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HMR-I Is an Origin of Replication and a Silencer in *Saccharomyces cerevisiae*

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ABSTRACT

There appear to be fundamental differences between the properties of the silencers at *HML* and *HMR*, with some being origins of replication and others not. Moreover, past studies have suggested that *HMR*-I's role in silencing may be restricted to plasmid contexts. This study established that *HMR*-I, like *HMR*-E and unlike either *HML* silencer, is an origin of replication. Moreover, both *HMR*-E and *HMR*-I contribute to silencing of a chromosomal *HMR* locus. In addition, we found that Abf1p plays no unique role in silencer function.

REPRESSION of transcription can occur by mechanisms that act locally, blocking the expression of a single gene, or regionally, blocking the expression of a cluster of genes. Transcriptional repression can inactivate genes for a brief period or can persist through many rounds of cell division. Position effects and genomic imprinting are two mechanisms of transcriptional repression that are clonally heritable even in the absence of the event that led to initial repression.

In the case of position effects, particular regions of eukaryotic chromosomes are assembled into heterochromatin, and the genes in those regions are inactivated. Conceptually, the formation of position effects can be thought of as involving an establishment event followed by other processes that allow clonal inheritance of the repressed state. The most dramatic example of a position effect, X-chromosome inactivation in female mammals, illustrates this formalism with the establishment event occurring early in embryogenesis, and inheritance operating at each subsequent cell division for the life of the individual.

Saccharomyces cerevisiae provides the opportunity for genetic analysis of a position effect that is known as silencing. Genes in three regions of the yeast genome, the *HML* and *HMR* silent-mating-type loci and the regions adjacent to the telomeres, are silenced (Laurenson and Rine 1992; Rivier and Rine 1992b; Loo and Rine 1994; Grunstein 1997, 1998; Lustig 1998). The *SIR2*, *SIR3*, and *SIR4* genes are essential for silencing at *HML*, *HMR*, and telomeres (Rine and Herskowitz 1987; Aparicio *et al.* 1991). These three *SIR* proteins associate with each other and with the core histones to form heterochromatin in the *HML*, *HMR*, and telomeric regions (Hecht *et al.* 1995, 1996; Strahl-Bolsinger *et al.* 1997). A related form of silencing that depends on *SIR2* but not *SIR3* or *SIR4* silences transcription of genes inserted into the repeated rDNA genes (Bryk *et al.* 1997; Smith and Boeke 1997).

In addition to the structural components of heterochromatin, silencing at the *HML* and *HMR* loci requires flanking regulatory elements known as silencers (Abraham *et al.* 1984; Feldman *et al.* 1984; Brand *et al.* 1985). Two silencers are found at each silent locus: the *HML*-E and *HML*-I silencers flank the mating-type genes at *HML*, and the *HMR*-E and *HMR*-I silencers flank the mating-type genes at *HMR*. Each of the four silencers contains binding sites for different combinations of three proteins that contribute to silencing (Shore and Nasmyth 1987; Buchman *et al.* 1988a,b; Foss *et al.* 1993; Loo *et al.* 1995). These proteins are known as ORC (the replication initiator protein) and two transcriptional activator proteins known as Rap1p and Abf1p. *HMR*-E, the most well-characterized silencer, contains an ARS consensus sequence element (ACS) that is the ORC-binding site, a binding site for Rap1p, and a binding site for Abf1p, whereas the *HMR*-I silencer contains an ACS and Abf1p-binding site but lacks a Rap1p-binding site.

At least one role of the silencers and their associated proteins is the recruitment of the *SIR* proteins to the silent regions. Two-hybrid analysis indicates that Rap1p binds Sir3p and Sir4p, suggesting that Rap1p acts to recruit Sir3p and Sir4p to the silencer region (Moretti *et al.* 1994). Sir1p is also recruited to the silencer through interactions with ORC (Triolo and Sternglanz 1996; Gardner *et al.* 1999). Sir1p plays a role in the establishment of silencing at *HML* and *HMR* but is not required for the inheritance of silencing, suggesting that the role of Sir1p may be limited to the initial assembly of heterochromatin (Pillus and Rine 1989).

Other aspects of silencer function are less well under-

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stood. For instance, although deletion of the *HMR*-E silencer abolishes silencing at *HMR*, none of the individual elements of the *HMR*-E silencer are required for silencing (Brand *et al.* 1987; Kimmerly and Rine 1987). In particular, mutation of the ACS, the Rap1p-binding site, or the Abf1p-binding site results in little or no silencing defect. However, mutation of any pairwise combination of these three elements results in loss of silencing. Thus, the *HMR*-E silencer elements appear to be functionally redundant. The molecular basis for this redundancy is not known.

In addition to its essential role in silencing, the *HMR*-E silencer is also a chromosomal origin of replication (Rivier and Rine 1992a). This observation raises the possibility that DNA replication, initiated at the silencers, may in some way contribute to either the establishment or inheritance of silencing. ORC's role in replication and silencing can functionally be separated by alleles that affect one process but not the other (Dillin and Rine 1997). However, it is still possible that ORC has an additional function(s) common to both processes.

The role of *HMR*-I in silencing and replication is unclear. Initial studies of *HMR* on a plasmid indicated that *HMR*-I contributed to silencing (Abraham *et al.* 1983). Further, *HMR*-I has ARS activity when inserted into a plasmid (Abraham *et al.* 1984; Kimmerly and Rine 1987). Thus, *HMR*-I was initially believed to contribute to silencing and to be an origin of replication. However, subsequent analysis raised doubt about the significance of these observations. In particular, deletion of *HMR*-I from the chromosome does not result in a detectable silencing defect (Brand *et al.* 1985). Furthermore, only some ARS elements are chromosomal origins (Dubey *et al.* 1991; Newlon and Theis 1993). In addition, both the *HML*-E and *HML*-I silencers have ARS activity when inserted into plasmids but are not origins of replication in the chromosome (Dubey *et al.* 1991). Thus, the contribution of *HMR*-I to silencing and replication initiation in the normal chromosomal context is not known. Hence, two questions emerge: (1) Is *HMR*-I a chromosomal origin? and (2) Does *HMR*-I normally contribute to silencing at *HMR*? The experiments presented here resolve both questions.

MATERIALS AND METHODS

Two-dimensional analysis of chromosomal replication intermediates: Genomic DNA was isolated from 10^{10} log-phase cells in an asynchronous culture and digested to completion with *Eco*RV and *Xba*I. The *HMR*-I silencer was located in the center of this 3065-bp *Xba*I-*Eco*RV fragment (1451–1756 bp from the *Xba*I site). In addition, this fragment contains the **a**1 gene and part of the **a**2 gene but lacks the *HMR*-E silencer. DNA replication intermediates were enriched by chromatography on benzoylated naphthoylated DEAE cellulose (BND; Sigma, St. Louis), which preferentially binds single-stranded DNA, as described previously (Rivier and Rine 1992a). Because the DNA at the replication forks is partially single stranded, this procedure enriches for replication intermediates. The resulting DNA was subjected to two-dimensional electrophoresis, transferred to Zeta probe GT membrane (Biorad, Richmond, CA), and hybridized with a probe to the **a**1 gene as described previously (Rivier and Rine 1992a). Because the strains tested had the *MAT*α genotype, this probe was unique to the *HMR*-I region (see Table 1).

Quantitative mating analysis: Cells were grown to a final OD_{600} of 0.5–1.0 in rich media supplemented with adenine. Serial dilutions of test strains were mixed with 2.2×10^7 *MAT*α cells (JRY2726) and plated onto minimal medium supplemented with adenine. Equivalent dilutions of test strains were plated onto rich medium. Mating efficiencies were calculated as the number of diploids formed per viable cell plated.

Patch mating analysis: Test strains were patched onto rich medium, grown overnight, and replica plated onto a lawn of *MAT*α cells (JRY2726) on minimal solid medium supplemented with adenine.

Construction of *HMR::ADE2* alleles: *HMR::ADE2* was constructed in two steps. First, a *Bgl*II fragment containing the *ADE2* gene was inserted into pRS202 (Sikorski and Hieter 1989) digested with *Bam*HI, resulting in plasmid pDR78. Plasmid pDR162 was made by replacing a *Bgl*II-partial *Xba*I fragment of *HMR* with a *Bgl*II-*Spe*I fragment of pDR78. This construct replaces the *MAT***a**-genes at *HMR* with the *ADE2* gene such that the *ADE2* promoter is proximal to the *HMR*-E silencer. Plasmids with different silencer alleles were constructed as follows. Plasmids pDR275 and pDR168 were constructed by replacing an *Eco*RI-*Eco*NI fragment of pDR162 containing the *HMR*-E silencer with the *Eco*RI-*Eco*NI fragment of either pDR130 (p8ΔE) or pDR60 (*HMR-SS*), respectively. Plasmid pDR279 was created by replacing the *Bgl*II-*Hin*dIII fragment of pDR162 containing the *HMR*-I silencer with the *Bgl*II-*Hin*dIII fragment of pDR55 that contains a 305-bp deletion of *HMR*-I (described below). The *HMR::ADE2* alleles were digested with *Eco*RI and *Pst*I and integrated into strain DRY724.

Construction of *HMR::URA3pr-ADE2* alleles: A construct for inserting reporter genes at *HMR* was made in four steps. First, the Bluescript polylinker was cut with *Xba*I-*Pst*I, filled in, and religated to create pDR546. Second, a *Hin*dIII-*Kpn*I fragment of pDR546 was replaced by a *Not*I site contained in the oligonucleotides, 5′ CCCAAGCTTGCGGCCGCGGTACCTAGCCC 3′ and 5′ GGGCTAGGTACCGCGGCCGCAAGCTTGGG 3′, to create pDR570. Third, a partial *Xba*I-*Bgl*II fragment of *HMR* containing the **a**1 and **a**2 genes was replaced by *Bam*HI and *Sal*I sites contained in oligonucleotides 5′ GCTCTAGAGGATCCCGGGGTCGACCGAGATCTTC 3′ and 5′ GAAGATCTCGGTCGACCCCGGGATCCTCT AGAGC 3′, to create pDR550. Fourth, the *Eco*RI-*Hin*dIII fragment of pDR570 was inserted into *Eco*RI-*Hin*dIII-

TABLE 1

Strains used in this study

Strain	Genotype[a]	Source
DBY703[b]	*his3 trp1 ura3-52 [cir°]*	C. Holm
DRY470	*HMR ΔE his3 lys2-6 trp1 ura3-52*	
DRY645	*HMR-SS his3 trp1 lys2-6 ura3-52*	
DRY647	*HMR-SS ars-his3 trp1 lys2-6 ura3-52*	
DRY502	*HMR-SS rap1-his3 trp1 lys2-6 ura3-52*	
DRY528	*HMR-SS abf1-1 his3 trp1 lys2-6 ura3-52*	
DRY536	*HMR-SS abf1-2 his3 trp1 lys2-6 ura3-52*	
DRY482	*HMR ΔI his3 trp1 lys2-6 ura3-52*	
DRY604	*HMR ΔE ΔI his3 lys2-6 trp1 ura3-52*	
DRY463	*HMR-SS ΔI his3 lys2-6 trp1 ura3-52*	
DRY514	*HMR-SS ars-ΔI his3 trp1 lys2-6 ura3-52*	
DRY490	*HMR-SS rap1-ΔI his3 trp1 lys2-6 ura3-52*	
DRY556	*HMR-SS abf1-1 ΔI his3 trp1 lys2-6 ura3-52*	
DRY616	*HMR-SS abf1-2 ΔI his3 trp1 lys2-6 ura3-52*	
DRY1693	*HMR-SS (RAP-RAP)*[c] *his3 trp1 ura3-52*	C. Fox
DRY1694	*HMR-SS (RAP-RAP)*[c] *ΔI his3 trp1 ura3-52*	C. Fox
DRY426	*hmr::URA3 his3 lys2-6 trp1 ura3-52*	
JRY2334[d]	*ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 can1-100*	
DRY434	*HMR ΔI ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 can1-100*	
DRY707	*MAT***a** *his3-11 leu2-3,112 lys2::hisG trp1-1 ura3-1*	
DRY1667	*HMR::URA3pr-ADE2 ade2::HIS3 his3-11 leu2-3,112 trp1-1 ura3-1*	
DRY1665	*HMR::URA3pr-ADE2 ΔI ade2::HIS3 his3-11 leu2-3,112 trp1-1 ura3-1*	
DRY827	*HMR-ΔE::ADE2 ade2::HIS3 his3-11 leu2-3,112 trp1-1 ura3-1*	
DRY829	*HMR::ADE2 ade2::HIS3 his3-11 leu2-3,112 trp1-1 ura3-1*	
DRY826	*HMR::ADE2 ΔI ade2::HIS3 his3-11 leu2-3,112 trp1-1 ura3-1*	
DRY815	*HMR-SS::ADE2 ade2::HIS3 his3-11 leu2-3,112 trp1-1 ura3-1*	
DRY724	*hmr::URA3 ade2::HIS3 his3-11 leu2-3,112 trp1-1 ura3-1*	
JRY2726	*MAT***a** *his4*	

[a] All strains are *MAT*α unless otherwise indicated.
[b] Strains below are derivatives of DBY703.
[c] The Abf1p-binding site was replaced by a Rap1p-binding site.
[d] Strains below are isogenic to W303-1a.

cleaved pDR570. The resulting plasmid, pDR576, contained *HMR* flanked by *Not*I sites with a polylinker replacing the **a**-genes. An *HMRa* ΔI derivative of pDR576 was made by replacing the *Bgl*II-*Hin*dIII fragment with the same fragment from pDR62, resulting in pDR606. Construction of *HMR::URA3pr-ADE2* was as follows. A *Bam*HI-*Sal*I fragment of pURADE2 (generously provided by Oscar Aparicio) was inserted into a *Bam*HI-*Sal*I digest of either pDR606 (ΔI) or pDR610 (WT). The *URA3pr-ADE2* fragment was oriented with the *URA3* promoter proximal to the *HMR*-I silencer.

Deletion of *HMR*-I: The deletion of *HMR*-I was constructed from two existing linker insertion mutations (Abraham *et al.* 1984). The *Eco*RI-*Xho*I *HMR* fragment of Δ59 and the *Xho*I-*Hin*dIII *HMR* fragment of Δ296-39 were inserted into *Eco*RI-*Hin*dIII-cleaved pUC18, resulting in plasmid pJR1270. DNA sequence analysis revealed that pJR1270 contains a deletion of 305 bp. The sequence of this deletion allele has been submitted to GenBank (Table 2).

Construction of synthetic silencer alleles: The identity of each *HMR*-E allele has been submitted to GenBank and, hence, is only briefly described here. *HMR*-E alleles were constructed on plasmids (Table 2) and each allele was subsequently integrated into the chromosome. Each of the elements of the synthetic silencer, the ACS, the Rap1p-binding site, and the Abf1p-binding site is separated by unique restriction sites. To construct specific mutations at any site, a plasmid containing the synthetic silencer was restricted with the two enzymes that flank that site, and complementary synthetic oligonucleotides were inserted. The identity of these alleles was confirmed by DNA sequence analysis. Alleles of the synthetic silencer that alter the Abf1p-binding site were constructed in pJR1272. Construction of the *HMR-SS abf1-2* allele has been previously described (referred to as ssb in McNally and Rine 1991). Construction of the alleles of the synthetic silencer that alter the Rap1p-binding site were constructed in pJR1268. pJR1268 was constructed by cleaving pJR934 (McNally and Rine 1991) with *Hin*dIII and *Pst*I, filling in the overlapping ends with Klenow fragment and deoxynucleotides, and religating the ends.

Combinations of mutations at *HMR*-E and deletion of

TABLE 2

Plasmids used in this study

Plasmid	*HMR*-E allele	*HMR*-I allele	GenBank accession no.
pJR1571	Wild type	Wild type	
pJR1270	Wild type	Deleted	U56968
pJR1294	Deleted	Wild type	
pJR1271	Deleted (*hmr::URA3*)	Deleted	
pJR1272	*HMR-SS*	Wild type	U56969
pJR1273	*HMR-SS*	Deleted	
pJR1284	*HMR-SS acs-*	Deleted	U56970
pJR1274	*HMR-SS rap1-*	Wild type	U56971
pJR1572	*HMR-SS rap1-*	Deleted	
pJR1277	*HMR-SS abf1-1*	Wild type	U56972
pJR1281	*HMR-SS abf1-1*	Deleted	
pJR1279	*HMR-SS abf1-2*	Wild type	U56967
pJR1285	*HMR-SS abf1-2*	Deleted	
pJR1573	*HMR-SS (RAP-RAP)*	Wild type	U57436
pJR1574	*HMR-SS (RAP-RAP)*	Deleted	

HMR-I were constructed by inserting the *Bgl*II-*Hin*dIII fragment of pJR1270 that contained the *HMR*-I deletion into *Bgl*II-*Hin*dIII-cleaved plasmids that harbored the appropriate allele of *HMR*-E (Table 2).

Media and genetic manipulations: Rich medium (YPD) and minimal medium (YM) were as described previously (Sherman 1991). Medium containing 5-FOA was as described previously (Guthrie and Fink 1991). Transformation was by a modified lithium-acetate method (Gietz and Schiestl 1991). For integration of *in vitro*-constructed *HMR* alleles, linear fragments of *HMR* were cleaved from 5 μg of plasmid and transformed into yeast cells in which *HMR* had been replaced with *URA3* (*hmr::URA3*). A total of 2.6×10^7 cells of the transformed culture were plated on solid rich medium supplemented with adenine, grown for seven doublings (2.5–3 days at 30°), and resuspended in YPD. A total of 2×10^5 cells were replated on minimal medium containing 5-fluoroorotic acid (FOA) to select for cells in which the *hmr::URA3* allele had been replaced with the *in vitro*-constructed *HMR* allele. Allelic replacement at *HMR* was confirmed by DNA blot analysis. These conditions typically yield 20–200 colonies, the majority of which contain the appropriate gene replacement.

RESULTS

***HMR*-I was a chromosomal origin of replication:** In the earlier studies of silencers on plasmids, all four mating-type silencers had ARS activity, implying that they were origins of replication (Brand *et al.* 1987; Kimmerly and Rine 1987). In fact, *HMR*-E proved to be a chromosomal origin of replication (Rivier and Rine 1992a), whereas *HML*-E and *HML*-I are not (Dubey *et al.* 1991). Therefore, we tested whether *HMR*-I was a *bona fide* chromosomal origin. Initiation of chromosomal replication can be monitored by a two-dimensional gel electrophoresis and DNA blot assay that separates DNA replication intermediates on the basis of size and shape (Brewer and Fangman 1987). The replication intermediates of genome-derived restriction fragments that do not contain an origin of replication are fork-shaped and give rise to a particular arc pattern. The replication intermediates of genome-derived restriction fragments that contain an origin of replication are bubble-shaped and give rise to a distinct arc pattern. To determine whether *HMR*-I was a chromosomal origin of replication, the replication intermediates of a wild-type strain were analyzed for a fragment of the genome that contained the *HMR*-I silencer, but not the *HMR*-E silencer. Bubble-shaped replication intermediates were detected in this fragment, revealing that a chromosomal origin of replication was close to, or coincident with, *HMR*-I (Figure 1). To determine whether the origin colocalized with the *HMR*-I silencer, the replication intermediates

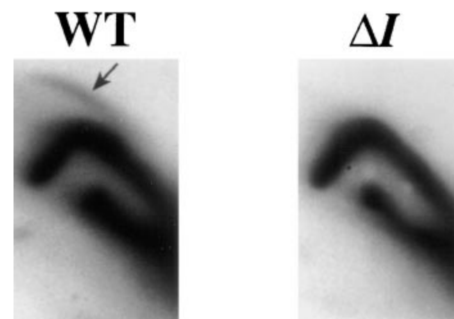

Figure 1.—A chromosomal origin of replication localized to *HMR*-I. DNA replication intermediates of strains JRY2334 (wild type, left) and DRY434 (*HMR* ΔI, right) are shown. The arrow indicates the arc of bubble-shaped replication intermediates.

of an isogenic strain, from which a 305-bp segment containing the *HMR*-I silencer had been deleted, were analyzed. The genome-derived restriction fragments harboring the deletion of *HMR*-I contained only fork-shaped replication intermediates and did not contain bubble-shaped intermediates (Figure 1). Thus, *HMR*-I, like *HMR*-E, was a *bona fide* chromosomal origin of replication.

Analysis of a synthetic silencer: A 138-bp synthetically constructed silencer was previously shown to be sufficient to bring about silencing at *HMR* when inserted into an 868-bp deletion that removed the wild-type *HMR*-E silencer (McNally and Rine 1991). This synthetic silencer contains an ACS, a Rap1p-binding site, and an Abf1p-binding site in the same orientation and relative positions as in the wild-type silencer. The DNA sequence between these elements was altered so that the sequence similarity between the synthetic silencer and the wild-type silencer was restricted to the elements known to contribute to silencing. The initial analysis of this synthetic silencer suggested that the elements of the synthetic silencer may not be redundant (McNally and Rine 1991). In particular, mutation of the ACS of the synthetic silencer caused a severe silencing defect.

To determine whether the Rap1p-binding site of the synthetic silencer was required for silencing, a mutant version of this site was created and a synthetic silencer harboring this mutation was inserted into the chromosome in place of the natural *HMR*-E. In particular, two bases of the Rap1p-binding site were changed. Previous analysis of the wild-type silencer demonstrated that the C-to-A transversion in the fifth nucleotide of the Rap1p-binding site alone was sufficient to prevent Rap1p binding *in vitro* and to abolish silencing *in vivo* in combination with a mutation in the Abf1p-binding site of the natural silencer (Kimmerly *et al.* 1988). The role of the Rap1p-binding site of the synthetic silencer in silencing of *HMR* was judged by a mating-type assay. *MAT*α *HMR***a** strains in which *HMR* is silent display the α-mating phenotype, whereas *MAT*α *HMR***a** strains in which *HMR* is not silent display a nonmating phenotype. The 2-bp substitution in the Rap1p-binding site of the synthetic silencer reduced mating efficiency by four orders of magnitude as judged by a quantitative mating assay (Figure 2). Hence, the Rap1p-binding site of the synthetic silencer, like the ACS of the synthetic silencer, provided a nonredundant function that was required for silencing.

A similar approach was taken to determine whether the Abf1p-binding site of the synthetic silencer was required for silencing. Initially an A-T insertion mutation was created in the center of this site. In the context of the wild-type silencer, this mutation abolishes Abf1p binding *in vitro* and abolishes silencing *in vivo* in combination with a mutation in the Rap1p-binding site of the natural silencer (Kimmerly *et al.* 1988). In the context of the synthetic silencer, this Abf1p-binding site mutation (*HMR-SS abf1-1*) resulted in only a slight decrease in silencing (Figure 2). To assess the role of the Abf1p-binding site more rigorously, the Abf1p-binding site was destroyed by a cluster of point mutations that changed each of the nucleotides of the consensus sequence (*HMR-SS abf1-2*). Similar to the 1-bp insertion, obliteration of the Abf1p-binding site also resulted in only a slight silencing defect as judged by quantitative mating (Figure 2). [A previous description of an Abf1p site mutation in the synthetic silencer having a strong effect on silencing (McNally and Rine 1991) was incorrect due to an error in strain construction (data not shown).]

Role of *HMR*-I in combination with the synthetic silencer: The results presented above suggested that the synthetic silencer might provide a sensitive context for testing the role of *HMR*-I in silencing. In particular, if *HMR*-I does not normally contribute to silencing at *HMR*, deletion of the *HMR*-I silencer would not result in a greater silencing defect in the presence of the synthetic *HMR*-E silencer or any of the mutant alleles described above. In contrast, if *HMR*-I normally contributes to silencing at *HMR*, deletion of *HMR*-I would likely result in a silencing defect in combination with at least some mutant alleles of the synthetic silencer. To test the role of *HMR*-I in silencing, the 305-bp region containing *HMR*-I was deleted from strains harboring various alleles of *HMR*-E. Deletion of *HMR*-I from a strain harboring the wild-type *HMR*-E did not result in a significant decrease in mating efficiency, as previously described (Brand *et al.* 1985) (Figure 2). Deletion of *HMR*-I from a strain harboring the synthetic silencer at *HMR*-E resulted in a slight decrease in mating efficiency. Hence, in this context, *HMR*-I contributed modestly to silencing but was not required for silencing. In contrast, deletion of *HMR*-I resulted in a dramatic silencing defect in combination with the mutations of the Abf1p-binding site of the synthetic silencer. In combination with either the *HMR-SS abf1-1* allele or the *HMR-SS abf1-2* allele, deletion of *HMR*-I resulted in a decrease in mating efficiency by three to four orders of magnitude relative to mutation of the Abf1p-binding site or deletion of *HMR*-I alone (Figure 2). Thus, *HMR*-I played a substantial role in silencing under normal growth conditions, implying that *HMR*-I normally contributes jointly with *HMR*-E to bring about silencing. In addition, in the context of the synthetic silencer, the Abf1p-binding site of *HMR*-E and *HMR*-I were redundant elements, just as the Rap1- and Abf1p-binding sites are redundant in wild-type *HMR*-E.

The mutations of the ACS or Rap1p-binding site of the synthetic silencer reduced silencing as judged by mating efficiency to 10^{-4} of wild type, but did not completely abolish silencing because deletion of *HMR*-E results in a 10^{-6}-fold reduction of mating ability (Figure 2). The residual silencing ability contributed by the mutant *HMR*-E alleles depended on a contribution from *HMR*-I. Specifically, deletion of *HMR*-I in combination

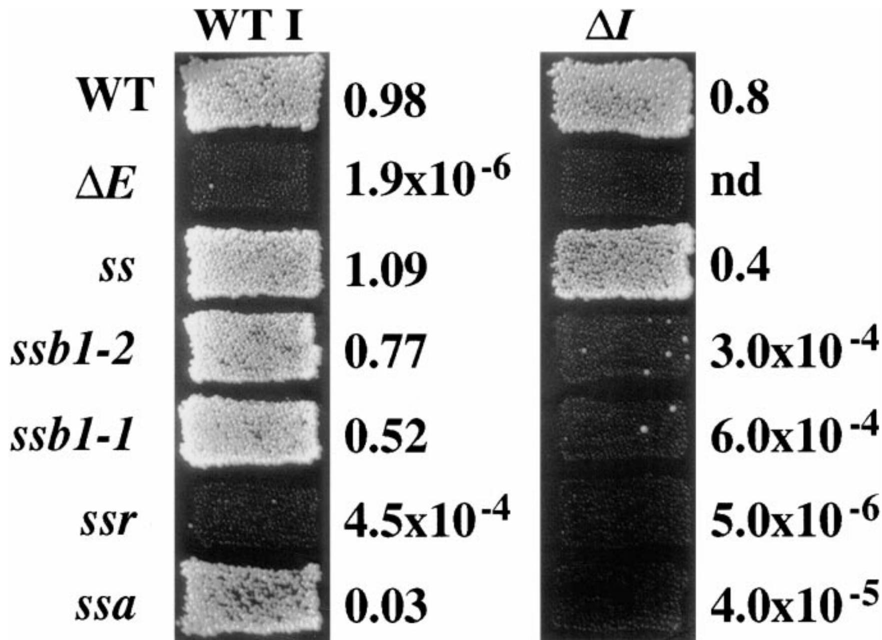

Figure 2.—*HMR*-I contributed to silencing in combination with alleles of the synthetic silencer. Shown are patch mating and quantitative mating analysis for strains with *HMR*-I (left) or lacking *HMR*-I (right). *HMR*-I strains are (top to bottom): DBY703 (wild type), DRY470 (*HMR* ΔE), DRY645 (*HMR-SS*), DRY536 (*HMR-SS abf1-2*), DRY528 (*HMR-SS abf1-1*), DRY502 (*HMR-SS rap1-*), and DRY647 (*HMR-SS acs-*). Strains lacking *HMR*-I are (top to bottom): DRY482 (*HMR* ΔI), DRY604 (*HMR*-ΔE ΔI), DRY463 (*HMR-SS* ΔI), DRY616 (*HMR-SS abf1-2* ΔI), DRY 556 (*HMR-SS abf1-1* ΔI), DRY490 (*HMR-SS rap1*-ΔI), and DRY514 (*HMR-SS acs*-ΔI).

with mutations in the ACS of the synthetic silencer or the Rap1p-binding site of the synthetic silencer reduced mating efficiency by an additional two orders of magnitude (Figure 2). Thus, *HMR*-I contributed to silencing at *HMR.*

***HMR*-I and *HMR*-E contributed jointly to silencing reporter genes inserted at *HMR*:** Deletion of *HMR*-I did not result in a silencing defect in an otherwise wild-type *HMR* locus as judged by quantitative mating assays. We used a more sensitive assay of silencing at *HMR* to examine the contribution of *HMR*-I in wild-type cells more clearly. The *ADE2* gene has been used as a sensitive reporter gene for monitoring either telomeric silencing or silencing at *HMR* (Gottschling *et al.* 1990; Sussel *et al.* 1993). Cells that express *ADE2* at wild-type levels give rise to white colonies on rich medium, whereas cells that do not express *ADE2* give rise to red colonies. Insertion of *ADE2* into the *HMR* (*HMR::ADE2*) locus in place of the mating-type genes results in silencing of *ADE2* as judged by a dark pink colony color, as reported previously (Figure 3). Deletion of the *HMR*-E silencer resulted in white colonies, indicating that the pink colony color resulted from silencing of *ADE2* (Figure 3). Replacement of wild-type *HMR*-E with the synthetic silencer resulted in the formation of light pink colonies, indicating that the synthetic silencer was not as efficient at silencing as wild-type *HMR*-E (Figure 3). Furthermore, the *ADE2* reporter gene was a more sensitive measure of silencing than mating-type assays since quantitative mating assays did not reveal a difference in silencing efficiency between the wild-type and synthetic silencers. Deletion of *HMR*-I from a strain with the wild-type allele of *HMR*-E and the *HMR::ADE2* reporter gene resulted in the formation of light pink colonies, indicating that silencing of the *HMR::ADE2* gene was dependent on both *HMR*-E and *HMR*-I. Thus, the full extent of silencing at *HMR* resulted from the combined action of both the wild-type *HMR*-E and *HMR*-I silencers.

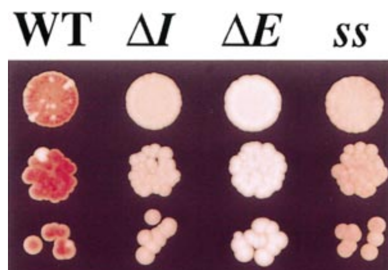

Figure 3.—*HMR*-I contributed to silencing of *HMR::ADE2.* Shown are a series of three serial dilutions of cells of strains DRY829 (*HMR::ADE2*), DRY826 (*HMR::ADE2 ΔI*), DRY827 (*HMR-ΔE::ADE2*), and DRY815 (*HMR-SS::ADE2*).

As a second test of whether the wild-type *HMR*-E and *HMR*-I silencers both contribute to complete silencing at *HMR*, we assessed the role of *HMR*-I with a different reporter gene inserted at *HMR* in place of the mating-type genes. Previous analysis revealed that activated transcription and silencing are competitive processes (Renauld *et al.* 1993; Aparicio and Gottschling 1994). To avoid this competition, we utilized a chimeric reporter gene in which the promoter region of the *URA3* gene was used to direct transcription of the *ADE2* coding region (*URA3pr-ADE2*). In addition to the colony color assay described above, *ADE2* expression can also be monitored by growth on minimal medium lacking adenine. Since the *ADE2* gene is required for adenine biosynthesis, cells that do not express *ADE2* are adenine auxotrophs. However, in the absence of adenine, the *ADE2* gene is induced and, therefore, would likely be expressed even when inserted into silent regions of the genome. In contrast, the *URA3* promoter is induced in the absence of uracil, and is not induced in the absence

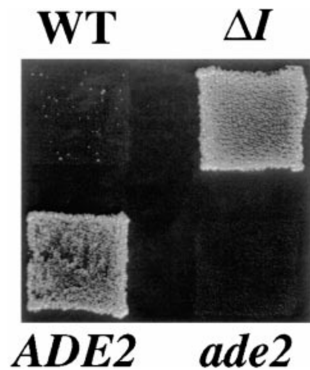

Figure 4.—*HMR*-I contributed to silencing of *HMR::URA3pr-ADE2.* Cells were patched onto rich medium, grown overnight, and replica plated onto minimal medium lacking adenine. Strains shown are DRY1667 (*HMR::URA3pr-ADE2*), DRY1665 (*HMR::URA3pr-ADE2 ΔI*), DRY707 (*HMR***a** *ADE2*), and JRY2334 (*HMR***a** *ade2-1*).

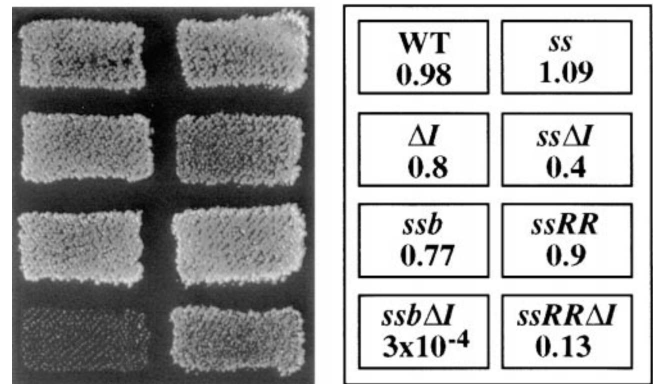

Figure 5.—Abf1p- and Rap1p-binding sites provided redundant silencing functions. Shown is patch mating analysis (left) and quantitative mating analysis (right) of strains JRY2334 (WT), DRY1691 (*HMR-SS*), DRY482 (*HMR ΔI*), DRY463 (*HMR-SS ΔI*), DRY536 (*HMR-SS abf1-2*), DRY1693 [*HMR-SS (RAP-RAP)*], DRY616 (*HMR-SS abf1-2 ΔI*), and DRY1694 [*HMR-SS* (*RAP-RAP*) *ΔI*].

of adenine (Roy *et al.* 1990). Hence, in medium containing uracil the chimeric *URA3pr-ADE2* gene is transcribed at the basal level. Cells with the *URA3pr-ADE2* reporter gene inserted into *HMR* in place of the **a**-mating-type genes (*HMR::URA3pr-ADE2*) were adenine auxotrophs on minimal medium supplemented with uracil (Figure 4). In contrast, *HMR::URA3pr-ADE2* cells from which the *HMR*-I silencer had been deleted were adenine prototrophs on minimal medium supplemented with uracil (Figure 4). These results confirmed that *HMR*-I was required for complete silencing of the *HMR::URA3pr-ADE2* reporter gene. Taken together, these observations on reporter genes indicate that, in conjunction with *HMR*-E, *HMR*-I brings about complete silencing at *HMR.*

Functional redundancy among elements: Deletion of either the Rap1p-binding site or the Abf1p-binding site of the wild-type *HMR*-E silencer has little or no effect on silencing, whereas deletion of both binding sites results in substantial loss of silencing. There are at least two ways to think about this apparent redundancy. One view is that Rap1p and Abf1p each make a unique contribution to silencing. In this case, the apparent redundancy could be explained if, in the absence of either the Rap1p-binding site or the Abf1p-binding site, both Rap1p and Abf1p are retained in the protein complex at the silencer via protein-protein interactions, whereas in the absence of both binding sites either Rap1p, Abf1p, or both are not retained in the complex. The other possibility is that Rap1p and Abf1p each provide a qualitatively similar activity that can contribute to silencing and that either protein alone can provide that activity.

A strain containing the synthetic silencer and lacking *HMR*-I offered the opportunity to test a prediction of the model that Abf1p and Rap1p provide qualitatively similar activities. In this strain both the Rap1p- and Abf1p-binding sites are required for silencing. If Rap1p and Abf1p provide qualitatively similar functions in silencing, then a synthetic silencer composed of an ACS and two Rap1p-binding sites or an ACS and two Abf1p-binding sites would be expected to function as a silencer. In contrast, if Rap1p and Abf1p provided qualitatively distinct functions that are required for silencing, synthetic silencers composed of an ACS and two Rap1p sites or an ACS and two Abf1p sites would not be expected to function as silencers. To test whether Rap1p and Abf1p may provide similar functions in silencing, two synthetic silencers were created. In the first, the Abf1p-binding site was replaced with a Rap1p-binding site such that the spacing among the silencer elements was maintained [*HMR-SS (RAP-RAP)*]. This synthetic construct was capable of silencing the mating-type genes at *HMR* in the absence of the *HMR*-I silencer (Figure 5). This observation suggested that Abf1p does not provide a unique function that is required for silencing at *HMR.* Furthermore, the ability of a second Rap1p-binding site to substitute for the Abf1p-binding site suggested that Rap1p provided a silencing function that was qualitatively similar to that of Abf1p. However, an alternate possibility is that the Rap1p-binding site provides a function that is qualitatively distinct from the Abf1p-binding site and that a quantitive increase in that function can bring about efficient silencing. A synthetic silencer in which the Rap1p site was replaced with a second Abf1p site, such that the spacing was maintained among the silencer elements, was not capable of silencing the mating-type genes at *HMR* in the absence of *HMR*-I (data not shown).

DISCUSSION

This work investigated the role of *HMR*-I in silencing and DNA replication. Two lines of evidence indicated that *HMR*-I contributed to silencing. First, deletion of *HMR*-I resulted in silencing defects of the *MAT***a***1* gene

at *HMR* when assayed in combination with mutant alleles of the *HMR*-E silencer. Second, deletion of *HMR*-I resulted in silencing defects of two different reporter genes inserted at *HMR* and flanked by a wild-type copy of *HMR*-E.

Moreover, the *HMR*-E and the *HMR*-I silencers appeared to contribute jointly to complete silencing of *HMR.* The most striking example of this joint contribution was seen with alleles of the synthetic silencer with a mutated Abf1p-binding site. Cells with this *HMR*-E allele and wild-type *HMR*-I silenced *HMR* to approximately wild-type levels. However, in cells with this *HMR*-E allele that lacked *HMR*-I, silencing was reduced by four orders of magnitude as judged by quantitative mating assays. Thus, elements at both *HMR*-E and at *HMR*-I contributed to silencing. Analysis of a reporter gene at *HML* has led to the idea that the *HML*-E and *HML*-I silencers contribute jointly to silence *HML* (Boscheron *et al.* 1996). Although the molecular basis for collaboration is unknown, it has been proposed that the *HML*-E and *HML*-I silencers physically interact with each other, perhaps transiently. In this model, the two silencers at *HMR* and at *HML* would more properly be viewed as a single silencer with two separate parts that interact over a distance, rather than as two autonomous silencers that contribute additively to silencing.

This work also addressed the molecular nature of the "redundancy" of the *HMR*-E silencer. In the absence of *HMR*-I, efficient silencing could be brought about by a synthetic silencer that contained an ACS, a Rap1p-binding site, and an Abf1p-binding site, or by a synthetic silencer that contained an ACS and two Rap1p-binding sites. These observations suggest that silencing was not brought about by a single type of protein complex at each of the silencers and telomeres. Rather, these observations suggest that distinct combinations of ORC, Rap1p, and Abf1p proteins can recruit the *SIR* proteins.

HMR-I was also found to be a chromosomal origin of replication. Thus, both silencers at *HMR* are chromosomal origins of replication and both function in silencing. Despite this relationship, a variety of evidence suggests that initiation of replication from the silencers is not required for silencing. For instance, essential roles of ORC in silencing and in initiation of replication are genetically separable (Dillin and Rine 1997). Further, artificial telomeres that do not contain ACS elements, and therefore are presumably not origins of replication, are capable of silencing nearby genes (Gottschling *et al.* 1990). Similarly, initiation of chromosomal replication does not occur at a detectable frequency at the *HML* silencers (Dubey *et al.* 1991). Thus, if initiation of replication at the silencers plays a role in the establishment or inheritance of silencing, that role can also be provided in other ways.

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