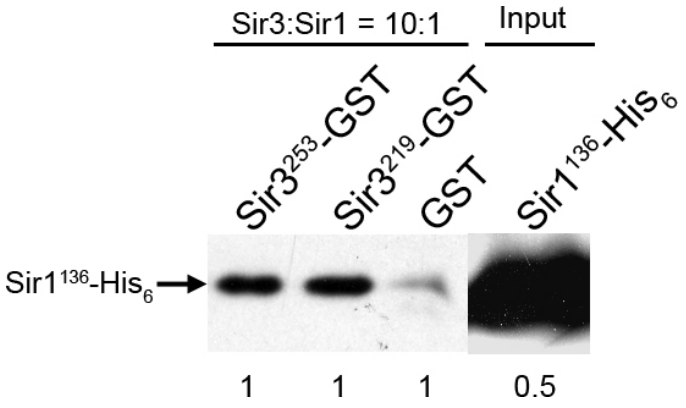

FIG 3-5 The Sir1 N-terminal domain interacted with the Sir3 BAH domain. Equal amounts of $Sir3^{253}$-GST, $Sir3^{219}$-GST and GST control purified from *E. coli* were used to pull down the purified $Sir1^{136}$-His_6. The molar ratio between the Sir3 fragments and $Sir1^{N}$ was 10 to 1. The immobilized proteins were resolved on an SDS-PAGE gel and visualized by anti-His western. The number below indicates the relative amounts of purified $Sir1^{136}$-His_6 used to perform the binding assay. Coomassie staining on a separate gel (data not shown) showed equal amount of the GST proteins were used for the binding assay.

5. Sir1 over-expression did not lead to its spreading into silent chromatin

It was previously shown by chromatin immunoprecipitation (ChIP) that Sir1 was predominantly localized at the *E* silencers of both *HM* loci, while little Sir1 was detected at the *I* silencers and the internal region, especially for the *HMR* locus (147). However, a recent study showed similar enrichment of Sir1 at both the *HMR-E* silencer and the internal locus *HMR***a***1* (93). In order to clarify the localization of Sir1 protein on the silent chromatin, we constructed a strain (JRY12) containing $FLAG_3$-tagged *SIR1* at the chromosomal locus for an anti-FLAG ChIP assay. A sensitive assay of silencing the *hmr::TRP1* locus was used to confirm the function of the tagged Sir1 protein (FIG 3-6A). The enrichment of Sir1 at different loci within *HMR* was compared with the signal from a non-heterochromatic *PMA1* locus. The resulting ratio was then normalized to the corresponding ratio from the untagged control strain W303-1a. My data, as shown in FIG 3-6B, showed restricted localization of Sir1 to the *HMR-E* silencer, which was about 2-fold more than that at the internal region, and thus was in agreement with the former observation (147).

Because the protein level of endogenous Sir1 is very low (about 20 molecules per cell (25)), we suspected it to be a limiting factor for spreading. Therefore, we introduced $FLAG_3$- tagged *SIR1* on a 2-micron plasmid and tested the localization of Sir1 when it was over-expressed (FIG 3-6C). The increased expression level was confirmed by anti-FLAG immunoblotting. The Sir1 protein level in cells containing both genomic

tagged Sir1 and 2-micron plasmid borne copy was about 4-fold more than in cells containing the genomic copy alone. However, the increased amount of protein didn't change the pattern of restricted localization, as the majority of the Sir1 protein associated with the *HMR-E* silencer. On the other hand, there was a slight increase of enrichment at *HMR***a***2* locus. However, it might be an artifact caused by the resolution of ChIP analysis. The resolution of ChIP analysis is determined by the size of the DNA fragments. Before the immunoprecipitation, the formaldehyde cross-linked chromatin is sheared to generate DNA fragments ranging from 0.2 to 1.2 kb in length, as shown in FIG 3-6D. Although the length of DNA fragments peaked at 0.5 kb, there was a small portion of the fragments which were about 1.2 kb or even slightly longer. Therefore, the slight increase of association observed at *HMR***a***2* (~1.2 kb downstream of the *HMR-E* silencer) may be due to the increase at *HMR-E*, rather than through direct association to *HMR***a***2*. Such an artifact disappeared as the testing locus was further away; i.e., at the *HMR***a***1*/**a***2* promoter region, which is ~1.4 kb downstream of *HMR-E* silencer, no such increase of Sir1 association was observed.

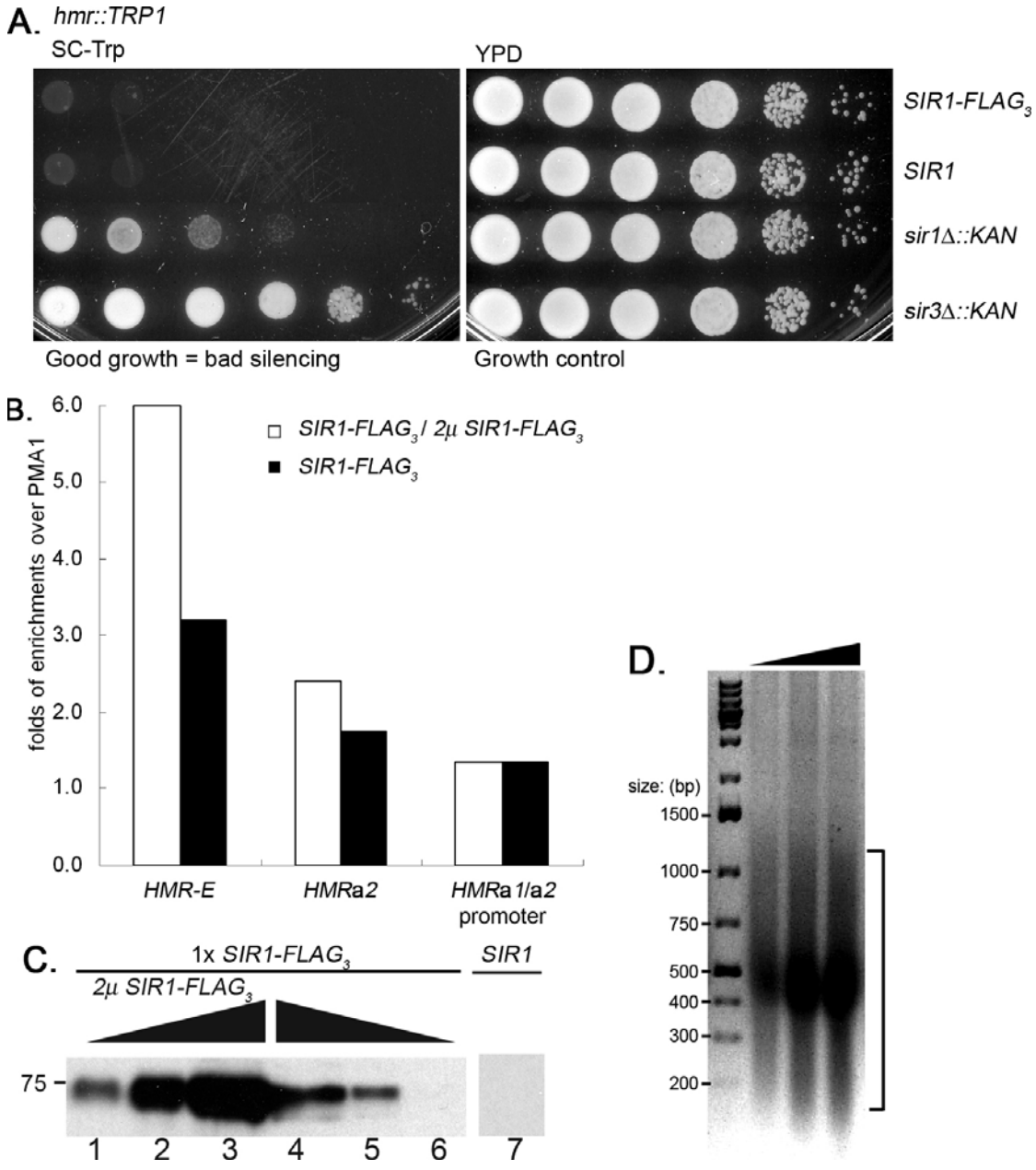

FIG 3-6 Sir1 over-expression did not lead to its spreading into the internal region of the *HMR* locus. (A) An *hmr::TRP1* silencing assay of Sir1 protein function. Strains carrying $FLAG_3$-tagged *SIR1* at the chromosomal locus (*SIR1-FLAG*$_3$), *SIR1* without tag (*SIR1*) as a positive control, *sir1Δ::Kan* as a negative control, and *sir3Δ::Kan* as an example of complete derepression were tested for silencing *hmr::TRP1* locus. Serial ten-fold dilutions were plated on YPD (right) as a growth control and the supplemented synthetic medium (SC-Trp, left) to monitor growth as an indicator of derepression. (B) A ChIP analysis for Sir1 localization in wild-type (*SIR1-FLAG*$_3$) and over-expressed (*SIR1-FLAG*$_3$ [*2μ SIR1-FLAG*$_3$]) strains. The enrichment of Sir1 at the

HMR-E silencer, *HMR***a***2* or the *HMR***a***1*/**a***2* promoter was compared with the signal from a non-heterochromatic *PMA1* locus. The resulting ratio was then normalized to the corresponding ratio from the untagged control strain W303-1a, and plotted as "folds of enrichment over *PMA1*". (C) The Sir1 protein level. Three-fold serial dilutions of total proteins extracted from strains expressing FLAG-tagged $Sir1^{FL}$ protein both from the genomic locus and the two-micron plasmid (*SIR1-FLAG*$_3$ [*2*μ *SIR1-FLAG*$_3$]) (lane 1-3), only from the genomic locus (*SIR1-FLAG*$_3$) (lane 4-6), and no-tag control (lane 7) were resolved by SDS-PAGE, transferred to the membrane and detected with anti-FLAG monoclonal antibodies (M2, Sigma^{TM}). (D) Analysis of chromatin shearing. DNA fragments prepared for ChIP was electrophoresed on 2% agarose gel; and the ethidium bromide-stained gel is shown. The size of DNA marker (GeneRuler® DNA ladder plus) was labeled on the left. The range of DNA fragment size is denoted with a bracket on the right.

6. The Sir1 C-terminal domain interacted with specific regions of the Sir4 N-terminus

It is known that $Sir1^{C}$ interacts with Sir4, facilitating the establishment of silencing (3, 130). Moreover, the minimal region of the Sir1 C-terminus for the interaction with Sir4 has been defined (3). However, the domain of Sir4 that mediates this interaction was not well characterized.

A previous study from our lab demonstrated the two-hybrid interaction between LexA-$Sir1^{322-654}$ and GAD-$Sir4^{FL}$ (130). However, I found this interaction was lost when testing various C-terminal fragments of Sir4 instead of the full-length protein (data not shown), indicating that the N-terminus of Sir4 was critical for interacting with $Sir1^{C}$. Moreover, Marshall *et al*. found that a plasmid-borne Sir4 C-terminal fragment (45% of Sir4) could trans-complement a mutant strain containing 26% of Sir4 from its N terminus

at its chromosomal locus (82). These facts prompted us to consider the first 26% amino acids of Sir4 as an independent domain possibly responsible for the $Sir1^{C}$ interaction.

Triolo *et al*. found that LexA-$Sir1^{322-654}$ was slightly auto-active in a *sir4* null mutant two-hybrid strain, producing colonies with a light blue/green color in the beta-galactosidase assay when co-transformed with the Gal4 activation domain (GAD) alone(130). But the same LexA-$Sir1^{322-654}$ was not auto-active in a $Sir4^{+}$ strain, suggesting that the endogenous Sir4 protein interacted with LexA-$Sir1^{322-654}$ and masked its auto-activity (130). Although the signal from the slight auto-activity was much weaker than that from a real two-hybrid interaction, in order to eliminate any possible dilution by the endogenous Sir4 and get a better signal, the following two-hybrid assays were carried out in a *sir4*Δ background.

To test if Sir4 interacts with Sir1 through its N-terminal domain and to further delineate the critical regions for this interaction, we constructed a variety of Sir4 N-terminal fragments fused to the Gal4 activation domain (GAD). By testing the two hybrid interaction of these fragments with LexA-$Sir1^{322-654}$, we identified the N-terminal 360 amino acids (aa) as mediating the $Sir1^{C}$-Sir4 association. Moreover, we further delineated this N-terminal domain and found that two regions, aa 91~164 and aa 261~309, were required for this interaction. GAD-$Sir4^{91-309}$ was the minimal fragment that showed equal, if not stronger, interaction with the Sir1 C-terminal domain as the full-length Sir4 did. Absence of aa 261~309 greatly reduced the two hybrid signal, and lacking of aa

91~164 abolished it.

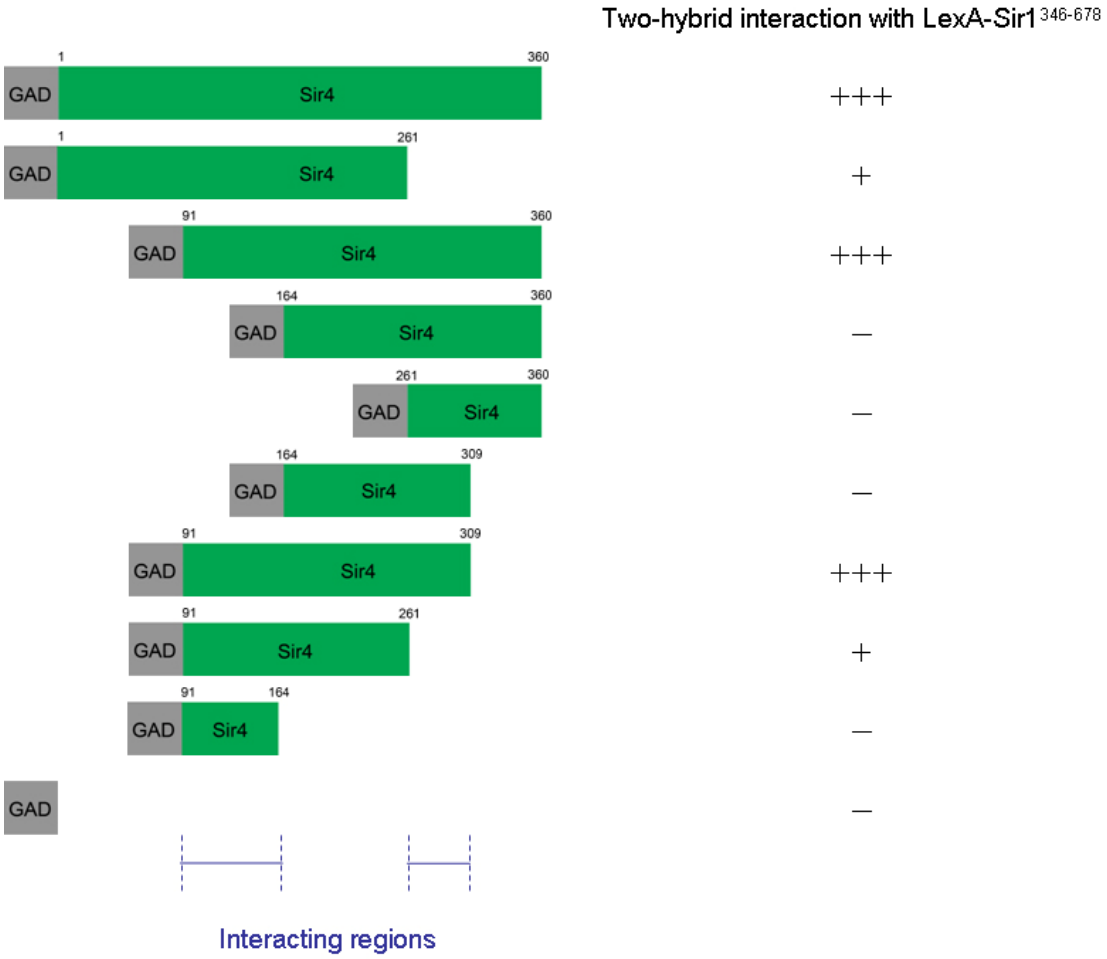

FIG 3-7 Two minimal regions were required for SIR4 to interact with Sir1 through its N-terminal domain in the two-hybrid system. Shown are various hybrid N-terminal fragments of Sir4 fused to the Gal4 activation domain (GAD) that were tested against LexA-Sir1$^{322\text{-}654}$, the strength of the transcriptional activation of the *lacZ* reporter gene is indicated (+++, cells turned blue with X-gal indicator in 1 hour; +, cells turned green in 1 hour; –, no detectable color within 1 hour).

III. Discussion and future directions

1. Functions of the Sir1 N-terminal domain

The ability of over-expressed Sir1 full-length protein to strengthen silencing in some extremely compromised circumstances, such as BAH silencing, or silencing with a

temperature sensitive sir3-8 protein, or in a background with *nat1/ard1*Δ and a weakened Δ*Ehmre* silencer, stands out from its dispensable physiological role in a wild-type cell (13-14, 123). Moreover, the fact that the C-terminal fragment alone does not have such ability prompted us to attribute it to the N-terminal domain. Previous evidence with $sir1^{\Delta N}$ fragment was flawed by deletion of its promoter at the same time, leaving the identity and amount of the putative Sir1 fragment in question. We clarified this ambiguity by expressing $sir1^{127\text{-}654}$ (ΔN) from its own promoter and confirmed that $Sir1^{N}$ is essential for its suppressing ability. Although the Sir1 fragment lacking the N-terminal 126 aa can complement the *sir1* null phenotype, it cannot establish BAH silencing or suppress the *sir3-8* mutation at a non-permissive temperature, while full-length protein can when over-expressed (FIG 3-1). Therefore, the N-terminal fragment is important for silencing, especially in certain severely defective situations.

In the current model of silencing at *HM* loci, Sir1 facilitates the establishment of heterochromatin by being recruited to the silencer through its C-terminal interaction with Orc1, and in turn, bringing in Sir4 also via its C terminal domain (45, 47, 123, 130, 147). Accordingly, we propose two possible ways for its N-terminal domain to fit in the whole picture of silencing. First, it may contribute indirectly by affecting the protein level of the full-length protein. Second, the N-terminal domain may interact with the Sir3 BAH domain and promote the assembly of heterochromatin directly. In the following parts, we will explore both possibilities.

2. The Sir1 N-terminal domain stabilizes the full-length protein.

To test the first model that the Sir1 N-terminal domain affects the protein level, we compared the amount of the $FLAG_3$-tagged full-length protein and the protein without the N-terminus (aa 127-654) when they were over-expressed from the *SIR1* promoter on 2μ plasmids. We found that the protein level of $Sir1^{\Delta N}$ was about 7-fold lower than that of the full-length protein (FIG 3-2). This stabilization effect of the N-terminal domain is also supported by the observation of Hou et al (46). They constructed a *sir1* mutant with aa 35-IDGWLVD-41 changed to 35-IAAALVA-41. Compared to the wild type protein, the mutant was less abundant when expressed either from its own promoter at the chromosomal locus or from an *ADH1* promoter on a 2μ plasmid when fused with Gal4 DNA binding domain (GBD). Therefore, several lines of evidence demonstrated that the Sir1 N-terminal domain is important for stabilizing the full-length protein. Nevertheless, it will still be helpful to incorporate the $FLAG_3$-tagged $Sir1^{FL}$ and $sir1^{\Delta N}$, which we used for over-expression, into the chromosomal locus and test if a similar pattern of protein levels is observed.

However, the protein dosage effect caused by its N-terminal domain is not always reflected by Sir1's silencing ability. Instead, the impact of protein dosage depends on how compromised the strain background is for silencing. It is the least obvious in a wild-type setting, where even the deletion of the whole Sir1 protein only caused mild silencing defects, or rather in a small portion of the cells (96). Then, the importance of the Sir1

protein becomes visible only in strains with some leaky mutations, such as *eso* mutants. Apparently, a relatively low level of Sir1 is sufficient to suppress *eso* mutations (24, 122). Finally, when the silencing machinery is severely compromised, such as in BAH silencing (14), or in a *sir3-8*^{ts} mutant strain at the non-permissive temperature (13, 122), a large amount of Sir1 protein is needed to suppress the defect. When the N-terminal domain is absent, the suppression cannot be fulfilled by the reduced amount of protein. Nevertheless, whether or not stabilization is the only way the Sir1 N-terminal domain contributes to silencing in such circumstances is still an open question. One way to address this question is to artificially increase the amount of Sir1$^{\Delta N}$ to the same level when full-length protein showed the suppressing ability. It may be achieved by expressing Sir1$^{\Delta N}$-G_8-$FLAG_3$ from the *GPD* promoter, a much stronger promoter than Sir1's own promoter, thus producing a similar amount of protein comparable to that of Sir1^{FL}-G_8- $FLAG_3$ from its own promoter. If the elevated protein level still cannot render the suppressing ability to Sir1$^{\Delta N}$, it will indicate that the N-terminal domain has other functions in addition to stabilization. It is an interesting question why the Sir1 N-terminal domain is important for protein stability. It is possible that removal of the N-terminal domain exposes a sequence that triggers degradation.

3. The Sir1 N-terminal domain may facilitate silencing through its weak association with the Sir3 BAH domain.

The high sequence conservation between the Sir1 N- and C-terminal domains (14)

and the existing C-terminal structure (45, 47) enabled us to predict the N-terminal structure. And indeed, it is well conserved at the tertiary level, especially, for the part mediated the interaction with the Orc1 BAH domain (FIG 3-4). On the other hand, the features for Sir4 binding are not well conserved (FIG 3-4). All these lines of evidence point to similarity in binding partners for $Sir1^{N}$ and $Sir1^{C}$, i.e., a BAH domain. The Sir3 BAH domain is similar to the Orc1 BAH domain, but carries some differences on the corresponding $Sir1^{C}$-interacting surface. Therefore, we investigated if the Sir1 N-terminal domain binds to the Sir3 BAH domain. The GST pull-down assay demonstrated the interaction *in vitro*; however, it was weak. We are going to investigate the specificity of this weak interaction by testing against Orc1 BAH domain and Sir4 fragments.

The interaction between $Sir1^{1-136}$-His and $Sir3^{BAH}$-GST we observed by pull-down assays is supported by recently published two hybrid interactions. A slightly different Sir1 N-fragment ($Sir1^{1-131}$-GBD) was tested against GAD-$Orc1^{BAH}$ and GAD-$Sir3^{BAH}$, and showed weak interaction with both (46). In contrast, the Sir1 C-terminal domain ($Sir1^{449-654}$-GBD) showed a strong preference to GAD-$Orc1^{BAH}$ and no interaction with GAD-$Sir3^{BAH}$ (46). The weak $Sir1^{N}$-$Sir3^{BAH}$ interactions showed by two different assays, the GST pull-down and two-hybrid assays, are consistent. However, the similarly weak $Sir1^{N}$-$Orc1^{BAH}$ interaction was not expected based on the surface difference of the two BAH domains. It would be important to test this interaction with our GST pull-down system. A plasmid expressing $Orc1^{1-219}$-GST has been constructed and could be tested

against Sir1$^{1-136}$-His with Sir1$^{456-586}$-His as a positive control.

Nevertheless, the weak and non-discriminative binding ability of the Sir1 N-terminal domain to the BAH domains raises question about if and how it contributes to the silencing ability of the full-length Sir1 protein. One hypothesis is that this "weak and non-discriminative" feature is needed for a "scanning" function. There are a large number of ORC binding sites over the genome (143), whereas Sir1 protein only co-localizes with ORC within the *HM* loci *in vivo* (25, 93). Given the strong *in vitro* binding of the Sir1 C-terminal domain to Orc1^{BAH}, it is likely that the C-terminal domain is somehow excluded in the initial interaction between Sir1 and the chromatin, so that Sir1's concentration won't be titrated by the tight association with ORC outside the *HM* loci. Instead, my hypothesis is that the N-terminus "scans" the chromatin and generates unstable association with the BAH domains. Only at *HM* loci, which has a high concentration of the BAH domains (from Orc1 and Sir3) and other Sir proteins (esp. Sir4), the "scanning" mode of the Sir1 N-terminal domain can be trigged to transfer to the strong association mode of the C-terminal domain. Therefore, in this model, the localization of Sir1 to *HM* loci is reduced in the absence of its N-terminal domain. As a consequence of decreased Sir1 concentration at *HM* loci, silencing is abolished in some already compromised backgrounds, such as BAH silencing. This hypothesis can be tested by comparing the enrichment of full-length Sir1 and Sir1$^{\Delta N}$ at silencers of *HM* loci and an ARS (ORC binding site) outside *HM* loci.

Another hypothesis to explain the importance of the Sir1 N-terminal domain for it's strong suppressing ability is that the interaction between $Sir1^{N}$ and $Sir3^{BAH}$ may facilitate the association between Sir3 and the nucleosome. As discussed in the introduction (Chapter One Part V), a possible common feature of sir3 eso mutants is the compromised Sir3-nucleosome interaction. As shown by Sampath et al., an eso mutant ($sir3^{219\ A2G}$-GST) demonstrated reduced retention of purified nucleosomes in a pull-down assay as compared to wild-type $Sir3^{219}$-GST (113). The Sir1 N-terminal domain may suppress *eso* mutations by strengthening the Sir3-nucleosome interaction through its binding to $Sir3^{BAH}$. In order to test this idea, we need to express $sir3^{BAH}$-GST carrying eso mutations, and test if nucleosomes show reduced binding to all or most of the eso mutants as compared to the wild-type $Sir3^{BAH}$- GST. Then we can test if addition of recombinant $Sir1^{1-136}$-His will increase the retention of nucleosomes in these GST pull-down assays.

Given the broad spectrum of pathways that *eso* mutations affect (FIG 1-7), it is possible that the Sir1 N-terminal domain has some yet unknown interaction. Such an interaction may function indirectly by increasing local Sir3 or Sir4 concentration, or play a role in forming higher-order chromatin structures, such as long-range interactions between *HML* and *HMR* (84), to facilitate the assembly of heterochromatin. Containing a duplication of the Sir1 C-terminal domain at its N-terminus and reserving some of its binding ability may be an advantage for Sir1 to serve as a bridge to mediate the long-range interaction between *HML* and *HMR* (84). Further investigation is needed to

address these hypotheses.

4. Sir1 localization at the *HMR* locus

There are contradictory results about whether or not Sir1 spreads from the silencer into the internal region of the silent chromatin (93, 147). Nevertheless, our result at the *HMR* locus supports the model for restricted localization of Sir1 to *HMR-E* even when it is over-expressed. It is possible that Sir1 is confined to the silencer because of its tight association with Orc1. This indicates that Sir1 carries out its physiological function through its association with the silencer. However, it is still possible that the pattern of Sir1 distribution changes in a compromised background. For example, in BAH silencing, when full-length Sir3 is not available, Sir1 may need to spread into the internal region to facilitate the association between the Sir3 BAH domain and the nucleosomes. This spreading is probably mediated via Sir4, or even through its weak interaction with the Sir3 BAH domain. Testing of Sir1's localization under such compromised circumstance is needed to clarify this possibility.

However, when testing such defective silencing, the tagging strategy may affect the suppressing ability of Sir1. For example, direct tagging to the C-terminus and consequent changing to the *ADH1* terminator jeopardized BAH silencing; however, inserting an 8-Gly linker between the Sir1 C-terminus and the $FLAG_3$-epitope, while keeping Sir1's own terminator, preserved the suppressing ability (FIG 3-3). Therefore, using the direct tagging strategy has been a flaw for both others' and our observations. Instead,

SIR1-G$_8$-FLAG$_3$-T$_{SIR1}$ should be used in the future to reflect the real Sir1 localization.

5. Further characterization of the Sir1-Sir4 interaction

Based on previous (3, 82, 130) and our own observations, we defined the N-terminal 360 amino acids of Sir4 as a separate domain responsible for Sir1^C interaction. Further delineation of this region found that aa 91~164 and aa 261~309 were required for this interaction as tested by two-hybrid assays. A mutagenesis screening in this region would be important to identify the Sir4 residues important for this interaction. Moreover, the putative mutations should be tested to be sure that they don't affect Sir4's other functions. Such a mutation should abolish the Sir1^C-Sir4^N two hybrid interaction, but not have a significant silencing defect in the otherwise wild-type cells. That is, it should not have a greater silencing defect than a *sir1*Δ mutant, unless the Sir4 N-terminal domain has additional functions. Because Sir1 can interact with both Orc1 and Sir4, it has always been a question how each interaction contributes to silencing. Such *sir4* mutants will be very helpful for us to dissect Sir1's functions. For example, Sir1 can suppress *eso* mutations with wild-type Sir4; if it loses this ability in a *sir4* mutant background, it indicates that the Sir1-Sir4 interaction is critical for suppression.

In summary, I have clarified the importance of the N-terminal domain of Sir1, especially in some compromised silencing backgrounds. It contributes to silencing by stabilizing the full-length Sir1 protein. Moreover, the Sir1 N-terminal domain shows a weak interaction with the Sir3 BAH domain, which may serve in a "scanning" mode to

recruit Sir1 to the *HM* loci, followed by switching to the tight association with Orc1 BAH domain through the Sir1 C-terminal domain. A $Sir1^{N}$-$Sir3^{BAH}$ interaction may also function directly through strengthening the Sir3-nucleosome association. On the other hand, over-expressing Sir1 did not change the restricted localization of Sir1, which did not spread into the internal region of silent chromatin. Therefore, association with the *HMR-E* silencer is necessary and sufficient for Sir1 to carry out its physiological functions at the *HMR* locus. Further investigation is needed to figure out the underlying mechanism of the function of $Sir1^{N}$.

Chapter Four: Bur1 and Its Interacting Partners

I. Introduction

1. The Bur1/Bur2 complex and its function in transcription regulation

BUR1 and *BUR2* are two *BUR* (Bypass UAS Requirement) genes. In yeast, regulated transcription of most protein-encoding genes requires a UAS (upstream activating sequence), which is the functional equivalent of a transcriptional enhancer in mammals (34). Without a UAS, there is little or no transcription of these genes. However, in some mutant strains, transcription can still occur from the remaining core promoters without the UAS (127). Prelich and Winston screened for such mutants which bypass the requirement of the UAS of the *SUC2* gene, allowing the growth of a *suc2*Δ*uas* mutant on medium containing sucrose as the carbon source. They identified *BUR* genes along with several *SPT* (Suppressor of *Ty*) genes known to suppress transcription defects (97, 127). Similar to the *SPT* genes, these *BUR* genes turned out to have pleiotropic phenotypes, indicating general involvement in transcription (97), and were later confirmed as such (97, 144). Among them, *BUR1* and *BUR2* were found to encode a cyclin-dependent kinase (CDK) and the corresponding cyclin (144).

Transcription can be divided into three stages, i.e., initiation, elongation, and

termination. Each transcription stage encompasses various events which can be classified into two types: chromatin modification and remodeling, mRNA maturation and export. The transition and progression of each stage is tightly controlled. And CDKs play critical roles in this regulation, especially for initiation and elongation. In *Saccharomyces cerevisiae*, among all the CDKs/cyclins, there are four pairs important for transcription: Kin28/Ccl1, homologous to mammalian CDK7/cyclin H, are subunits of the general transcription factor TFIIH and phosphorylate the Pol II CTD at Serine 5 (S5) during transcription initiation and promoter clearance stages, which leads to recruitment of the mRNA capping enzyme and cap binding complex (CBC), as well as the sequential association of elongation factor Spt4/Spt5, the PAF complex, and the histone H3 K4 methyltransferase complex (COMPASS) (21, 57, 75, 88, 103, 108); Srb10/Srb11 (mammalian CDK8/cyclin C equivalent), subunits of the mediator complex, can phosphorylate several transcription factors to alter their activity (8, 41, 87), and may promote or inhibit initiation under different conditions (39, 75); the Ctk1/Ctk2/Ctk3 complex and Bur1/Bur2 complex both contribute to Pol II S2 phosphorylation (10, 56, 76), which is carried out by P-TEFb (CDK9) in higher eukaryotes. The predominant phosphorylation of Serine 2 (S2) is considered the critical switch from initiation to elongation, leading to recruitment of Set2 (histone H3 K36 methyltransferase) and other histone modification enzymes (reviewed in (114)) and mRNA polyadenylation and termination factors (1).

Defined as a complex, Bur1 and Bur2 physically interact with each other, as shown by two-hybrid interactions and co-immunoprecipitation assays (27, 144). And as the corresponding cyclin, Bur2 is required for the kinase activity of Bur1 (144). This functional relationship explained why *bur1* and *bur2* mutants had nearly identical phenotypes (97, 144). It has been found that the Bur1/Bur2 complex is recruited to the transcription machinery through phosphorylated S5 on the Pol II CTD during initiation, peaks at the 5'-end of the ORF and remains associated with the elongating polymerase (102). Once recruited, Bur1 phosphorylates Spt5 on its C-terminal repeat domain, which promotes subsequent association of the PAF complex (76). The PAF complex mediates the recruitment of various chromatin modification enzymes, including Rad6/Bre1 for histone H2B monoubiquitylation and COMPASS (Set1) for histone H3 K4 methylation (95, 114). Moreover, the phosphorylation of Rad6 S120 by Bur1 is required for histone H2B monoubiquitylation (140), which regulates the subsequent methylation of histone H3 at K4 and K79 (4, 18, 89, 125). This explains why the lack of Bur1 kinase activity causes reduced association of the PAF complex and decreased level of histone H3 K4 trimethylation, histone H2B K123 monoubiquitylation, all of which play important roles during initiation and the subsequent transition into elongation (68, 103, 140). At the transition to elongation, Bur1 and Ctk1 phosphorylate the Pol II CTD at S2, which recruits to the elongating polymerase a different set of chromatin modification enzymes, e.g., Set2 (histone H3 K36 methyltransferase) (reviewed in (114)), and mRNA

polyadenylation and processing factors (1). Finally, as the elongation proceeds downstream, it was proposed that Bur1/Bur2 may stimulate Ctk1's activity, which makes an increasingly larger contribution to the CTD S2 phosphorylation (102). In summary, the kinase activity of Bur1 targets to at least three substrates *in vivo,* i.e. Spt5, Rad6, and Pol II CTD S2; while *in vitro* kinase assays showed even more targets including Bur1 autophosphorylation and Pol II CTD S5 (144). These phosphorylations control important steps in various aspects of transcription regulation, especially during initiation and the transition into elongation stage.

On the other hand, although there is mounting evidence showing Bur1/Bur2 influences histone modifications coupled to transcription (18, 68, 76, 88-89, 95, 102-103, 108, 119, 125, 140), direct linkage of Bur1/Bur2 to the other type of co-transcriptional events, affecting mRNA maturation, is still missing. In the following part of this chapter, efforts to identify such a connection will be discussed.

2. Npl3 and its functions in transcription termination and mRNA export

Transcription, especially its elongation and termination stages, is coordinated with mRNA 3'-end processing and the formation of heterogeneous ribonucleoprotein complex for export (11, 40, 53, 71, 100-101, 109). In higher eukaryotes, the serine/arginine-rich (SR) family members play essential roles in the aforementioned coordination. They bind to RNA through their RNA recognition motif (RRM), mediating co-transcriptional alternative splicing of pre-RNA and the subsequent export of the ribonucleoparticles

(RNPs) (15, 133). Moreover, their reversible phosphorylation on the C-terminal serine/arginine-rich domain facilitates the shuttling of SR proteins between the nucleus and the cytoplasm (67).

In *Saccharomyces cerevisiae*, although alternative splicing is missing, there are three SR-like proteins, Npl3, Gbp2, and Hrb1, which carry out the conserved function of coordinating transcription, mRNA maturation, packaging and export. As a demonstration of the conservation, Npl3 was actually found to promote splicing in budding yeast (67). Similar to mammalian SR proteins, the yeast SR-like proteins Npl3 and Gbp2 can be phosphorylated by Sky1 (homologous to mammalian SR Protein Kinase 1) in the cytoplasm (30, 80, 137), which is required for the subsequent import by the Mtr10 transporter into the nucleus (121, 137, 146). Inside the nucleus, Npl3 is found in the same complex with RNA Pol II and is recruited co-transcriptionally yet independent of RNA (71). The other two SR-like proteins, Gbp2 and Hrb1, are also recruited to the transcription machinery through a different mechanism, via the transcription/export (TREX) complex (49). The TREX complex is recruited co-transcriptionally and couples transcription elongation to mRNA export (124). Therefore, SR-like proteins are all recruited co-transcriptionally and may contribute to both transcription and mRNA export, albeit through different mechanisms. In the case of Npl3, the phosphate group on S411 added by the cytosolic kinase Sky1 is later removed by the nuclear phosphatase Glc7 (29). In addition, it has been found another kinase, casein kinase 2, and multiple

phosphorylation sites were utilized to regulate Npl3's activity (17). Npl3 interacts with S2 phosphorylated CTD of Pol II and stimulates the elongation rate of the polymerase (17). Meanwhile, it antagonizes termination and mRNA 3'-end formation by competing for RNA binding with Rna15, a key component of the cleavage/polyadenylation machinery (6-7, 17). The dephosphorylation and RRM are both required for this function (6-7, 17). Moreover, Npl3's role in mRNA export also depends on the dephosphorylation, probably through promoting the association of the mRNA complex to Mex67, a component of the nuclear pore complex (NPC) (29-30). Nuclear Npl3 is also methylated by the Hmt1 arginine methyltransferase (70), which is recruited to the 5'-end of the ORFs (145), coincident with the recruitment of Npl3. The methylated Npl3 associates with the elongating mRNA transcript and leads to the assembly of mRNPs with other methylated RNA binding proteins (Yra1, Nab2 and Hrp1) for export by TREX complex via NPC (118). In the cytoplasm, the SR-like proteins remain associated with the translating mRNPs, and Npl3 was found to promote translation termination (20, 138). In summary, Npl3, although first identified as an mRNA export factor, contributes to various aspects of transcriptional and translational regulation as well. However, the detailed mechanism how Npl3 coordinates its own multiple roles is not clearly understood. It is likely that the interplay between arginine methylation and phosphorylation (81) regulates the localization of Npl3 and hence its interacting partners, so as to determine its function. Notably, in contrast to its well-established role in transcription termination and RNA

export, the contribution of Npl3 in early stages of transcription, i.e. elongation, was not appreciated until recently. The recruitment and modification of different proteins at each stage of transcription mentioned before is summarized in FIG 4-1. In the following part of this chapter, we are trying to understand more about it.

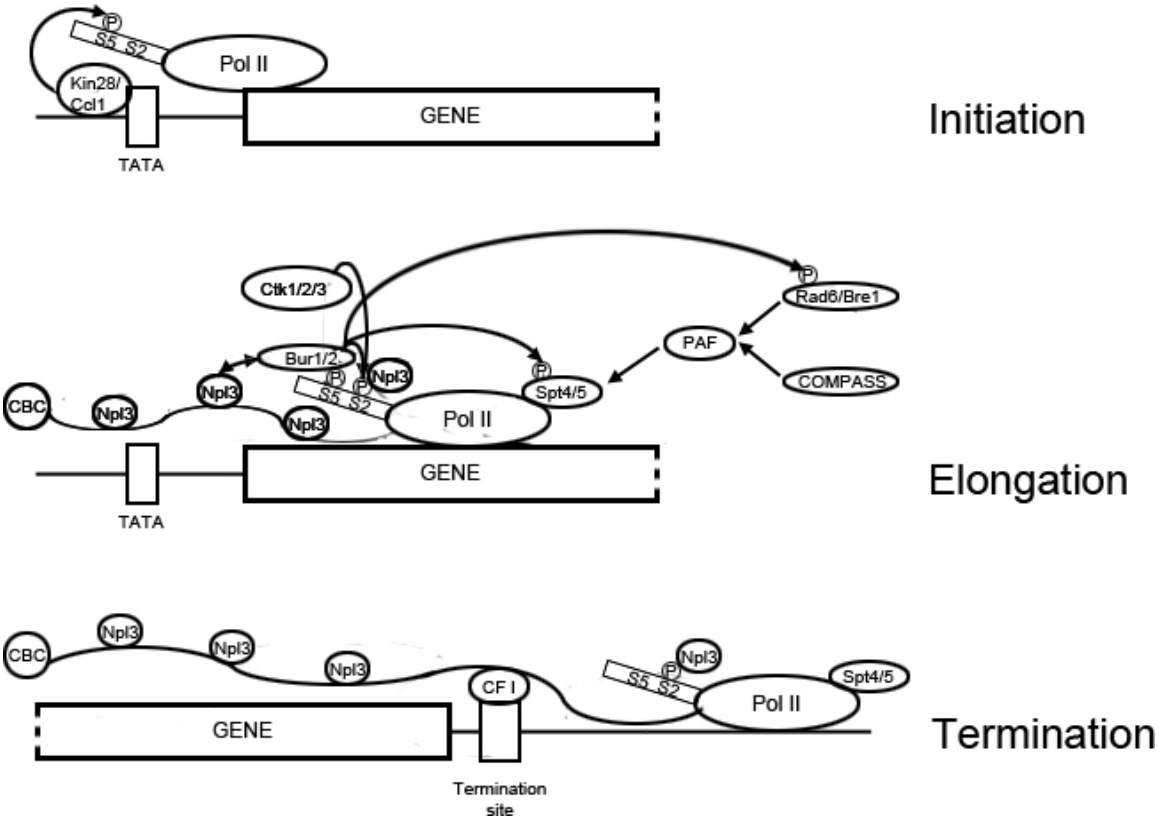

FIG 4-1 Co-transcriptional recruitment and phosphorylation of various proteins. The rectangular boxes represent DNA elements, the gene, TATA and termination site, respectively. Curved arrows link the kinases and their substrates. Straight arrows denote the sequential recruitment events to the transcription machinery. During the initiation and promoter clearance stage, the CDK/cyclin pair Kin28/Ccl1 phosphorylate the Pol II CTD at Serine 5 (S5). The phosphorylated S5 recruits the Bur1/2 complex, which in turn phosphorylate S2 of Pol II CTD. Ctk1/2/3 complex also contributes to S2 phosphorylation. The phosphorylation of S2 is considered the critical switch from initiation to elongation. Another substrate of the Bur1/2 complex is Spt5, which promotes subsequent association of the PAF complex. The PAF complex mediates the recruitment of various chromatin modification enzymes during elongation, including Rad6/Bre1 for histone H2B monoubiquitylation and COMPASS (Set1) for histone H3 K4 methylation. The phosphorylation of Rad6 is also carried out by Bur1 and is required for ubiquitylation. Npl3 binds to both the nascent mRNA and the phosphorylated S2 of Pol II CTD. Its interaction with Bur1 as denoted with a double arrow, will be discussed in the following part. As Pol II proceeds to the 3' end of the gene, the termination site recruits cleavage factor I (CF I) and signals for termination and mRNA 3'-end formation. During

the termination stage, Pol II CTD is only phosphorylated at S2, not at S5, thus retaining its association with Npl3 but not with Bur1.

II. Results

1. Searching for Bur1 interacting partners with the two-hybrid system

Bur1 has been shown to physically interact with Bur2 (27, 144) and an S5-phosphorylated Pol II CTD peptide (102). In order to find out if there are any other interacting partners to mediate Bur1's multiple functions, we carried out a two-hybrid screen using full-length Bur1 as the bait. Two reporter genes, *lacZ* and *HIS3*, were used to look for a two-hybrid interaction (130). The LexA-Bur1 bait did not have auto-activity, because cells co-transformed with LexA-Bur1 and Gal4 activation domain (GAD) alone did not turn blue in a beta-galactosidase assay (data not shown). The bait was also checked for its expression level by anti-LexA immunoblotting and shown to be expressed (data not shown). The screen generated about $2x10^6$ transformants, much higher than $4.6x10^5$ transformants required for a 99% chance to identify an interaction from the GAD-fusion library (9). About 600 $His3^+$ transformants were checked for beta-galactosidase activity. And finally two clones were isolated from the library as interacting strongly with the LexA-Bur1 bait. They were also tested for specificity against other nuclear protein baits, i.e. LexA-Sir4, LexA-lamin and LexA-Hst3, and showed no interaction. DNA sequencing showed one candidate encoded an in-frame fusion with Npl3 from aa 63 to the C-terminus, and the other with Gbp2 from aa 22 to

339. Both candidate fusions contained the two conserved RRM domains: aa 126 – 277 for Npl3 (16, 20), the only RRMs in Npl3; and aa 120 – 310 for Gbp2, which contains another less conserved RRM domain at the C-terminus (137). Identifying two SR-like proteins, both containing two conserved RRMs, strongly suggested the specificity of the two-hybrid interactions, and also suggested that Bur1 may interact with Npl3 and Gbp2 via these homologous RRM regions.

Although Bur1/Bur2 is identified as a functional complex, their individual mutants showed similar but not identical phenotypes. The *bur1*Δ mutant displayed a more severe growth defect than the *bur2*Δ mutant (97, 144). Also, it has been shown that over expressing Bur1 can suppress *bur2* null phenotype (144). One possible explanation is that Bur1 may utilize another cyclin for its cyclin-dependent kinase activity when Bur2 is not available to alleviate the defect. Ctk2, the cyclin subunit of C-terminal domain kinase 1 (CTDK-1), which also phosphorylates S2 of Pol II CTD as Bur1/Bur2 does, was proposed to be a substitute of Bur2. I tested GAD-Ctk2 for a two-hybrid interaction with LexA-Bur1, but the result was negative (data not shown). Therefore, Ctk2 is less likely to act in place of Bur2 to form a complex with Bur1 and to activate its kinase activity.

2. Interaction between Bur1 and Npl3 confirmed by co-IP

Since the two-hybrid interactions showed that Bur1 associates with Npl3 and Gbp2, we continued to investigate if they interact under physiological conditions. We obtained a strain in which Bur1 was N-terminally FLAG-tagged and expressed from a *CEN* plasmid,

while its genomic copy was deleted (RS2075). In the same strain background, either Npl3 or Gbp2 was C-terminal HA-tagged by me for detection after co-immunoprecipitation (co-IP) with FLAG-Bur1. As shown in FIG 4-2A, Npl3-HA co-precipitated with FLAG-Bur1 (lane 3); however, Gbp2-HA was not detected in the precipitates (lane 2). It is still possible that the Gbp2-HA did co-IP with FLAG-Bur1; however, its signal expected around 49 kDa could be masked by cross-reaction to the mouse heavy chain contamination at 55 kDa introduced by the anti-FLAG antibody. Another explanation is that Gbp2 has a relatively lower abundance, about $2.5x10^3$ molecules per cell, while cells contain about 30-fold more Npl3 molecules (28). Notably, although Npl3-HA is predicted to have a molecular weight of 45 kDa, it migrated much slower at about 60 kDa (lane 3), similar to what has been shown without a tag by others (67, 137). The same precipitate was immunoblotted for FLAG-Bur1 (FIG 4-2A lower panel); however, the signals (top band in lane 2-4) were very weak. There was also a consistently lower band of unknown identity as marked with an asterisk.

Then we tested the co-IP interaction in the other direction, i.e., anti-HA IP of Npl3 or Gbp2, followed by detection of FLAG-Bur1. Consistent with our previous result, Bur1 co-precipitated with Npl3 (FIG 4-2B lane 4). There was a very weak signal detected for Gbp2 co-precipitation (lane 5); however, it migrated faster than the usual Bur1 band. Therefore, Bur1 physically interacts with Npl3 as shown by both two-hybrid and co-IP assays. On the other hand, Gbp2 demonstrated two-hybrid interaction with Bur1, but this

putative interaction was not confirmed under physiological conditions.

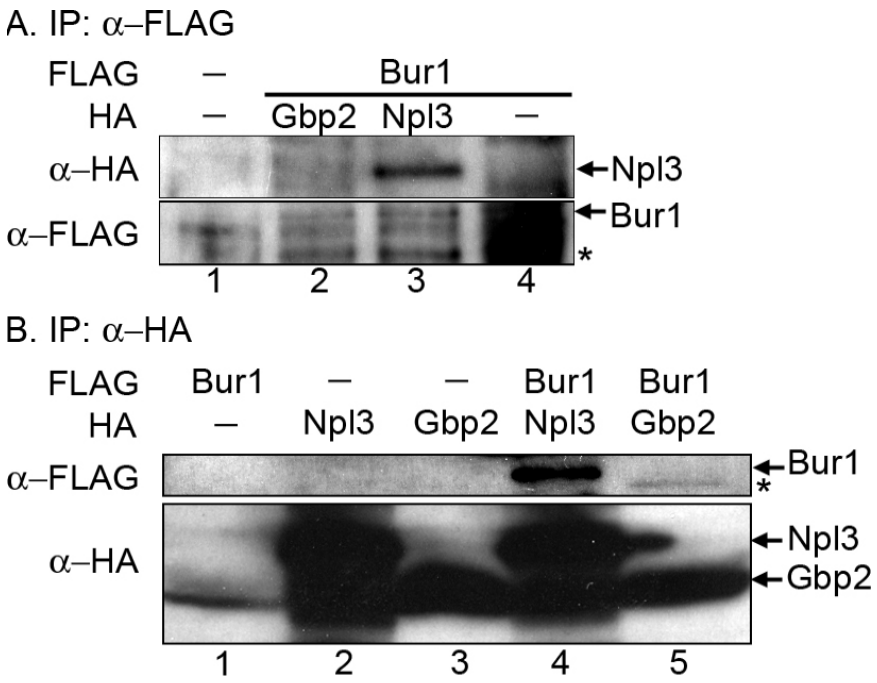

FIG 4-2 Bur1 physically interacted with Npl3. A) Co-immunoprecipitation assays using α-FLAG antibodies to pull down proteins from the indicated HA-tagged strains. Strains without HA- and/or FLAG-tagging were used as negative controls. Precipitates were eluted by boiling with sample buffer, resolved by SDS-PAGE and transferred to Hybond-P PVDF membrane. Blots were probed with α-HA antibodies for co-precipitated proteins, and also with α-FLAG antibodies for Bur1 loading control. B) Similar co-IP assays were carried out except that α-HA antibodies were used to pull down proteins, α-FLAG antibodies were used for probing co-precipitated Bur1, and also α-HA for the input.

3. Interaction between Bur1 and Npl3 depended on RNA partially

To further characterize the interaction between Bur1 and Npl3, we tried to identify if it was a direct interaction or mediated by other factors. Because Npl3 is an RNA-binding protein, and both Bur1 and Npl3 are recruited co-transcriptionally, we suspected that RNA may contribute to this interaction. Yeast whole-cell extracts were treated with

RNase before co-IP. As shown in FIG 4-3 (comparing lane 2 and 3 to lane 1), less Npl3 associated with Bur1 after RNase treatment. The amount of Bur1 protein in the precipitate was also probed as a loading control (lower panel); although there is a little fluctuation in lane 2, it didn't change the conclusion. Nevertheless, increasing amount of RNase didn't abolish the interaction completely. Therefore, RNA is important, but not essential for the interaction between Bur1 and Npl3.

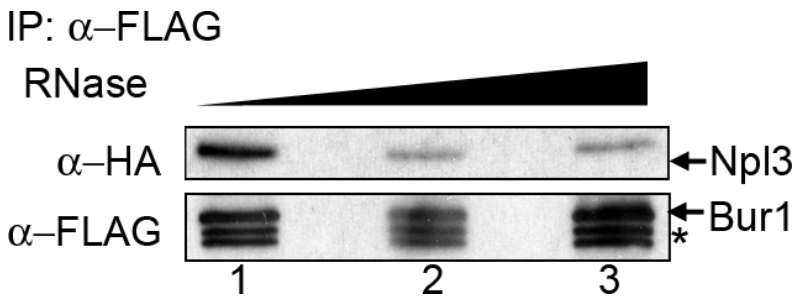

FIG 4-3 RNase treatment reduced the Bur1-Npl3 interaction. Yeast soluble extracts from cells expressing FLAG-Bur1 and Npl3-HA were pre-incubated without or with increasing amount of RNase (0, 13, and 50 ng RNase per 1 mg whole-cell extract) as described in Methods and Materials. These extracts were then subjected to α-FLAG co-immunoprecipitation assays as described in FIG 4-2A. Blots were probed with α-HA for the amount of Npl3 associated with Bur1, and also probed with α-FLAG for the amount of Bur1 as loading control.

4. The protein level of Bur1 and Npl3 was affected by the cap-binding complex (CBC)

Since RNA only contributes to the Bur1-Npl3 interaction partially, there should be other contributing factors, including direct interaction and/or other proteins which

mediate this interaction. In an affinity purification with TAP-tagged Npl3 followed by mass spectrometry analysis, Hurt and colleagues identified a set of proteins, including Cbp80 (Sto1) (large subunit of CBC) and Gbp2 (49). However, Bur1 was not included in their list of Npl3-associated proteins. Nevertheless, in a genome-wide study of protein complexes using affinity purification and mass spectrometry, Sto1 was found to attach to the Bur1/Bur2 complex (27). All these facts prompted us to propose that Sto1, which is recruited to the transcription machinery at the initial steps, probably earlier than Npl3 and Bur1, may facilitate their interaction afterwards. Therefore, I tested if deletion of Sto1 would abolish Bur1-Npl3 association by co-IP assays. First, *STO1* was deleted in a parent strain expressing FLAG-Bur1 and Npl3-HA. The parent strain already grew slower than wild-type strains, probably because the only source of Bur1 came from a *CEN* plasmid instead of its genomic locus. In combination with the growth defect caused by *STO1* deletion, the resulting strain was very sick and had a doubling time of about two days. Next, I checked the expression level of Bur1 and Npl3 in the absence of Sto1. As shown in FIG 4-4A, without Sto1, the amount of Npl3 dropped more than 27-fold. Although Bur1 was less affected, its level also reduced about 3-fold. It was possible that the transcription and translation defects caused by mutant CBC had a more profound impact on Npl3, because Npl3 is much more abundant than Bur1 and probably relies on CBC more. It was also likely that the association with CBC affected the protein stability of Npl3. Further analysis is needed to distinguish whether the loss of Sto1 caused a general

decrease of the transcriptome and the proteotome or a specific impact on a functional group of proteins. However, for my purpose of testing Bur1-Npl3 association in *sto1* mutants, the greatly reduced protein level of Npl3 and Bur1 made it difficult to compare the co-IP results with that in *STO1* strains. Nevertheless, as shown in FIG 4-4B, the amount of co-precipitated Npl3 was dramatically reduced, which can barely be detected (compare lane 3 and 4). But we cannot conclude that the Bur1-Npl3 association was disrupted because of the lack of CBC as a mediator. Instead, it might be due to the reduced protein level of the input (compare lane 1 and 2, lane 5 and 6).

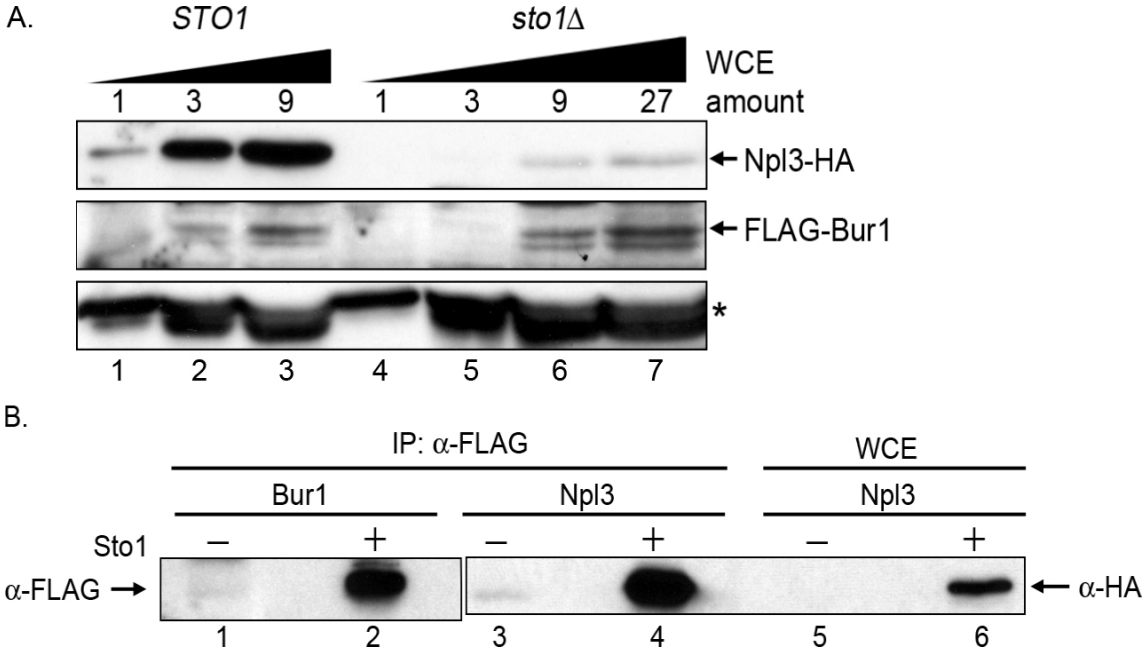

FIG 4-4 The protein levels and interaction of Bur1 and Npl3 were greatly reduced in the absence of Sto1. A) The protein levels of Bur1 and Npl3 in strains with or without Sto1. Three-fold serial dilutions of total proteins extracted from strains expressing FLAG-Bur1 and Npl3-HA with (lane 1-3) or without Sto1 (lane 4-7) were resolved by SDS-PAGE, transferred and detected with α-FLAG monoclonal antibodies (M2, Sigma^{TM}) for FLAG-Bur1 and with α-HA polyclonal antibodies (12CA5) for Npl3-HA. A band cross-reacting with α-FLAG antibodies was denoted with an asterisk and taken as a loading control. The relative amount of the whole-cell extract (WCE) was labeled on top. B) Co-immunoprecipitation assays using α-FLAG antibodies to pull down proteins from wild-type strain and *sto1*Δ mutant. Precipitates were eluted by boiling with sample buffer, resolved by SDS-PAGE and transferred, along with the WCE used for co-IP. Blots were probed with α-HA antibodies for input (lane 5 and 6) and co-precipitated Npl3 (lane 3 and 4), and also with α-FLAG antibodies for Bur1 (lane 1 and 2).

5. Interaction between Bur1 and Npl3 was not interrupted in *hmt1*Δ mutants

Next, we examined if the Bur1-Npl3 interaction depends on certain post-translational modification. Npl3 is a target of arginine methyltransferase Hmt1, which signals the nuclear export of Npl3 and the mRNA cargo (70, 145). However, it is

not clear if this methylation will affect the recruitment of Npl3 to the transcription machinery. And if the above assumption is right, Npl3 may not be in the vicinity of Bur1 for interaction. Therefore, we tested the two-hybrid interaction in an *hmt1*Δ strain background. We found equally robust interaction between LexA-Bur1 and GAD-Npl3 in the presence or absence of Hmt1 (data not shown), indicating that Hmt1, and hence arginine methylation of Npl3 was not required for its interaction with Bur1. It is likely that Npl3 was recruited co-transcriptionally when still unmethylated, interacting with Bur1, and followed by methylation before export.

6. The cyclin partner Bur2 was not required for its association with Npl3, and Npl3 was not likely to be a substrate of Bur1

Since Bur2 is required for Bur1's kinase activity (144), we wondered if Bur2 was required for the Bur1-Npl3 association as well. Initially, I tried to delete *BUR2* in a haploid strain, but could not get a viable transformant because of the severe growth defect. So I crossed two two-hybrid reporter strains L40 and AMR70, and deleted one copy of *BUR2* with *KanMX6* in the resulting diploid strain. After sporulation and tetrad dissection, I selected for Kan^r strains for the following two-hybrid assay. Although the *bur2*Δ mutant was sick and hence produced much smaller colonies for the beta-galactosidase assay, it turned blue within an hour as the big wild-type colonies did, indicating that the two-hybrid interaction between Bur1 and Npl3 does not require Bur2 or the associated kinase activity of Bur1.

Although the kinase activity was not required for this interaction, it was still possible that Npl3 was phosphorylated by Bur1 afterwards. In order to clarify this possibility, we tried to identify Bur1's substrates by kinase assay on its co-precipitates. Consistent with a previous observation (144), we also found two major phosphorylated proteins (FIG 4-5). As judged by the molecular weight, one was auto-phosphorylated Bur1 (~80 kDa) (144), and the other, which has a higher molecular weight, was likely to be Rpb1, the core Pol II subunit (76, 102). However, no band specific for phosphorylation was found migrating between 50~70 kDa, at the range of molecular weight of Npl3. Therefore, Npl3 was not likely to be a substrate of Bur1. This conclusion was confirmed by checking the phosphorylation status of Npl3 in *bur2*Δ and *bur1-8*^{ts} mutants. The lack of Bur1 kinase activity had no effect on Npl3 phosphorylation status (personal communication from Rebecca Holmes in Christine Guthrie's lab).

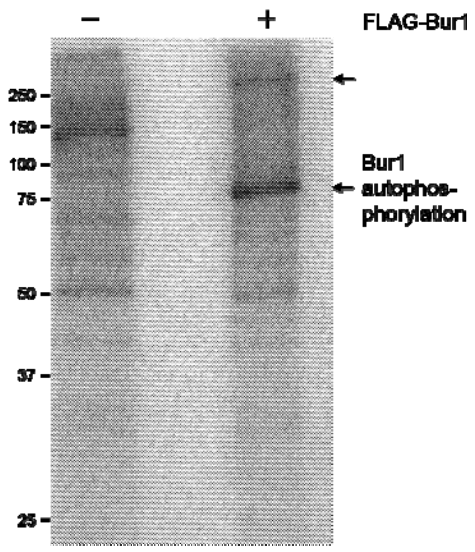

FIG 4-5 Npl3 may not be phosphorylated by Bur1. Total proteins were extracted from cells expressing FLAG-Bur1 (+) or not (–). Proteins were immunoprecipitated with FLAG-conjugated beads and washed extensively before [γ-^{32}P]ATP was added for kinase activity. An autoradiogram of the SDS-PAGE gel is shown with size marks indicated in kilodaltons on the left. The two major phosphorylated protein bands were indicated by arrows on the right.

7. There was no RNA export defect associated with *bur1-8* or *bur2*Δ mutants

In an effort to define the physiological role of the association between Npl3 and Bur1, we proposed that Npl3's function in RNA export might be regulated by Bur1. Bur1 might signal for mRNA maturation and export through its interaction with Npl3 after transition from initiation to elongation. On the other hand, in *bur1*^{ts} or *bur2* mutant strains where such signaling is not available, RNA processing cannot be coupled with transcription, and hence the severe growth defects. Therefore, we tested if there was any RNA export defect in *bur1* or *bur2* mutants in collaboration with Dr. F. Stutz's lab.

Mex67 is an mRNA transport receptor in the nuclear pore complex (NPC), which binds to mRNA and translocates mRNA cargo from nucleus to cytoplasm. In strains with mRNA export defects, such as those carrying a temperature sensitive allele of *mex67-6*, mRNA accumulates in the nucleus (FIG 4-6). However, no such accumulation was observed in Bur1 wild-type, *bur1-8*^{ts}, or *bur2*Δ mutant strains, indicating that mRNA export was not affected by the Bur1 kinase activity.

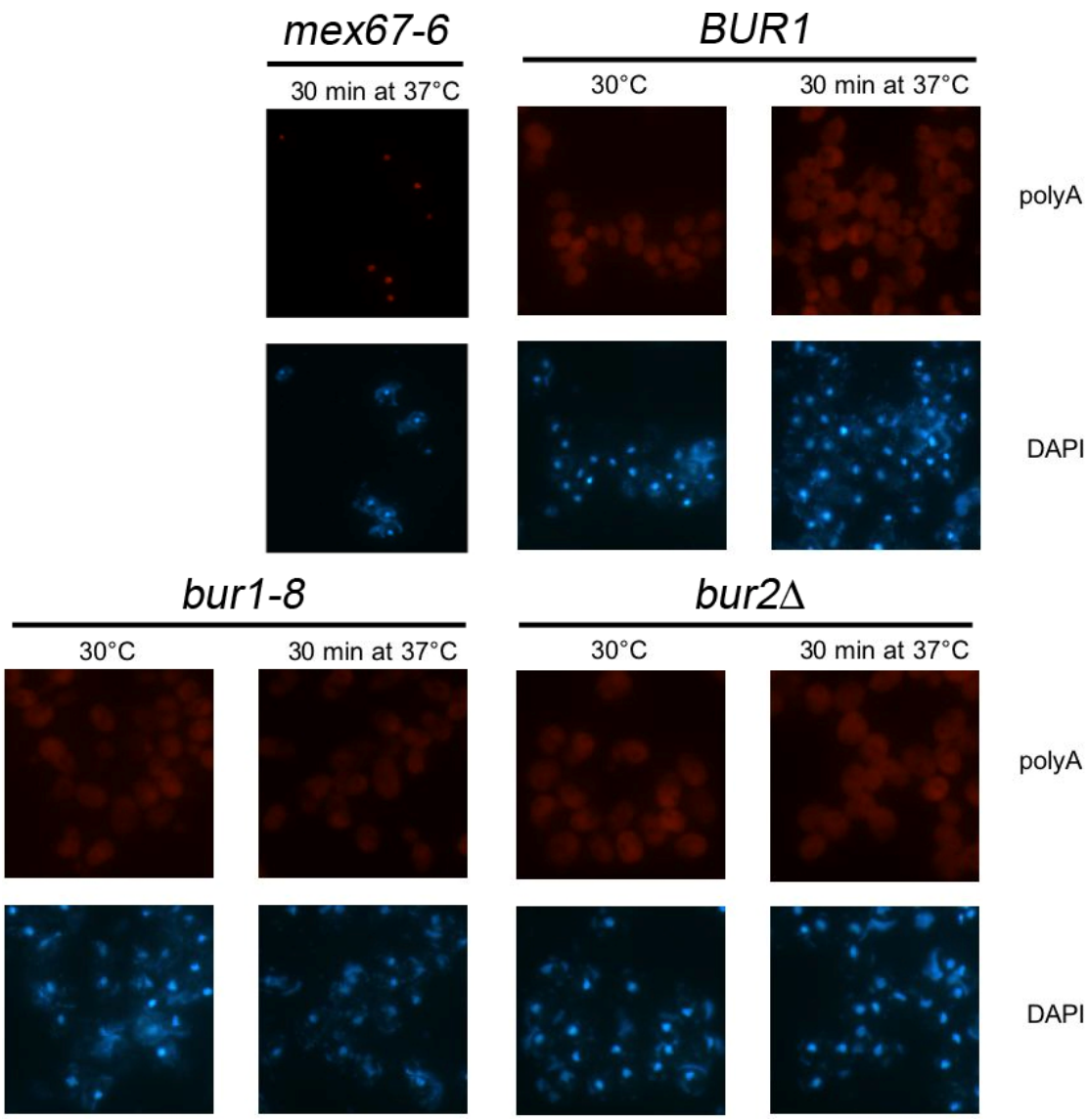

FIG 4-6 There was no mRNA export defect in *bur1-8* or *bur2*Δ mutants (data collected from F. Stutz's lab). Fluorescence *in situ* hybridization (FISH) experiments were performed as described in (35). Cells were grown in YPD until log phase before and then shifted to 37°C for 30 min for heat shock with a control group remaining at 30°C for 30min. PolyA mRNA was stained with rhodamin labeled oligo dT, while DNA was visualized by DAPI staining.

8. Screening for high copy suppressors of *bur1-8* mutation

It has been proposed that Bur1 kinase activity is required for the normal pattern of

histone methylation by Set2 (12). At non-permissive temperature, the strain with temperature sensitive *bur1-8* allele showed elevated di-methylation but reduced level of mono- and tri-methylation of histone H3 K36, which was suggested to lead to the lethality (12). Based on these observations, we performed a screen for a high copy suppressor of the lethality of *bur1-8* mutants. Since deletion of *SET2* reversed the defects caused by lack of Bur1 kinase activity, we rationalized that the hypothetical suppressor may function as a demethylase.

The screening for a high-copy suppressor was carried out as the flowchart shown in FIG 4-7. At the non-permissive temperature, *bur1-8* mutation was lethal. We transformed *bur1-8* mutants at an intermediate temperature (30^{o}C) with a library of 2 micron plasmids. Each library plasmid had a genomic insert fragment of 6~10 kb. The transformants were selected for revertants growing at 37^{o}C. The revertants went through two rounds of selection. First, they were tested whether or not it was the library plasmid, rather than genomic mutations, that caused the suppression. Upon losing the plasmid, they should not grow at non-permissive temperature. Second, the plasmid suppressing *bur1-8* lethality by over-expressing full-length Bur1 were excluded. Finally, three clones were identified, among which two carried different genomic fragments containing Bur2 and one encoded about 3/4 of Bur1 to the C-terminus. Therefore, we didn't identify a demethylase as initially expected. Instead, we found that over expressing Bur2 or the 75% C-terminal Bur1 suppress the lethality of *bur1-8* mutation.

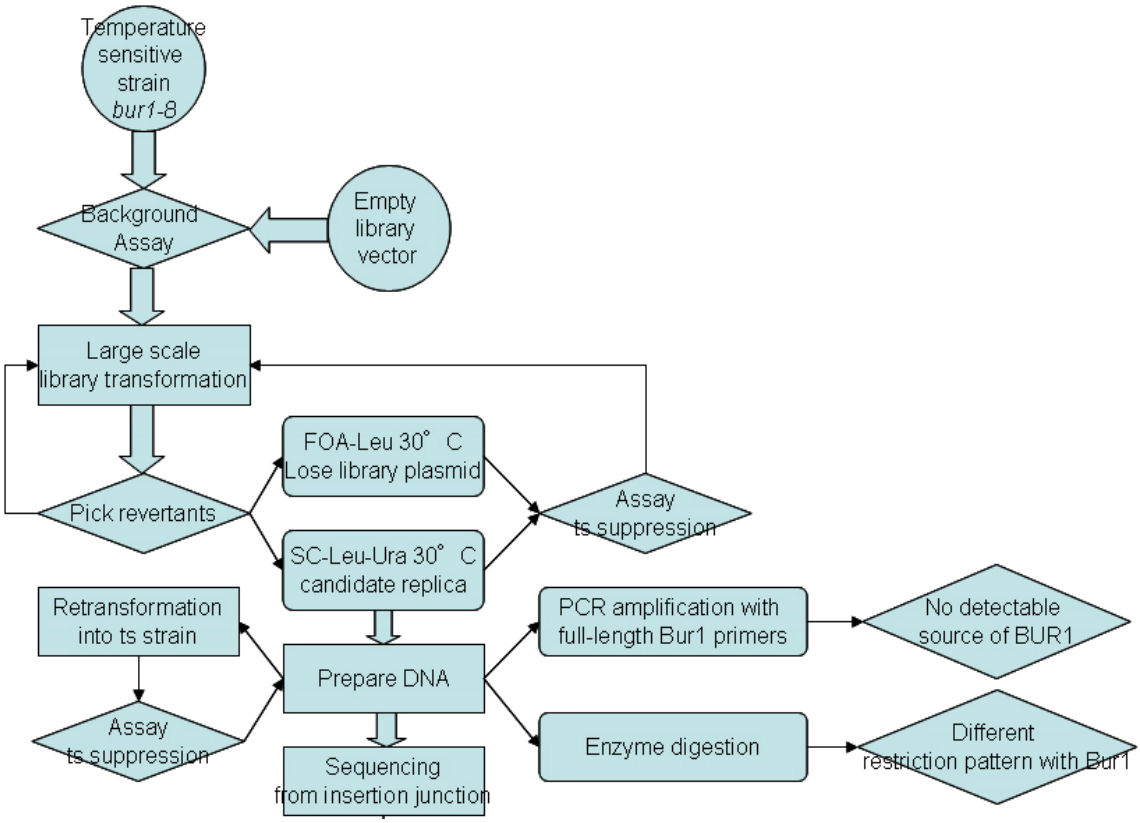

FIG 4-7 Screening for high-copy suppressors of *bur1-8* temperature sensitivity. Initially, empty library vector (yEP24) was checked and could not suppress *bur1-8* lethality. Then, the high-copy library was transformed into *bur1-8* mutants and revertants grown at 37°C were selected. In order to confirm that the suppression was due to the plasmid, revertants were re-streaked onto FOA-containing media to lose the library plasmid which carried a *URA3* marker and tested for temperature sensitivity. Plasmids from the revertants were isolated and checked that the repression was not caused by expressing full-length Bur1. PCR with Bur1 specific primers and restriction map generated by Pst1 digestion were utilized to exclude candidates with a full-length Bur1 insert. The final candidates were retransformed into *bur1-8* to confirm their growth at 37°C and then sent for sequencing.

III. Discussion and future directions

In order to study the underlying mechanism of Bur1's role in transcription, we searched for its interacting partners with a two-hybrid screen. Using full-length Bur1 as the bait, we identified two new interacting factors, Npl3 and Gbp2. Both are SR-like

proteins, containing two conserved RRMs and an SR-rich region. Based on the fusion fragments identified by the two-hybrid screen and our later finding that RNA contributes to this interaction, it is likely that RRMs mediate the interaction with Bur1. It will be interesting to delineate the interacting regions on each protein in the future. As for the Bur1-Npl3 interaction, we also confirmed it under physiological conditions by co-IP assays, but couldn't prove that for Bur1-Gbp2 co-IP, whose signal might be masked by that from the heavy chain of the antibody migrating with a similar molecular weight (FIG 4-2). A different immunoblotting strategy could be used, such as using a secondary antibody that doesn't recognize denatured heavy chain to avoid the masking signal.

Next, we tried to characterize the contributing factors of this interaction. RNA was found to be important for this interaction (FIG 4-2). Although Npl3 contains two RNA recognition motifs, there is no RNA binding domain found in Bur1. So it is likely that another RNA binding protein mediated this interaction. For example, the RNA polymerase II recruits Bur1 to the transcription machinery; meanwhile, Npl3 associates with the nascent transcripts. Thus, Bur1 and Npl3 reside in the same complex as mediated by RNA and polymerase II.

On the other hand, since RNase treatment cannot totally abolish the Bur1-Npl3 interaction, protein-protein interactions without RNA should also be involved. Bur1 and Npl3 may interact directly or through another factor. To address the issue of direct interaction, recombinant Bur1 and Npl3 need to be expressed, purified and tested by an *in*

vitro pull-down assay. As for possible protein mediators, Pol II again serves as a candidate, because both Bur1 and Npl3 can interact with Pol II directly. However, Bur1 bound to Ser5P-CTD peptides, not to Ser2P peptides (102); while Npl3 acted the opposite way (17), raising the question if these associations with different status of Pol II CTD can mediate the Bur1-Npl3 interaction. It might be answered by the aforementioned *in vitro* pull-down assay, testing if addition of Ser5P and/or Ser2P-CTD peptide can increase the Bur1-Npl3 interaction. Another candidate is Sto1 (Cbp80), the large subunit of the cap-binding complex (CBC). Sto1 is also co-transcriptionally recruited and co-purifies with Npl3 and Bur1 (27, 49). We found that the interaction between Bur1 and Npl3 can barely be detected in a *sto1* null mutant. However, another explanation is the great reduction in the protein amount of Npl3 and Bur1 caused by the deletion of *STO1* (FIG 4-4). So the effect of Sto1 on Bur1-Npl3 interaction need to be tested again with co-IP assays that pull down equal amount of FLAG-Bur1 with or without Sto1. In summary, except for RNA, it is still not clear if it is direct interaction or other proteins that mediated Bur1-Npl3 association.

Because Bur1 is a kinase and the function of Npl3 is regulated by the interplay of its phosphorylation and methylation, we also tested if certain post-translational modification would affect this interaction, or vice versa. We found that methylation of Npl3 was not required for this interaction as shown by the two-hybrid assay. It will be interesting to analyze if the methylation of Npl3 occurs downstream of its association with Bur1 or

upstream. As for the possible involvement of phosphorylation, neither did the Bur1-Npl3 interaction require the kinase activity of Bur1, nor did it change the phosphorylation status of Npl3 (FIG 4-5 and personal communication with R. Holmes and C. Guthrie).

In addition to the investigation of contributing factors of the Bur1-Npl3 association, we tried to figure out its physiological functions. Bur1 is well-documented for its role in early stages of transcription, i.e. promoting the transition form initiation to elongation; while Npl3 is often found important in later stages and coupling transcription to mRNA maturation and export. The association of these two proteins might be important for coordinating different stages and events of transcription. We proposed that Bur1, which phosphorylates Pol II for its full capacity to produce mRNA in elongation, signals for the downstream processing and export of mRNA through its interaction with Npl3 at the same time. Therefore, we tested if there was defective RNA export in strains lacking Bur1 kinase activity, but the results were negative (FIG 4-6). However, our proposed model may still be true because lack of Bur1 kinase activity cannot abolish its association with Npl3, and thus might not block the putative downstream function. A better way to test our model is to use a viable *bur1* mutant which cannot interact with Npl3, rather than lacking the kinase activity, and test for its phenotype for RNA export. To acquire such a mutant, we need to have better knowledge about the critical residues for the Bur1-Npl3 interaction.

Finally, we tried to understand the effect of Bur1 kinase activity on the methylation

pattern of histone H3 K36 generated by Set2. We hypothesized that there was a histone demethylation involved in the said pathway, and searched for it as a high-copy suppressor of *bur1-8* temperature sensitivity (FIG 4-7). However, the only high-copy suppressors we identified were Bur2 and the C-terminus of Bur1. With the recent discovery of histone demethylases for different methylation status of histone H3 K36, i.e. Rph1, Jhd1, Gis1 (66, 131-132), we should check if their over-expression can suppress *bur1-8* lethality to validate the assumption of our screen.

In summary, we have identified Bur1-Npl3 interaction and tested several possible contributing factors. Among them, RNA was found important for this interaction. Further investigation is needed before we can have a clear understanding of its function.

Chapter Five: Conclusions and Future Directions

I. The S phase requirement for establishment of silencing

I have demonstrated a difference in the S phase requirement for establishment of silencing at *HML* and *HMR*. While silencing cannot occur at the *HMR* locus without passage through S phase (22, 63-64, 69, 72, 85, 92) (FIG 2-1C), it can be established to a significant extent at the *HML* locus under the same conditions (FIG 2-1D). In order to understand this difference, several chimeric HM loci were tested (FIG 2-2, 3, 5) and the strength of the promoters at these loci (FIG 2-4) was identified as the determinant of the S phase requirement. To silence a locus with a strong promoter such as the **a***1*/**a***2* promoter required passage through S phase, while HM loci with weaker promoters such as the α*1*/ α*2* or *TRP1* promoter did not show this requirement. Thus, transcriptional activity counteracts the establishment of silencing but can be overcome by passage through S phase.

Therefore, we proposed two different S phase events which may facilitate the spreading of the Sir complex and allow it to overcome the competition from transcription. One is an S-phase dependent post-transcriptional modification of a Sir protein or a histone that would strengthen the association between the Sir complex and the

nucleosomes. The other could be the histone synthesis and deposition during S phase, which may facilitate silencing by providing more binding surface for the Sir complex. It will be very interesting to investigate these hypotheses and to discover the nature of the S phase requirement.

In order to provide some additional support to our conclusion that the transcription units, rather than the flanking silencers, dictate the need of the proposed S phase events, I'm currently constructing a hybrid *HMR*α locus where the α1/α2 transcription unit from *HML* locus is now flanked by the *HMR* silencers. This is a reciprocal experiment of that with a hybrid *HML***a** locus (FIG 2-2). If silencing can be established at this hybrid *HMR*α locus as it is at *HML* or at another hybrid *HMR* locus with a weak transcription unit (*hmr::TRP1*), it will further rule out the possibility that *HMR* silencers may influence the S phase requirement.

Another remaining question is how the amount of silencing observed for the population relates to that of the individual cell. When we observe a significant decrease in the amount of RNA as a measure of silencing, is it because a portion of the cells were fully silenced or because the entire population was partially silenced? To address this question, we need to insert a fluorescent reporter gene (e.g. GFP) into either *HM* locus and examine silencing by monitoring the fluorescent signal from individual cells by flow cytometry. Importantly, we can destabilize the fluorescent reporter proteins by fusion with a PEST-rich domain from the G1 cyclin Cln2, so as to observe dynamic changes in

expression.

II. The role of Sir1 in transcriptional silencing

My study clarified the importance of the Sir1 N-terminal domain, especially in some compromised silencing background (FIG 3-1). It contributes to silencing by stabilizing the full-length Sir1 protein (FIG 3-2). Moreover, the Sir1 N-terminal domain has high sequence similarity to the Sir1 C-terminal domain (14, 46) (FIG 1-8), and thus was predicted to have a tertiary structure homologous to the known C-terminal one (46) (FIG 3-4). As predicted, the Sir1 N-terminal domain did show a weak interaction with the Sir3 BAH domain (FIG 3-5). This interaction may allow Sir1 to function directly through strengthening the Sir3-nucleosome association, thus contributing to BAH silencing and the suppression of severe silencing defects. We also propose that the Sir1 N-terminal domain serves in a "scanning" mode to recruit Sir1 to the *HM* loci, followed by switching to the tight association with the Orc1 BAH domain through the Sir1 C-terminal domain. Further investigation is needed to figure out the underlying mechanism of $Sir1^{N}$ function.

Another part of this project dealt with the controversial localization of Sir1 on the silent chromatin (93, 147). I found that over-expressing Sir1 did not change the restricted localization of Sir1, which did not spread into the internal region of silent chromatin (FIG 3-6). Therefore, association with the *HMR-E* silencer is necessary and sufficient for Sir1 to carry out its functions at the *HMR* locus. Finally, I have delineated the Sir1-Sir4

interaction and found two Sir4 N-terminal domains important for this interaction (FIG 3-7).

III. The Bur1-Npl3 interaction

Through a two-hybrid screening, I found that Bur1 binds to two related proteins, Npl3 and Gbp2. Later, the Bur1-Npl3 interaction was confirmed by co-IP assays (FIG 4-2). RNA plays an important role in this interaction, but there should be other contributing factors (FIG 4-2). It will be important to test if Bur1 interacts with Npl3 directly, or if they interact indirectly through proteins other than RNA. Our analysis showed that the methylation status of Npl3 did not affect this interaction and neither did the kinase activity of Bur1. In addition, we tested two possible functions of this interaction: one is that Npl3 associates with Bur1 to get phosphorylated; the other is that Bur1 couples mRNA maturation and export to transcription elongation through its interaction with Npl3. However, our current results do not support these hypotheses (FIG 4-5, 4-6). Further tests and other possibilities should be investigated.

Chapter Six: Material and Methods

Yeast Strains and plasmids

Strains used in this study are listed in Table 1. Gene replacements were performed as described (77, 115), and were confirmed by PCR analysis for deletion, and sequenced for tagging and replacement in addition. In the case where a phenotype should be generated by the replacement, the resulting strain was checked for that phenotype.

Transformation of all yeast strains was performed by the standard lithium acetate method (116), except that an additional one-hour grow out period in yeast extract-peptone-dextrose medium (YPD) prior to plating was included for two-hybrid screening transformations.

A list of plasmids used and details on their construction can be found in Table 2.

Cell cycle synchrony

Cultures were generally incubated at 30^{o}C, except for those with temperature-sensitive alleles. For cell cycle synchrony, strains with the sir3-8 mutation (RS1230, RS1231, JRY19, JRY25, and JRY27) were all grown to early log-phase in YPD before synchronizing with 0.2 M hydroxyurea (HU) at non-permissive temperature, except that for JRY25, 150 nM alpha factor (αF) was used instead of HU to synchronize into late G1

phase, ahead of early S phase. Another exception is that for JRY27, before the final synchrony achieved by HU, αF was used for a better outcome. To restore silencing, strains were shifted back to permissive temperature either with HU (or with αF for JRY25) to prevent passage through S phase, or released into fresh YPD to allow for cell-cycle progression. Samples were taken at 1 hour intervals and subjected to DNA and RNA measurements.

Flow cytometry

Flow cytometry analysis of DNA content was performed as described (36). Briefly, cells were harvested and fixed with 70% ethanol. After sonication, cells were treated with RNaseA (Sigma) and pepsin (Sigma). Samples were stained with CYTOX Green (Invitrogen) and analyzed on a FACScan using Cell Quest Pro software (BD).

RT-PCR

Total RNA was extracted with a RiboPure™ – Yeast Kit (Applied Biosystems) followed by treatment with RNase-free DNase (Applied Biosystems). RT-PCR was performed with SuperScript™ II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions.

Real-time PCR and quantification

Real-time PCR was performed with a LightCycler® 480 SYBR Green I Master kit (Roche) according to recommended conditions. Experiments were conducted and analyzed in a Mastercycler® ep realplex^2 thermal cycler (Eppendorf) according to

manufacturer's instructions. Primer sequences for **a***1*, α*1*, *ACT1*, *18S*, *yEmRFP* are included in Table 3. For each set of samples, the RNA level at the 0 h time point, normalized to either an *ACT1* or *18S* internal control, was set to 1.0 and RNA levels from subsequent time points were normalized relative to the initial state. For comparison of the RNA level from derepressed *HMR***a***1* and *HML*α*1*, the relative amount of HMR**a**1 and HMLα1 RNA was measured by comparing to the corresponding locus in genomic DNA isolated from JRY30, which only contains one copy of each transcription unit. The relative amount of HMR**a**1 RNA was set to 1.0. For measurement of promoter strength, the RNA level of *yEmRFP* was normalized to the *ACT1* internal control, and the relative amount of transcript from **a***1* promoter was set to 1.0.

***hmr::TRP1* reporter assays**

A *sir1* null mutant strain with *hmr::TRP1* reporter (JCY36) was transformed with the following plasmids: full-length Sir1 with C-FLAG$_3$ tag (2μ Sir1^{FL}, pJR52, *SIR1-G$_8$-FLAG$_3$-T$_{SIR1}$*), sir1$^{127\text{-}654}$ with C-FLAG$_3$ tag (2μ sir1$^{\Delta N}$, pJR55, *sir1*$^{127\text{-}654}$*-G$_8$-FLAG$_3$ -T$_{SIR1}$*), and vector control (pRS316). Serial 10-fold dilutions were plated on SC-Ura medium to monitor growth and on SC-Ura-Trp medium to monitor silencing at the *hmr::TRP1* locus. All plates were grown for 3 days at 30^{o}C.

Semi-quantitative mating assays

For mating assays in *MAT***a** *sir3-8^{ts}* background (RS1232), cells transformed with the indicated plasmids were grown in SC-Ura medium at the non-permissive temperature

(37°C). Serial 10-fold dilutions were spotted onto a lawn of tester strain (DC17 *MAT*α) for mating at the permissive (24°C) and non-permissive temperature (37°C) for 5 h, followed by replica to SD plates and incubation at the indicated temperatures to measure silencing at *HML* locus.

BAH silencing assays

A *sir3* null mutant strain (JCY3) was co-transformed with LexA-Sir3^{BAH} (pJC14) and either one of the indicated plasmids. Serial 10-fold dilutions were spotted onto a lawn of tester strain (DC17 *MAT*α) for mating at 30°C for 20 h, followed by replica to SD plates and incubation for 2 d to measure BAH silencing at *HML* locus.

Recombinant protein purification

Sir1$^{1-136}$-His$_6$ was expressed from pJR70 in BL21 (DE3) codon$^+$ cells. When culture was grown at 37°C to an A_{600} of ~0.4, protein expression was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at room temperature. Cells were harvested by centrifugation, frozen at -80°C, resuspended at 4°C in a binding buffer [20 mM Tris-HCl (pH 8), 500 mM NaCl, and 5 mM imidazole] and lysed by sonication. The cell lysate was cleared by centrifugation, then loaded onto the Ni-agarose resin (His Bind Kit, Novagen), and washed extensively with the binding buffer containing 60 mM imidazole. Proteins were eluted with the binding buffer containing 150 mM imidazole and dialyzed against a buffer containing 50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 1 mM EDTA, and 10% glycerol. Protein concentrations were estimated by comparing

Coomassie Blue staining of samples to BSA standards, as well as quantitated with Bradford assays (Bio-Rad).

Sir3$^{1-253}$-GST, Sir3$^{1-219}$-GST and GST alone were expressed from pPY109, pEP14 and pJC82, respectively. When culture was grown at 37^{o}C to an A_{600} of ~0.4, protein expression was induced by the addition of 1 mM IPTG for 3 h at room temperature. The GST-tagged proteins and GST alone were purified by using glutathione Sepharose 4 fast flow resin (GE Healthcare), following the manufacturer's instructions. Purified proteins were dialyzed against a buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, and 10 % glycerol. Protein concentrations were estimated by comparing Coomassie Blue staining of samples to BSA standards, as well as quantitated with Bradford assays (Bio-Rad).

GST pull down assays

The purified GST-tagged proteins or GST alone was incubated with 60 μl of packed glutathione beads for 0.5 h at 4^{o}C in an assay buffer containing 50 mM Tris-HCl (pH 8), 1 mM EDTA, and 150 mM NaCl. The beads were spun at 3,000 rpm for 1 min, and the supernatant removed. Purified Sir1$^{1-136}$-His$_6$ protein of the indicated molar ratio was added to these beads bound with GST-tagged proteins, GST, or beads alone, and incubated in the assay buffer for 1 h at 4^{o}C with rocking. The beads were spun down again, and the supernatant removed by using a syringe with a 30-gauge needle. The beads were washed twice with 300 μl of wash buffer [50 mM Tris-HCl (pH 8), 1 mM EDTA,

300 mM NaCl, and 0.05% NP-40]. The bound proteins were released by boiling in 1X SDS-gel loading buffer. A fraction of the bound proteins were analyzed on a 12% SDS-PAGE gel, transferred and immunoblotted, using anti-His antibody (Santa Cruz) as the primary antibody and anti-rabbit immunoglobulin G-horseradish peroxidase as the secondary antibody, and visualized by using an ECL Plus system (GE Healthcare). Another fraction of the bound proteins were also analyzed on a 12% SDS-PAGE gel, and stained by Coomassie blue staining to visualize the GST-tagged proteins and GST control.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed from strains JRY12 with or without pJR50 and RS1230 cultured at 24^{o}C and 30^{o}C as described before (73). Briefly, cell extracts from cross-linked cultures were sonicated for 18 cycles of 10 s each in an Ultrasonics, Inc., sonicator (model W220-F). 25 μl anti-FLAG M2 affinity gel (Sigma A2220), or 1 μg anti-histone H3 antibody (Abcam ab1791) followed by incubation with protein A agarose beads (Roche) was used to immunoprecipitated 2.5 mg of total protein extract. Input and immunoprecipitated DNA samples were analyzed real-time PCR using a Mastercycler® ep realplex^{2} thermal cycler and a LightCycler® 480 SYBR Green I Master kit (Roche) as described earlier in this chapter. The primer used were listed in Table 3. The result was analyzed and presented as described in the figure legends.

Co-immunoprecipitation (co-IP) assays

For the Bur1-Npl3 interaction, strain JRY5 and JRY7 were used; for the Bur1-Gbp2 interaction, strain JRY6 was tested. Cultures were grown to an A_{600} of 1.0. 50 ml of cells were pelleted and washed with 10 ml of PBS. Cells were resuspended in 300 µl IP lysis buffer [50 mM HEPES-KOH (pH 7.5), 10 mM $MgCl_2$, 150 mM KCl, 0.1 mM EDTA, 10% glycerol, 0.1% NP-40, and protease inhibitors]. Cells were lysed with glass beads for 8 cycles of 30 s each in a mini bead-beater at 4^{o}C. The extract was recovered from the glass beads and spun at 13,200 rpm in a microcentrifuge for 20 min at 4^{o}C to clarify the extract. The Bradford assay (Bio-Rad) was performed on extracts to determine the protein concentration. 3 mg of protein was added to a total volume of 500 µl IP buffer [25 mM HEPES-KOH (pH 7.5), 12.5 mM $MgCl_2$, 150 mM KCl, 1 mM EDTA, 0.1% NP-40, and protease inhibitors] and incubated at 4^{o}C with rotation over-night with anti-FLAG M2 affinity gel (Sigma A2220) to pull down FLAG-Bur1, or with anti-HA polyclonal antibodies (12CA5) followed by addition of protein A agarose (Roche) to pull down Npl3-HA. Beads were spun down at 3,000 rpm for 1 min at 4^{o}C and supernatant was removed using a syringe with a 30-gauge needle. Beads were then washed in 15 ml cold IP buffer for 4 times of 15 min each. The precipitated proteins were released by boiling in 1X SDS-gel loading buffer. A fraction of the bound proteins were analyzed on a 10% SDS-PAGE gel, transferred and immunoblotted. FLAG-Bur1 was detected by using anti-FLAG antibody (Sigma, M2) as the primary antibody and anti-mouse immunoglobulin G-horseradish peroxidase as the secondary antibody, and visualized by

using an ECL Plus system (GE Healthcare). Npl3-HA or Gbp2-HA was detected by using anti-HA polyclonal antibody (12CA5) as the primary antibody and anti-rabbit immunoglobulin G-horseradish peroxidase as the secondary antibody, and visualized by using an ECL Plus system (GE Healthcare).

For co-IP assays with RNA treatment, whole cell extracts were incubated with indicated amount of RNase A for 30 min at the room temperature. Afterwards, immunoprecipitation was carried out similarly as described.

***In vitro* kinase assays**

Anti-FLAG immunoprecipitation assays were performed as described in this chapter and *in vitro* kinase assays were carried as described in (144). Briefly, precipitates bound to anti-FLAG M2 affinity gel (Sigma) were washed and incubated in 30 μl of kinase buffer [25 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 0.1% Tween 20, and 1 μCi of [γ-^{32}P]ATP] for 30 min at 30^{o}C. Proteins were separated by 7.5% SDS-PAGE and dried, and ^{32}P-labeled products were detected by autoradiography.

Two-hybrid analysis

Screening was performed essentially as described in (42, 126). Two-hybrid interactions were screened by growth on medium selective for plasmid transformed and on the same medium lacking histidine for *HIS3* reporter, and then assayed for production of beta-galactosidase. L40, which contains LexA operator sequences upstream of *HIS3* and *LacZ* reporter genes, was used as the reporter strain in all cases unless otherwise

stated. All bait plasmids were assayed for auto-activity by co-transformation with pGAD424. The yeast genomic library screened was constructed by James *et al.* (52). Direct two-hybrid assays were performed as described above, except instead of a library, the specific GAD fusion plasmid was transformed. Growth was selected on medium selective for plasmids transformed as well as the same medium lacking histidine. Beta-galactosidase assays were performed on both.

Table 1 Yeast strains used in this study

Strain	Genotype[a]	Source
W303-1a	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein
L40	*MAT***a** *his3Δ200 trp1-901 leu2-3, 112 ade2 lys2-801am ura3 LYS2:(lexAop)$_8$:HIS3 URA3::(lexAop)$_8$:lac*	(42)
AMR70	*MATα his3Δ200 trp1-901 leu2-3, 112 ade2 lys2-801am ura3 LYS2:(lexAop)$_8$:HIS3 URA3::(lexAop)$_8$:lac*	R. Sternglanz
RS3	*HMLα mat::LEU2 hmr::TRP1 trp1-1 leu2 ura3 his3 can 1-100 ade2-1*	R. Sternglanz
RS547	*HML***a** *MAT***a** *HMR***a** *leu2-1 can1-100 met trp1-1 his3, his4, ade2-1*	R. Sternglanz
RS1230	*MATα sir3-8 ade2 trp1-1 ura3 leu2 his3 his4*	R. Sternglanz
RS1231	*MAT***a** *sir3-8 ade2 trp1-1*	R. Sternglanz
RS1232	*MAT***a** *sir3-8 ade2 trp1-1 ura3 leu2 his3 his4 lys2*	R. Sternglanz
RS2075	MAT**a** *bur1::TRP1 ura3 trp1 leu2 hisΔ4-912 lysΔ2-128 suc2Δuas* [Amp^r *CEN LEU2 FLAG-BUR1*]	G. Prelich
RS2076	MAT**a** *bur1::TRP1 ura3 trp1 leu2 hisΔ4-912 lysΔ2-128 suc2Δuas* [Amp^r *CEN LEU2 FLAG-bur1-8^{ts}*]	G. Prelich

RS2139	MAT**a** *trp1 hisΔ4-912 lysΔ2-128 suc2Δuas*	G. Prelich
RS2141	MAT**a** *trp1 hisΔ4-912 lysΔ2-128 suc2Δuas ura3-52 bur2Δ::URA3*	G. Prelich
JCY3	W303-1a *MAT**a** sir3Δ::kanMX6*	J. Connelly
JCY36	RS3 *sir1Δ:: kanMX6*	J. Connelly
XRY19	RS3 *sir3Δ::kanMX6*	X. Wang
JRY5	RS2075 *NPL3-HA$_3$*	This study
JRY6	RS2075 *GBP2-HA$_3$*	This study
JRY7	JRY5 *sto1::URA3*	This study
JRY8	L40 *hmt1::kanMX6*	This study
JRY9	L40 *bur2Δ::kanMX6*	This study
JRY11	RS3 *SIR1-FLAG$_3$*	This study
JRY12	W303-1a *SIR1-FLAG$_3$*	This study
JRY17	*HML**a** mat::LEU2 hmr::TRP1 sir3Δ::kanMX6 bar1Δ::S.p.his5$^+$ trp1-1 leu2 ura3 his3 can 1-100 ade2-1*	This study
JRY19	*HML**a** mat::LEU2 hmr::TRP1 sir3-8 bar1Δ::S.p.his5$^+$ trp1-1 leu2 ura3 his3 can 1-100 ade2-1*	This study
JRY25	*HML**a** mat::LEU2 hmr::TRP1 sir3-8 bar1Δ::S.p.his5$^+$ trp1Δ::kanMX6 leu2 ura3 his3 can 1-100 ade2-1*	This study

JRY27	*HML-P***a** *mat*::*LEU2 hmr*::*TRP1 sir3-8 bar1*Δ::*S.p.his5*$^{+}$ *trp1*Δ::*kanMX6 leu2 ura3 his3 can 1-100 ade2-1*	This study
JRY30	*HML*α *mat::kanMX6 HMR***a** *sir3-8 ade2 trp1-1 ura3 leu2 his3 his4*	This study

[a] *S.p.his5*$^{+}$, *Schizosaccharomyces pombe his5*$^{+}$ gene.

Table 2 Plasmid list

Name	Construct	Vector	Insert	Remarks
pJR50	$Sir1^{FL}$	yEp352	P_{SIR1}- *SIR1-FLAG*$_3$-T_{ADH1}	Amp^r *URA3 2*μ
pJR52	$Sir1^{FL}$	yEp352	P_{SIR1}-*SIR1-G*$_8$-*FLAG*$_3$-T_{SIR1}	Amp^r *URA3 2*μ
pJR55	$sir1^{\Delta N}$	yEp352	P_{SIR1}-*sir1*127-654-*G*$_8$-*FLAG*$_3$-T_{SIR1}	Amp^r *URA3 2*μ
pES13b	$Sir1^{FL}$	yEP352	P_{SIR1}-*SIR1*-T_{SIR1}	Amp^r *URA3 2*μ
pJR66	*HML-P***a**	pRS306	*HML* region (Chr III 10025-14984) with Yα region (Chr III 12944 to 13244) substituted with *HMR* sequences from the Y**a** region (Chr III 293734 to 293819)	
pJR67	$P_{\mathbf{a}1}$– *yEmRFP*	yEPGAP-Cherry	$P_{\mathbf{a}1}$-*yEmRFP*	Amp^r *URA3 2*μ
pJR68	$P_{\alpha 1}$– *yEmRFP*	yEPGAP-Cherry	$P_{\alpha 1}$-*yEmRFP*	Amp^r *URA3 2*μ
pJR69	P_{TRP1}– *yEmRFP*	yEPGAP-Cherry	P_{TRP1}-*yEmRFP*	Amp^r *URA3 2*μ
pJR70	$Sir1^{136}$-His_6	pET23a	P_{T7}-$SIR1^{1-136}$-HIS_6	Amp^r
pEP14	$Sir3^{219}$-GST	pET28a	$P_{T7(lac)}$-$SIR3^{219}$-*GST*	Kan^r
pPY109	$Sir3^{253}$-GST	pET28a	$P_{T7(lac)}$-$SIR3^{253}$-*GST*	Kan^r
pJC82	GST	pET28a	*GST*	Kan^r
pTT44	LexA-$Sir1^{322-654}$	pSTT91	P_{ADH1}-LexA-$Sir1^{322-654}$	Amp^r *TRP1 2*μ
pJR23	GAD-$Sir4^{1-360}$	pGAD424	P_{ADH1}-GAD-$Sir4^{1-360}$ into BamHI-SalI sites	Amp^r *LEU2 2*μ
pEP30	GAD-$Sir4^{1-261}$	pGAD424	P_{ADH1}-GAD-$Sir4^{1-261}$ into BamHI-SalI sites	Amp^r *LEU2 2*μ
pEP31	GAD-$Sir4^{91-360}$	pGAD424	P_{ADH1}-GAD-$Sir4^{91-360}$ into BamHI-SalI sites	Amp^r *LEU2 2*μ
pEP32	GAD-	pGAD424	P_{ADH1}-GAD-$Sir4^{91-261}$ into	Amp^r

	Sir4$^{91-261}$		BamHI-SalI sites	*LEU2 2μ*
pEP34	GAD-Sir4$^{262-360}$	pGAD424	P_{ADH1}-GAD-Sir4$^{262-360}$ into BamHI-SalI sites	Amp^{r} *LEU2 2μ*
pEP35	GAD-Sir4$^{91-309}$	pGAD424	P_{ADH1}-GAD-Sir4$^{91-309}$ into BamHI-SalI sites	Amp^{r} *LEU2 2μ*
pEP36	GAD-Sir4$^{164-309}$	pGAD424	P_{ADH1}-GAD-Sir4$^{164-309}$ into BamHI-SalI sites	Amp^{r} *LEU2 2μ*
pEP37	GAD-Sir4$^{164-360}$	pGAD424	P_{ADH1}-GAD-Sir4$^{164-360}$ into BamHI-SalI sites	Amp^{r} *LEU2 2μ*
pEP38	GAD-Sir4$^{91-164}$	pGAD424	P_{ADH1}-GAD-Sir4$^{91-164}$ into BamHI-SalI sites	Amp^{r} *LEU2 2μ*
pJR02	LexA-Bur1	pSTT91	P_{ADH1}-LexA-Bur1 into BamHI-SalI sites	Amp^{r} *TRP1 2μ*
pJR01	GAD-Npl3	pGAD424	P_{ADH1}-GAD-Npl3$^{63-415}$ from library	Amp^{r} *LEU2 2μ*
pJR04	GAD-Gbp2	pGAD424	P_{ADH1}-GAD-Gbp2$^{22-339}$ from library	Amp^{r} *LEU2 2μ*
pJR10	GAD-Ctk2	pGAD424	P_{ADH1}-GAD-Ctk2 into BamHI-SalI sites	Amp^{r} *LEU2 2μ*

Table 3 Primer list

Name	Locus*	Coordinates	Sequence
JRP53	α*1* F	Chr III 13313-13336 or 200469-200492	AGAACAAAGCATCCAAATCATACA
JRP54	α*1* R	Chr III 13422-13399 or 200578-200555	GAGTGGTCGAATAATATTGAAGCA
JRP93	*ACT1* exon1 F	Chr VI 54707-54686	ACTGAATTAACAATGGATTCTG
JCP122	*ACT1* exon2 R	Chr VI 54256-54275	CATGATACCTTGGTGTCTTG
YY43F	*RDN18-1* F	Chr XII 456332-456315	GCCGATGGAAGTTTGAGG
YY43R	*RDN18-1* R	Chr XII 456083-456106	TACTAGCGACGGGCGGTGT
JRP102	**a***1* exon 2 F	Chr III 294112-294129	CAATATCACCCCAAGCAC
JRP103	**a***1* exon 2-3# R	Chr III294302-294287 + 294235-294226	CGTTTATTTATGAAC CAAACTCTTA

JRP132	*yEmRFP* F		AACTATGGGTTGGGAAGC
JRP133	*yEmRFP R*		CACCTGGTAATTGAACTG
JRP47	*HMR-E F*	Chr III 292695 – 292715	TGCAAAAACCCATCAACCTTG
JCP113	*HMR-E R*	Chr III 292922 - 292903	ACCAGGAGTACCTGCGCT TA
JCP107	*HMR-A R*	Chr III 293761 - 293742	CAGTTTCCCCGAAAGAACAA
JCP108	*HMR-A F*	Chr III 293471 - 293489	CCATCCGCCGATTTATTTT
JRP51	*HMR-P F*	Chr III 293745 - 293769	TTCTTTCGGGGAAACTGTATAAAA C
JRP52	*HMR-P R*	Chr III 293844 – 293819	AAATATCATCCATGTTGTCCTTCTT G

* "F" stands for forward primer, "R" stands for reverse primer

JRP103 was designed to span the **a**1 exon2 and exon3.

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