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 (Abranham et al. 1984; Felman et al. 1984; Brand et al. 1985). Two silencers are found at each silent locus: the HMR-E and HMR-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 Stern-glanz 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 (Bradford et al. 1987; Kimmery 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 et al. 1997). Moreover, 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; Kimmery 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 (Bradford 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 EcoRV and XbaI. The HMR-I silencer was located in the center of this 3065-bp XbaI-EcoRV fragment (1451–1756 bp from the XbaI site). In addition, this fragment contains the a1 gene and part of the a2 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 electrophoreses, transferred to Zeta probe GT membrane (Biorad, Richmond, CA), and hybridized with a probe to the a1 gene as described previously (Rivier and Rine 1992a). Because the strains tested had the MATα 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 MATα 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 MATα cells (JRY2726) on minimal medium supplemented with adenine.

Construction of HMR::ADE2 alleles: HMR::ADE2 was constructed in two steps. First, a BglII fragment containing the ADE2 gene was inserted into pRS202 (Sikorski and Hieter 1989) digested with BamHI, resulting in plasmid pDR78. Plasmid pDR62 was made by replacing a BglII-partial XbaI fragment of HMR with a BglII-SpeI fragment of pDR78. This construct replaces the MATα 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 EcoRI-EcoNI fragment of pDR162 containing the HMR-E silencer with the EcoRI-EcoNI fragment of either pDR130 (p8E) or pDR60 (HMR-SS), respectively. Plasmid pDR279 was created by replacing the BglII-HindIII fragment of pDR162 containing the HMR-I silencer with the BglII-HindIII fragment of pDR55 that contains a 305-bp deletion of HMR-I (described below). The HMR::ADE2 alleles were digested with EcoRI and PstI 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 XbaI-PstI, filled in, and religated to create pDR546. Second, a HindIII-KpnI fragment of pDR546 was replaced by a NotI site contained in the oligonucleotides, 5’ CCAAGCTTCTCGCGCCGTAATCGACGCC 3’ and 5’ GGCTAGAGTACCAGGGCGACCAGAGCTC 3’, to create pDR570. Third, a partial XbaI-BglII fragment of HMR containing the a1 and a2 genes was replaced by BamHI and SalI sites contained in oligonucleotides 5’ GCTTCTAGAGGATCCGGGCGTCGAGGATCTTC 3’ and 5’ GGAAGATCTCGTGCTACGCCGGGATCTCTAG 3’, to create pDR550. Fourth, the EcoRI-HindIII fragment of pDR570 was inserted into EcoRI-HindIII.
cleaved pDR570. The resulting plasmid, pDR576, contained HMR flanked by NcoI sites with a polylinker replacing the \( a \)-genes. An HMR \( \Delta I \) derivative of pDR576 was made by replacing the BglII-HindIII fragment with the same fragment from pDR62, resulting in pDR606. Construction of HMR::URA3pr-ADE2 was as follows. A BamHI-Sall fragment of pURADE2 (generously provided by Oscar Aparicio) was inserted into a BamHI-Sall digest of either pDR606 (\( \Delta I \)) or pDR610 (WT). The URA3pr-ADE2 fragment was oriented with the URA3 promoter proximal to the HMR-1 silencer.

**Deletion of HMR-I**: The deletion of HMR-I was constructed from two existing linker insertion mutations (Abraham et al. 1984). The EcoRI-XhoI HMR fragment of \( \Delta 59 \) and the XhoI-HindIII HMR fragment of \( \Delta 296-39 \) were inserted into EcoRI-HindIII-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 pJR1268. pJR1268 was constructed by cleaving pJR934 (McNally and Rine 1991) with HindIII and PstI, filling in the overlapping ends with Klenow fragment and deoxynucleotides, and religating the ends.

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

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### Table 1

**Strains used in this study**

<table>
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<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
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<td>his3 trp1 ura3-52 [cir&lt;sup&gt;b&lt;/sup&gt;]</td>
<td>C. Holm</td>
</tr>
<tr>
<td>DRY470</td>
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<tr>
<td>DRY645</td>
<td>HMR-SS his3 trp1 lys2-6 ura3-52</td>
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<tr>
<td>JRY2726&lt;sup&gt;e&lt;/sup&gt;</td>
<td>MATa his4</td>
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<sup>a</sup> All strains are MATa unless otherwise indicated.  
<sup>b</sup> Strains below are derivatives of DBY703.  
<sup>c</sup> The Abf1p-binding site was replaced by a Rap1p-binding site.  
<sup>d</sup> Strains below are isogenic to W303-1a.
### TABLE 2

Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>H M R-E allele</th>
<th>H M R-I allele</th>
<th>GenBank accession no.</th>
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<td>Wild type</td>
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</tr>
<tr>
<td>pJR1270</td>
<td>Wild type</td>
<td>Deleted</td>
<td>U56968</td>
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<tr>
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<td>Deleted</td>
<td>Wild type</td>
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<tr>
<td>pJR1271</td>
<td>Wild type (hmr::URA3)</td>
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<tr>
<td>pJR1272</td>
<td>H M R-SS</td>
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<td>pJR1273</td>
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<tr>
<td>pJR1284</td>
<td>H M R-SS acs-</td>
<td>Deleted</td>
<td>U56970</td>
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<tr>
<td>pJR1274</td>
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<tr>
<td>pJR1277</td>
<td>H M R-SS rap1-</td>
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<td>pJR1281</td>
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<td>pJR1278</td>
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</table>

H M R-I were constructed by inserting the BglII-HindIII fragment of pJR1270 that contained the H M R-I deletion into BglII-HindIII-cleaved plasmids that harbored the appropriate allele of H M R-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 H M R alleles, linear fragments of H M R were cleaved from 5 μg of plasmid and transformed into yeast cells in which H M R had been replaced with URA3 (hmr::URA3). A total of 2.6 × 10⁷ cells of the transformed culture were plated on solid rich medium supplemented with adenine, grown for seven doublings (2.5–3 days at 30°C), and resuspended in YPD. A total of 2 × 10⁶ 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 H M R allele. Allelic replacement at H M R was confirmed by DNA blot analysis. These conditions typically yield 20–200 colonies, the majority of which contain the appropriate gene replacement.

### RESULTS

**H M R-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, H M R-E proved to be a chromosomal origin of replication (Rivier and Rine 1992a), whereas H M L-E and H M L-I are not (Dubey et al. 1991). Therefore, we tested whether H M R-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 H M R-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 H M R-I silencer, but not the H M R-E silencer. Bubble-shaped replication intermediates were detected in this fragment, revealing that a chromosomal origin of replication was close to, or coincident with, H M R-I (Figure 1). To determine whether the origin colocalized with the H M R-I silencer, the replication intermediates

![Figure 1](image_url)
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. MATα HMR-Ra strains in which HMR is silent display the α-mating phenotype, whereas MATα HMR-R 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 (Br 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 10⁻⁶ 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
resulted in white colonies, indicating that the pink color is the result of silencing of the synthetic silencer. Shown are patch mating and quantitative mating analysis for strains with HMR-I (left) and HMR-I (right). HMR-I strains are (top to bottom): DBY703 (wild type), DRY470 (HMR DE), DRY645 (HMR-SS), DRY536 (HMR-SS abf1-2), DRY528 (HMR-SS abf1-1), DRY502 (HMR-SS rap1-1), and DRY574 (HMR-SS acs-1). Strains lacking HMR-I are (top to bottom): DRY482 (HMR D), DRY604 (HMR DE), DRY631 (HMR-SS D), DRY626 (HMR-SS abf1-2 D), DRY574 (HMR-SS abf1-1 D), DRY490 (HMR-SS rap1-D), and DRY514 (HMR-SS acs-D).

**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 DE), DRY645 (HMR-SS), DRY536 (HMR-SS abf1-2), DRY528 (HMR-SS abf1-1), DRY502 (HMR-SS rap1-1), and DRY574 (HMR-SS acs-1). Strains lacking HMR-I are (top to bottom): DRY482 (HMR D), DRY604 (HMR DE), DRY631 (HMR-SS D), DRY626 (HMR-SS abf1-2 D), DRY574 (HMR-SS abf1-1 D), DRY490 (HMR-SS rap1-D), and DRY514 (HMR-SS acs-D).

HMR 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 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.

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 (Renaud 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 of adenine.

**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::ADE2 ΔE), and DRY815 (HMR-SS::ADE2).
**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), DRY1692 (HMR ΔI), DRY1693 (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 α-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 quantitative 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 MATα1 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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