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A role for a replicator dominance mechanism in silencing

Madeleine A.Palacios DeBeer and Catherine A.Fox[1]

Department of Biomolecular Chemistry, 587 MSC 1300 University Avenue, University of Wisconsin, Madison, WI 53706-1532, USA

[1]Corresponding author
e-mail: cfox@facstaff.wisc.edu

The role of the natural *HMR*-E silencer in modulating replication initiation and silencing by the origin recognition complex (ORC) was examined. When natural *HMR*-E was the only silencer controlling *HMR*, the silencer's ORC-binding site (ACS) was dispensable for replication initiation but essential for silencing, indicating that a non-silencer chromosomal replicator(s) existed in close proximity to the silencer. Further analysis revealed that regions flanking both sides of *HMR*-E contained replicators. In contrast to replication initiation by the intact silencer, initiation by the non-silencer replicator(s) was abolished in an *orc2-1* mutant, indicating that these replicators were extremely sensitive to defects in ORC. Remarkably, the activity of one of the non-silencer replicators correlated with reduced silencing; inactivation of these replicators caused by either the *orc2-1* mutation or the deletion of flanking sequences enhanced silencing. These data were consistent with a role for the ORC bound to the *HMR*-E silencer ACS in suppressing the function of neighboring ORC molecules capable of inhibiting silencing, and indicated that differences in ORC-binding sites within *HMR* itself had profound effects on ORC function. Moreover, replication initiation by natural *HMR*-E was inefficient, suggesting that closely spaced replicators within *HMR* contributed to an inhibition of replication initiation.

Keywords: ORC/origins/replication/silencers/yeast

Introduction

One characteristic of eukaryotic DNA replication is that individual chromosomes initiate replication at more than one distinct position, or replication origin, during the S-phase of the cell cycle (Hand, 1978; Fangman and Brewer, 1991; DePamphilis, 1996). Although many individual origins are used during a given S-phase, studies in yeast indicate that many of these origins are not required for efficient replication of the eukaryotic genome (Dershowitz and Newlon, 1993), suggesting that individual origins may play roles in chromosome function which extend beyond their direct contribution to chromosome duplication. Consistent with this view is the observation that individual yeast replication origins display unique characteristics. For example, some yeast origins are efficient, initiating once per cell cycle, whereas others are inefficient, initiating in only a fraction of cell divisions (Newlon *et al*., 1993; Newlon, 1996). Each origin initiates at a specific time during S-phase, with some origins initiating at the beginning and others at the end, after most of the genome has been replicated (Reynolds *et al*., 1989; Friedman *et al*., 1997; Yamashita *et al*., 1997). Moreover, there exists a small class of specialized origins which is closely associated with elements that control the expression of nearby genes (Loo and Rine, 1995). Identifying features that contribute to origins' distinct characteristics should provide insights into the relationships between chromosome maintenance and expression.

At some level, the regulation of individual origin function must involve the origin recognition complex (ORC), the protein complex that binds to the conserved sequence, the autonomously replicating sequence (ARS) consensus sequence (ACS), which is common to yeast replicators. A replicator is defined as a genetic element that controls origin activity (Jacob *et al*., 1963; Stillman, 1993), and in yeast it appears that replicators and their origins are very close together if not coincident (Bielinsky and Gerbi, 1998). The ACS is an 11-bp AT-rich sequence that is necessary, but not sufficient, for replicator function (Van Houten and Newlon, 1990; Deshpande and Newlon, 1992; Marahrens and Stillman, 1992; Rivier and Rine, 1992; Huang and Kowalski, 1993). The ORC is a six-subunit protein complex identified by its ability to bind to the ACS of yeast replicators in an ATP-dependent manner (Bell and Stillman, 1992). Several independent studies indicate that ORC is the best candidate for the eukaryotic replication initiator. For example, each of the genes encoding the ORC subunits is essential in yeast (Foss *et al*., 1993; Li and Herskowitz, 1993; Micklem *et al*., 1993; Bell *et al*., 1995), and mutations in *ORC* genes cause initiation defects at individual chromosomal origins (Fox *et al*., 1995; Liang *et al*., 1995). In addition, ORC homologs have been identified in a number of eukaryotic organisms including humans (Gavin *et al*., 1995; Gossen *et al*., 1995), and in some systems have been shown to be required for DNA replication *in vitro* (Carpenter *et al*., 1996; Walter *et al*., 1998). Thus, it is probable that ORC is required for the fundamental function of replicators: the ability to direct origin unwinding. The distinct features of an individual origin may be influenced by a number of factors, including direct interactions between ORC and the DNA comprising a particular replicator (Lee and Bell, 1997), and/or by interactions between ORC and other proteins that bind near the ACS of a particular replicator.

The *HMR*-E silencer in yeast is a member of the small class of replicators associated with elements that control the transcription of nearby genes (Loo and Rine, 1995). Specifically, *HMR*-E is a DNA element required for

repression of the *HMR* silent mating-type locus, one of two silent mating-type loci that act as storage cassettes for copies of the yeast mating-type genes. In yeast, mating type is controlled by the genes present at the transcriptionally expressed *MAT* locus (Herskowitz *et al*., 1992). Copies of the mating-type genes also reside at *HMR* and *HML*, where they are repressed by a mechanism known as silencing. Silencing involves the assembly of a specialized chromatin structure analogous to heterochromatin and requires the action of DNA elements that flank each locus, called the E and I silencers (Loo and Rine, 1995). Several proteins are also required for silencing and these fall into two classes. The first includes the silencer-binding proteins ORC, Rap1p and Abf1p, which bind silencer DNA directly through their binding sites within the silencer itself (Shore, 1994; Dillin and Rine, 1995; Loo and Rine, 1995; Loo *et al*., 1995b). These silencer-binding proteins recruit the second class of proteins, characterized by the four Sir proteins, to silent mating-type cassettes through protein–protein interactions. In particular, evidence from several studies indicates that ORC functions in the recruitment of the Sir1p to the silencer through direct interactions (Chien *et al*., 1993; Triolo and Sternglanz, 1996; Fox *et al*., 1997; Gardner *et al*., 1999), and, in turn, Sir1p helps recruit the three other Sir proteins to *HMR* and *HML*, where they function as structural components of silent chromatin (Hecht *et al*., 1995, 1996; Grunstein, 1997; Strahl-Bolsinger *et al*., 1997). Significantly, the E and I silencers at both *HMR* and *HML* contain an ACS and can provide for autonomous replication of plasmids, indicating an association between silencer and origin function (Loo and Rine, 1995). In particular, the *HMR*-E silencer also functions as a replicator in its chromosomal context (Rivier and Rine, 1992). Studies of a simplified version of the *HMR*-E silencer called the synthetic silencer provide evidence for ORC's role in both the silencing and replication origin functions at *HMR* (Fox *et al*., 1995; Dillin and Rine, 1997).

Although the synthetic version of *HMR*-E has been useful in the identification and initial characterization of *ORC* genes (McNally and Rine, 1991; Foss *et al*., 1993; Fox *et al*., 1995; Loo *et al*., 1995a; Dillin and Rine, 1997), evidence indicates that the natural *HMR*-E silencer possesses additional features that could provide further insights into the mechanisms modulating ORC function at this chromosomal domain. The initial characterization of *ORC* genes in silencing and replication at *HMR* exploited strains harboring an *HMR* locus that contained the synthetic silencer version of *HMR*-E, in place of the natural *HMR*-E silencer and a deletion of the *HMR*-I silencer element. In this relatively simplified context, mutations in the synthetic silencer's ORC-binding site (ACS), or mutations in individual *ORC* genes abolish or reduce both replication initiation and silencing at *HMR* (McNally and Rine, 1991; Rivier and Rine, 1992; Foss *et al*., 1993; Fox *et al*., 1995). In contrast, neither mutations in the natural *HMR*-E silencer's ACS (Brand *et al*., 1987) nor mutations in individual *ORC* genes reduce silencing at *HMR* (Foss *et al*., 1993; Fox *et al*., 1995), suggesting that additional elements at *HMR* substitute for ORC's silencing function. However, it is important to note that two different variables in these previous experiments prevent identification of the individual features of natural *HMR*-E that contribute to this redundancy. First, early studies indicating that the natural *HMR*-E silencer's ACS is not necessary for silencing *HMR* were performed in strains containing the *HMR*-I element. Although the *HMR*-I silencer is not sufficient for silencing *HMR*, it can modulate silencing efficiency at this locus (Abraham *et al*., 1984; Fox *et al*., 1995; Rivier *et al*., 1999) and it acts as a chromosomal origin (Rivier *et al*., 1999). Thus, the presence of *HMR*-I makes it difficult to analyze the role of the natural *HMR*-E silencer's ACS, or the role that the ORC plays in the natural *HMR*-E element's functions. Secondly, the natural *HMR*-E silencer itself contains near flanking sequences that were removed in the construction of the synthetic silencer, which may contribute to both silencing and replication initiation at *HMR* (McNally and Rine, 1991). For example, even in the absence of the *HMR*-I silencer, the replication and silencing functions of natural *HMR*-E are not reduced by the same mutations in *ORC* genes that abolish these functions in the synthetic silencer (Fox *et al*., 1995). Furthermore, in the absence of *HMR*-I, *HMR*-E is a more effective silencer than the synthetic silencer (Fox *et al*., 1995). Intriguingly, sequences that flank *HMR*-E contain several near matches to the ACS (Loo and Rine, 1995), and an early study indicates that a second region near *HMR*-E, but distinct from the silencer ACS, can confer autonomous replication to plasmids (Brand *et al*., 1987). A simple hypothesis to explain these observations is that the redundancy at natural *HMR* is due, in part, to sequences flanking the *HMR*-E silencer, which can bind additional ORC molecules and substitute for the role of the ORC bound to the *HMR*-E silencer's ACS. A test of this hypothesis requires examination of the functions of the natural *HMR*-E silencer within a chromosomal *HMR* locus that lacks the *HMR*-I silencer.

In this report, we initiated an analysis of natural *HMR*-E's silencer and replication origin functions. To focus on the function of natural *HMR*-E and its flanking sequences, the *HMR*-I element was deleted from all strains examined. We addressed three issues relevant to the behavior of natural *HMR*-E. First, we determined whether the DNA region adjacent to natural *HMR*-E contributed to ORC's function in replication initiation at *HMR*. Secondly, we determined whether this DNA region contributed to ORC's role in silencing *HMR*. Thirdly, we examined replication initiation efficiency within an *HMR* locus under the control of the natural *HMR*-E silencer and determined how the silencer's ACS affected this efficiency. Our data provided evidence that ORC bound to the silencer ACS suppressed the function of neighboring non-silencer replicators that could direct efficient initiation from *HMR*, suggesting a second role for ORC in silencing beyond its previously characterized role in recruiting the Sir1 protein.

Results

Previous studies that established a requirement for ORC in both silencing and origin function at *HMR* exploited a simplified version of the *HMR*-E silencer called the synthetic silencer because it is particularly sensitive to defects in ORC. Specifically, in two-dimensional origin-mapping experiments to measure initiation on the chromosome, initiation by the synthetic silencer replicator is severely reduced in an *orc2-1* mutant yeast strain

(Fox *et al*., 1995; Figure 1A, panels 2 and 3). In addition, the mating properties of isogenic *MAT*α strains indicate that silencing at *HMR* under the control of the synthetic silencer is reduced significantly in an *orc2-1* mutant (Foss *et al*., 1993; Fox *et al*., 1995; Figure 2). In contrast, natural *HMR*-E's replicator and silencer functions are unaffected by the same *orc2-1* mutation that reduces these functions of the synthetic silencer (Fox *et al*., 1995; Figures 1B and 2). In fact, in a two-dimensional origin-mapping experiment a slight, but reproducible, increase in the number of replication bubble intermediates, relative to small forks, is observed for the natural *HMR*-E in an *orc2-1* mutant compared with a wild-type *ORC* strain, suggesting that the initiation frequency of natural *HMR*-E is enhanced slightly by defects in ORC (Fox *et al*., 1995; Figure 1B, panels 1 and 2). The mating properties of isogenic *MAT*α strains indicate that silencing controlled by the natural *HMR*-E silencer is also not reduced in an *orc2-1* mutant (Fox *et al*., 1995; Figure 2). Thus, in contrast to the synthetic silencer, natural *HMR*-E's silencing function is not reduced by the *orc2-1* mutation. Furthermore, in contrast to synthetic silencer replicator and other replicators examined in an *orc2-1* mutant (Fox *et al*., 1995; Liang *et al*., 1995), initiation by the natural *HMR*-E replicator is not reduced by a defect in ORC caused by the *orc2-1* mutation.

One feature of the origin associated with *HMR*-E that distinguishes it from most other origins is that it resides within a region of DNA, the *HMR* locus, that is assembled into a repressive, or silenced, form of chromatin. Therefore, to determine whether the transcriptional state at *HMR* influenced the effects of the *orc2-1* mutation on replication initiation by natural *HMR*-E, we evaluated replication initiation by *HMR*-E in a set of isogenic strains containing a deletion of *SIR2* (Figure 1C). The *SIR2* gene encodes the Sir2 protein, one of four Sir proteins essential for silencing *HMR* (Loo and Rine, 1995). In the absence of *SIR2*, *HMR* is assembled into a transcriptionally active form of chromatin. Deletion of *SIR2* caused a slight enhancement in replication initiation frequency at *HMR*-E in a two-dimensional origin-mapping experiment (Figure 1C, compare panel 1 with panel 2), indicating that the silenced state did inhibit initiation within *HMR* somewhat. However, even in absence of *SIR2*, the *orc2-1*

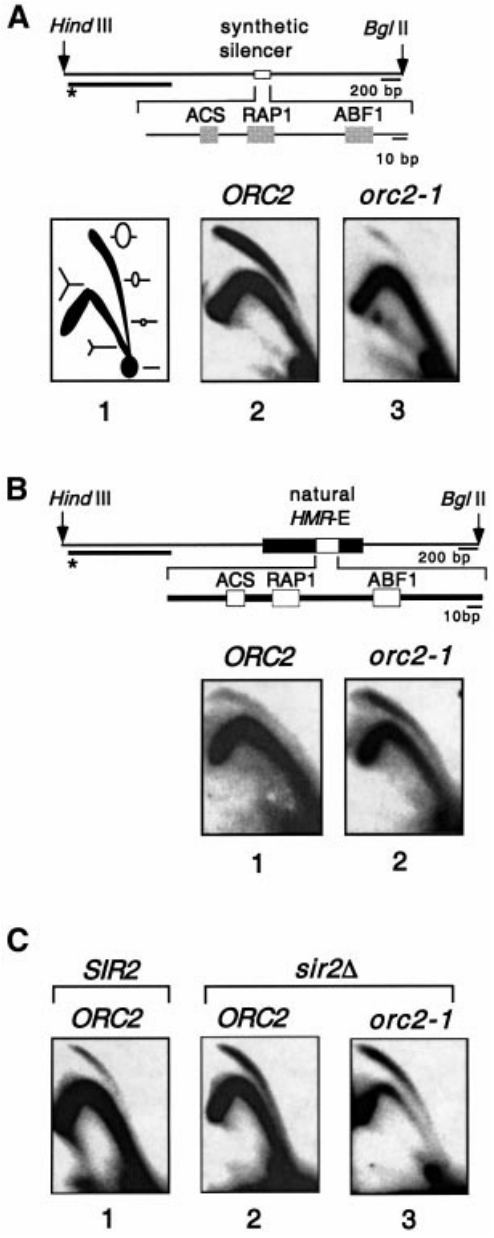

Fig. 1. Replication initiation by the synthetic silencer, but not natural *HMR*-E, was sensitive to defects in *ORC* caused by the *orc2-1* mutation. (**A**) Replication initiation was monitored at the synthetic silencer in two-dimensional origin-mapping experiments. Diagram of the *Hin*dIII–*Bgl*II *HMR* fragment containing the synthetic silencer examined in these experiments. The white box represents the synthetic silencer shown in expanded form below the fragment. The gray boxes represent the individual elements of the silencer: an ORC-binding site (ACS), a Rap1p-binding site and an Abf1p-binding site.
Panel 1: Representation of how two-dimensional origin mapping gels distinguish replication initiation bubbles from replication forks. A qualitative measure of origin efficiency is reflected by the ratio of replication bubbles to small forks; all other factors being equal, a more efficiently used replication origin will have a larger replication bubble to small forks ratio (Fangman and Brewer, 1991). Panels 2 and 3: The results of two-dimensional origin-mapping experiments of a pair of isogenic strains containing the synthetic silencer at *HMR* and either wild-type *ORC* or an *orc2-1* mutation (CFY36, CFY285).
(**B**) Replication initiation was monitored at natural *HMR*-E in two-dimensional origin-mapping experiments. Diagram of the *Hin*dIII–*Bgl*II *HMR* fragment examined in these experiments. The thick black portion of the fragment flanking the silencer represents the region present in natural *HMR*-E but deleted from *HMR* containing the synthetic silencer. The white box represents the natural silencer which is shown in expanded form below the fragment. The binding sites within the silencer itself are represented by white boxes to indicate that the exact sequences differ from the analogous binding sites in the synthetic silencer represented by gray boxes in (A) (McNally and Rine, 1991). In addition, the line representing the natural silencer is thicker than that used to represent the synthetic silencer in (A) to indicate that the sequences in between the binding sites themselves differ between the natural and synthetic silencer (McNally and Rine, 1991). Panels 1 and 2: The results from two-dimensional origin-mapping experiments of a pair of isogenic strains containing the natural *HMR*-E origin at *HMR* and either wild-type *ORC* or an *orc2-1* mutation (CFY37, CFY290). (**C**) The results from two-dimensional origin-mapping experiments of a set of isogenic strains containing natural *HMR*-E at *HMR* and either wild-type *ORC* or an *orc2-1* mutation. The strains used to generate panels 2 and 3 also contain a null mutation in *SIR2* (CFY37, CFY393, CFY391). The probe used to detect both the synthetic silencer and natural *HMR*-E-containing fragments is shown by a line marked with an asterisk below the fragment representations in (A) and (B). The primers used to generate the probe DNA fragment complementary to *HMR* were CTGGTCCTCACAGTTCGCAG and CAAGAAGTTCCCCTTGAAG.

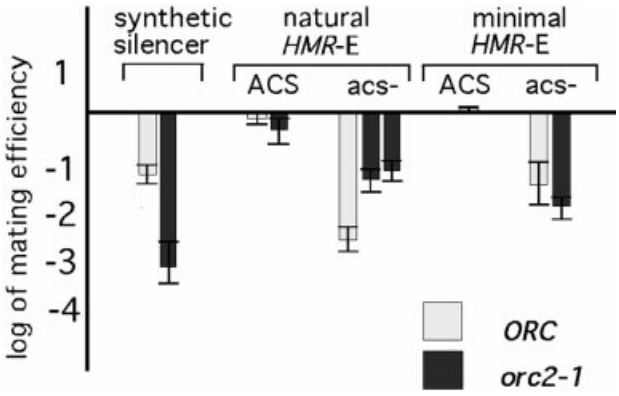

Fig. 2. Results of quantitative mating assays performed with the strains used for this study. The data are presented as the log of the mating efficiencies of each strain. *MAT*α strains to be tested were mated with an excess of **a** mating-type cells (CFY616) and diploids were selected at 27°C. The mating of *MAT*α yeast cells with *MAT***a** cells can be selected for by selecting for the growth of diploids on selective media. Defects in silencing *HMR***a** in the *MAT*α strains being tested cause defects in the ability of those *MAT*α cells to mate and form diploids that can grow on the selective media. Mating efficiencies are equal to the number of cells that mated divided by the number of viable cells for each strain, and provide a quantitative measure of silencing at *HMR***a**. All the strains were isogenic *MAT*α, lacked the *HMR*-I silencer at *HMR***a**, and contained the version of the *HMR*-E silencer indicated together with either wild-type *ORC* or the *orc2-1* mutation. The synthetic-silencer-containing strains were either *ORC2* (gray bar, CFY36) or *orc2-1* (black bar, CFY285). The natural *HMR*-E-containing strains with a wild-type ACS were either *ORC2* (gray bar, CFY37) or *orc2-1* (black bar, CFY290). The natural *HMR*-E-containing strains with a mutation in the silencer ACS (acs-) were either *ORC2* (gray bar, CFY108) or *orc2-1* (black bar, CFY201 or CFY143, left to right on the figure, respectively). The minimal *HMR*-E silencer-containing strain was *ORC2* (ACS, gray bar, CFY3). The minimal *HMR*-E silencer-containing strains with a mutation in the silencer ACS (acs-) were either *ORC2* (gray bar, CFY140) or *orc2-1* (black bar, CFY244).

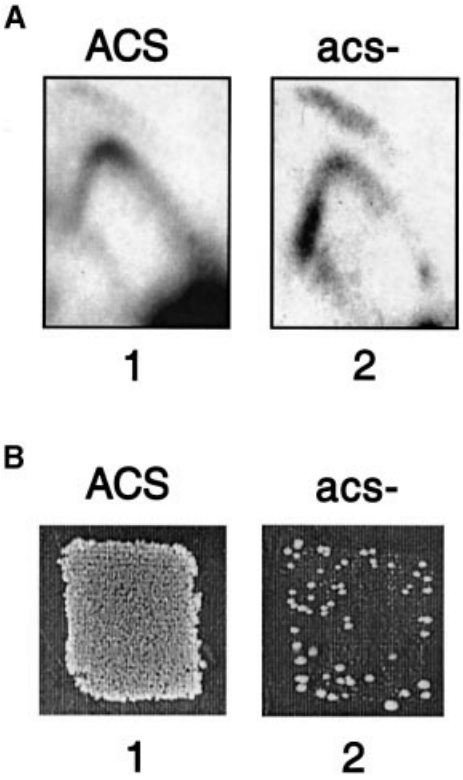

Fig. 3. The ACS within the natural *HMR*-E silencer was dispensable for initiation but was required for silencing. (**A**) The results from two-dimensional origin-mapping experiments of a pair of isogenic strains containing natural *HMR*-E at *HMR* with either a wild-type ACS or a mutant ACS (acs-) within the silencer (CFY37, CFY108). The probe used for this experiment was the same as that described in Figure 1. (**B**) The results from mating experiments with the same strains used in (A). The *MAT*α strains were grown on rich media at 23°C for 24 h and then replica-plated into minimal media containing a lawn of *MAT***a** cells at 27°C. Growth of diploid cells on minimal media reflected the extent of mating and thus the degree of silencing at *HMR***a**.

mutant yeast cells initiated replication within *HMR* more frequently than isogenic *ORC2* cells, based on the results of a two-dimensional origin-mapping experiment (Figure 1C, compare panel 2 with panel 3). Thus, the transcriptional state at *HMR* did not significantly influence the effect of the *orc2-1* mutation on replication initiation from *HMR*-E. Since the *orc2-1* mutation causes obvious defects in replication initiation efficiency at several origins (Fox *et al*., 1995; Liang *et al*., 1995), the effect of this mutation on initiation by natural *HMR*-E was exceptional. A simple hypothesis to explain the unusual behavior of the natural *HMR*-E origin was that sequences adjacent to natural *HMR*-E could substitute for the role of the ORC-binding site within the defined *HMR*-E silencer itself.

Natural HMR contained non-silencer replicator activity

If the DNA region immediately adjacent to natural *HMR*-E contained an additional ORC-binding site(s), which could substitute for the functions of the silencer ORC-binding site (ACS), then the natural *HMR*-E silencer's ACS would be dispensable for both replication initiation and silencing at *HMR*. Therefore, we mutated the ACS within the defined *HMR*-E silencer and determined the effect of this mutation on replication initiation and silencing at *HMR* (Figure 3A).

Mutation of the ACS in *HMR*-E [*HMR*-E(acs-)] caused no reduction in replication initiation efficiency at *HMR*, as measured by a two-dimensional origin-mapping experiment (Figure 3A). This behavior was in contrast to that of the synthetic silencer, which requires its ACS for replicator function (Rivier and Rine, 1992). Therefore the single exact match to an ACS within *HMR*, the ACS within the defined *HMR*-E silencer itself, was not required for chromosomal origin function at an *HMR* locus controlled by natural *HMR*-E. Thus, natural *HMR* contained an additional ORC-binding site(s) in the close vicinity of the *HMR*-E silencer, as reflected by replication initiation, which occurred independently of the *HMR*-E silencer ACS, and a pattern of replication intermediates indistinguishable from that formed by wild-type *HMR*-E.

If the additional replicator activity at natural *HMR* was also providing ORC-dependent silencing activity, then the ACS in natural *HMR*-E would not be required for silencing at *HMR*. Therefore, silencing by the mutant silencer was measured by comparing the mating properties of an isogenic pair of *MAT*α strains containing either the natural wild-type *HMR*-E silencer or the natural *HMR*-E silencer with a mutant ACS at *HMR***a** (Figures 2 and 3B). Significantly, the mutant *HMR*-E(acs-) silencer failed to provide for efficient silencing at *HMR***a**. Therefore, the replicator activity that was independent of the silencer ACS was referred to as the non-silencer replicator. Since the ACS

in *HMR*-E is dispensable for silencing in the presence of the *HMR*-I silencer (Brand *et al*., 1987), these data provided additional evidence for a role of the *HMR*-I element in modulating silencing (Abraham *et al*., 1984; Fox *et al*., 1995; Rivier *et al*., 1999). More importantly, the origin activity that remained at *HMR* in the absence of the silencer ACS indicated that the remaining ORC(s), which bound DNA in the close vicinity of the silencer and provided for replicator function, failed to provide for efficient silencing, even though the other elements of the silencer, the Rap1p- and Abf1p-binding sites, were still present.

Reduced ORC function enhanced silencing by the mutant HMR-E silencer

Although silencing by the mutant *HMR*-E(acs-) silencer was reduced significantly, it was not abolished. One possibility was that the ORC that functioned at the non-silencer replicator contributed a small amount of residual silencing activity. If this were true then the *orc2-1* allele, which reduces the amount of functional ORC in a cell (Bell *et al*., 1993), might reduce further the small amount of silencing at an *HMR***a** locus controlled by the mutant *HMR*-E(acs-) silencer. Therefore, the effect of the *orc2-1* mutation on silencing at *HMR***a** was measured by comparing the mating properties of an isogenic set of *MAT*α strains containing the mutant *HMR*-E(acs-) silencer combined with wild-type *ORC* or the *orc2-1* allele (Figures 2 and 4A).

Surprisingly, the *orc2-1* mutation significantly improved silencing at an *HMR***a** locus controlled by the mutant *HMR*-E(acs-) silencer (Figures 2 and 4A). The ability of the *orc2-1* mutation to enhance silencing by the mutant *HMR*-E(acs-) silencer was recessive, as are the other phenotypes caused by the *orc2-1* mutation (Foss *et al*., 1993), strongly suggesting that a loss of ORC function was responsible for the enhanced silencing phenotype (M.A.Palacios DeBeer and C.A.Fox, unpublished data). Since the *orc2-1* mutation causes defects in replication initiation at chromosomal origins, these data provided evidence that ORC's replication function could inhibit the formation of silent chromatin at *HMR*.

Initiation by non-silencer replicator was abolished by the orc2-1 mutation

The above data provided evidence that, in the absence of the silencer ACS, ORC's replication function could inhibit the assembly of silent chromatin at *HMR*. If this ORC-dependent inhibitory activity was a result of ORC bound in the near vicinity of *HMR*, then it was possible that ORC function at the non-silencer replicator itself actually inhibited silencing. If this were true, then initiation controlled by the non-silencer replicator might be reduced by the *orc2-1* mutation. Therefore, non-silencer replicator activity was evaluated directly in isogenic *ORC2* and *orc2-1* strains (Figure 4B). Strikingly, by two-dimensional origin-mapping experiments, the *orc2-1* mutation caused a drastic reduction in non-silencer replicator activity at *HMR*. Thus, in the absence of the silencer ACS, the remaining origin activity at *HMR*, which was at least as robust as the origin activity of the intact silencer as measured by two-dimensional origin mapping gels (Figure 4B, compare panel 1 with panel 2), was extremely

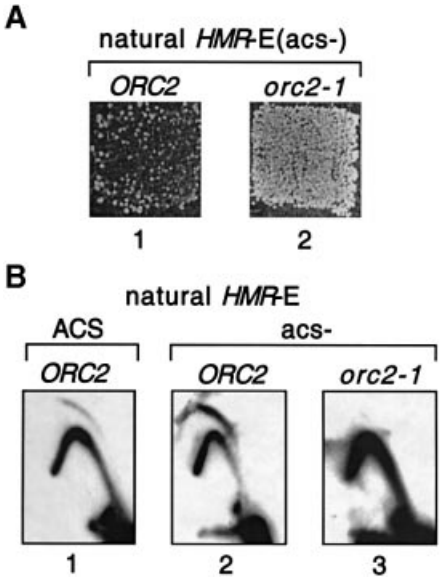

Fig. 4. Reduced ORC function enhanced silencing by the mutant *HMR*-E(acs-) silencer and abolished initiation by the non-silencer replicator(s). (**A**) The results from patch-mating experiments of isogenic *MAT*α strains containing the mutant natural *HMR*-E(acs-) silencer at *HMR***a** and either wild-type *ORC* or an *orc2-1* mutation (CFY108, CFY201). The strains were grown on rich media at 23°C for 24 h and then replica-plated to minimal media containing a lawn of *MAT***a** cells at 27°C. Growth of diploid cells on minimal media reflected the extent of mating and thus the degree of silencing at *HMR***a**. (**B**) The results from two-dimensional origin-mapping experiments of a set of isogenic *MAT*α strains containing the natural *HMR*-E origin at *HMR* with either a wild-type or mutant ACS and wild-type *ORC* or an *orc2-1* mutation (CFY37, CFY108, CFY201). The probe used for this experiment was the same as that described in Figure 1.

sensitive to a reduction in ORC activity caused by the *orc2-1* mutation.

A region adjacent to HMR-E was required for both non-silencer replicator activity and ORC-dependent inhibition of silencing

The data described above were consistent with the view that the *orc2-1* mutation enhanced silencing by the mutant silencer by reducing the function of the non-silencer replicator at *HMR*. If this view were correct, deletion of the non-silencer replicator would also enhance silencing by the mutant *HMR*-E(acs-) silencer. At first it seems as though the synthetic silencer could be used to address this issue, because an *HMR* locus controlled by this silencer lacks the non-silencer replicator; mutation of the synthetic silencer ACS abolishes all origin function at *HMR*, indicating that the synthetic silencer is the only functional replicator in the vicinity of an *HMR* locus lacking the *HMR*-I element (Rivier and Rine, 1992; Fox *et al*., 1995). However, the synthetic silencer also differs in a number of other ways from natural *HMR*-E (McNally and Rine, 1991). Therefore, to focus on the region adjacent to *HMR*-E, we constructed a minimal version of *HMR*-E (minimal *HMR*-E; Figure 5A). The minimal *HMR*-E silencer was identical to natural *HMR*-E except that it lacked the region of DNA surrounding the defined silencer (compare Figure 1B with 5A).

As expected, the minimal *HMR*-E silencer functioned as both a replication origin and a silencer, as measured

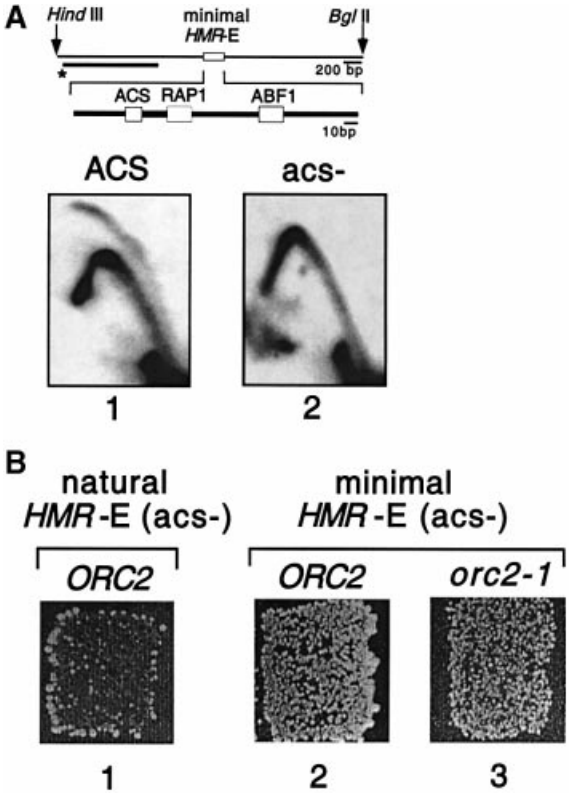

Fig. 5. The minimal *HMR*-E silencer required its ACS for efficient initiation but not for silencing. (**A**) Diagram of the *Hin*dIII–*Bgl*II *HMR* fragment examined in these experiments. Note that minimal *HMR*-E was identical to natural *HMR*-E except that it lacked the adjacent sequences (compare diagrams in Figures 1B and 5A, and see Materials and methods). The region of DNA immediately adjacent to the defined silencer is represented by the thick black bar in Figure 1B and is missing in Figure 5A to indicate that this region was deleted from minimal *HMR*-E. Panels 1 and 2: Replication initiation was monitored at *HMR***a** containing the minimal *HMR*-E silencer with a wild-type or mutant ACS (acs-) in two-dimensional origin-mapping experiments (CFY3, CFY140). The probe used to detect the minimal *HMR* fragment was the same as that used for the experiments in Figure 1 and is shown with a line marked with an asterisk below the fragment representation. (**B**) The results from mating experiments of isogenic *MAT*α strains containing the mutant natural *HMR*-E(acs-) silencer or the minimal *HMR*-E(acs-) silencer at *HMR***a** and either the wild-type *ORC* or an *orc2-1* mutation (CFY108, CFY140, CFY244). The strains were grown on rich media at 23°C for 24 h and then replica-plated to minimal media containing a lawn of *MAT***a** cells at 27°C. Growth of diploid cells on minimal media reflected the extent of mating and thus the degree of silencing at *HMR***a**.

by two-dimensional origin-mapping and yeast mating experiments, respectively (Figures 2 and 5). Importantly, mutation of the ACS within minimal *HMR*-E significantly reduced initiation at *HMR* (Figure 5A, compare panel 1 with panel 2), indicating that sequences adjacent to the natural *HMR*-E silencer did indeed provide for the function of a chromosomal replicator that was distinct from the silencer replicator itself (compare Figure 3A with 5A).

If non-silencer replicator activity contributed to the low silencing efficiency of the mutant natural *HMR*-E(acs-) silencer, then removal of a region required for this activity should enhance silencing. Therefore, the level of silencing conferred by the minimal *HMR*-E silencer with an ACS mutation should be greater than that conferred by the natural *HMR*-E silencer harboring the identical ACS mutation, and silencing conferred by a minimal *HMR*-E

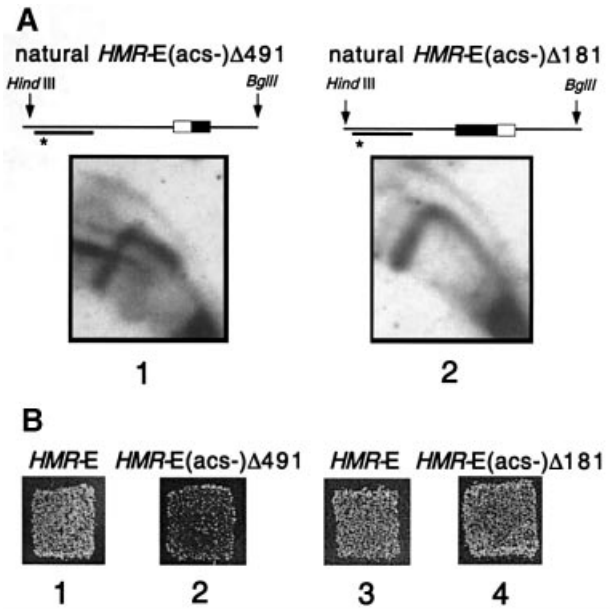

Fig. 6. The regions centromere-proximal and telomere-proximal to *HMR*-E each contained replicators. (**A**) Diagram of the *Hin*dIII–*Bgl*II *HMR* fragment examined in these experiments and the results of two-dimensional origin-mapping experiments. The region of DNA immediately adjacent to the defined silencer (represented by the white box) is represented by a thick black bar. Natural *HMR*-E(acs-)Δ491 (CFY693) lacks the 491 bp region centromere-proximal to *HMR*-E, but contains the 181 bp telomere-proximal region, represented by the thick black bar in the diagram above the corresponding two-dimensional origin-mapping gel in panel 1. Natural *HMR*-E(acs-)Δ181 (CFY692) lacks the 181 bp region telomere-proximal to *HMR*-E but contains the 491 bp centromere-proximal region, represented by the thick black bar in the diagram above the corresponding two-dimensional origin-mapping gel in panel 2. The probe used to detect the *HMR* fragments was the same as that used for the experiments in Figure 1 and is shown by a line marked with an asterisk below the fragment representation. (**B**) Results from mating experiments of isogenic *MAT*α strains containing natural *HMR*-E (CFY37; panels 1 and 3), or natural *HMR*-E(acs-)Δ491(CFY693; panel 2), natural *HMR*-E(acs-)Δ181(CFY692; panel 4) at *HMR***a**. The strains were grown on rich media at 23°C for 24 h and then replica-plated into minimal media containing a lawn of *MAT***a** cells at 27°C. Growth of diploid cells on minimal media reflected the extent of mating and thus the degree of silencing at *HMR***a**.

with a mutant ACS was determined (Figures 2 and 5B). As predicted, silencing by the minimal *HMR*-E silencer was less sensitive to mutation of its ACS than the natural *HMR*-E silencer, consistent with the view that sequences adjacent to *HMR-E* could inhibit silencing. Significantly, the *orc2-1* mutation did not enhance silencing by the minimal mutant *HMR*-E(acs-) silencer (Figures 2 and 5B). This observation was consistent with the view that *orc2-1* enhanced silencing by reducing the function of a non-silencer replicator adjacent to *HMR*-E.

Natural HMR contained at least three replicators

To begin mapping the regions required for chromosomal non-silencer replicator function in the vicinity of *HMR*, replicator activity was measured by two-dimensional origin mapping in an isogenic pair of yeast strains. The first strain lacked the adjacent 491 bp region centromere-proximal to *HMR*-E [natural *HMR*-E(acs-)Δ491; Figure 6A, panel 1], and the second strain lacked the 181 bp region telomere-proximal to *HMR*-E [natural *HMR*-E(acs-)Δ181; Figure 6A, panel 2]. Both strains

contained the same mutation in the silencer ACS used in the experiments described above. Interestingly, both strains contained similar levels of replicator activity as measured by two-dimensional origin-mapping gels. Therefore, natural *HMR* contained at least three potential replicators in the vicinity of the *HMR*-E silencer: the silencer replicator itself, which required the silencer ACS (Figure 5A, panels 1 and 2); the telomere-proximal replicator (Figure 6A, panel 1); and the centromere-proximal replicator (Figure 6A, panel 2).

The region required for ORC-dependent inhibition of silencing included the telomere-proximal replicator

To determine whether sequences required for the *ORC2*-dependent inhibition of silencing mapped to the regions containing either of the two non-silencer replicators described above, silencing was measured by comparing the mating properties of three isogenic *MAT*α strains: one strain contained the natural *HMR*-E silencer (Figure 6B, panels 1 and 3), one strain contained the natural *HMR*-E(acs-) silencer that lacked the centromere-proximal replicator [*HMR*-E(acs-)Δ491; Figure 6B, panel 2], and one strain contained the natural *HMR*-E(acs-) silencer that lacked the telomere-proximal replicator [*HMR*-E(acs-)Δ181; Figure 6B, panel 4]. Significantly, the 181 bp telomere-proximal region caused a significant reduction in silencing as measured by mating (Figure 6B, panel 2). Furthermore, this inhibition of silencing was reduced in an *orc2-1* mutant (M.A.DeBeer and C.A.Fox, data not shown), indicating that these 181 bp contained the *ORC2*-dependent sequences that could inhibit the assembly of silent chromatin at *HMR*. Thus, the 181 bp region telomere-proximal to *HMR*-E contained sequences that provided for both non-silencer replicator function and *ORC*-dependent inhibition of silencing.

Replication initiation at HMR controlled by natural HMR-E occurred in only a fraction of cell divisions and was enhanced by mutation of the silencer ACS

The data described above indicated the presence of at least three potential replicators within the natural *HMR* locus, each of which alone was capable of initiating replication with a similar efficiency as measured by two-dimensional origin-mapping experiments. One issue raised by these data concerned the frequency of replication initiation within the natural *HMR* locus. It was possible that the presence of multiple potential replicators increased the probability of an initiation event occurring at *HMR*, such that initiation occurred at a high frequency in this chromosomal region. For example, previous studies established that multiple origins can enhance the probability of an initiation event occurring in a plasmid context (Hogan and Koshland, 1992). However, the low ratio of bubble intermediates to small forks observed in two-dimensional origin-mapping experiments of the intact natural *HMR*-E origin suggested that initiation at *HMR* actually occurred at a low frequency (Rivier and Rine, 1992; Fox *et al*., 1995; Figures 1B, 3A and 4B). However, other factors, in addition to origin efficiency, can cause a low ratio of bubble intermediates relative to small forks in a two-dimensional origin-mapping experiment, including several independent initiation events occurring over a broad zone (Fangman and Brewer, 1991). Therefore, two-dimensional fork-migration analysis was performed to examine the efficiency of replication initiation within an *HMR* locus under the control of natural *HMR*-E (Fangman and Brewer, 1991; Figure 7A).

A two-dimensional fork-migration experiment indicated that replication initiation at the natural *HMR* locus under the sole control natural *HMR*-E silencer was relatively inefficient (Figure 7B and C, panel 1). Analysis of the fragment centromere-proximal to *HMR*-E indicated that *HMR* was replicated by a fork emanating from a neighboring centromere-proximal origin in a significant number of cell divisions (Figure 7B, panel 1). Based on phosphoimager analysis of the two forks, we estimate that the fork coming from outside *HMR* on this fragment was responsible for replicating *HMR* in ~20–30% of cell cycles. Similar analysis of the *HMR* fragment telomere-proximal to *HMR*-E indicated that *HMR* was replicated by a fork emanating from a neighboring telomere-proximal origin in a significant fraction of cell divisions (Figure 7C, panel 1). Fork-migration experiments from this region of *HMR* were consistently difficult to analyze by phosphoimager analysis because of the low signal-to-noise ratio. Nevertheless, we consistently observed that replication forks emanated from either side of this telomere-proximal fragment with approximately equal frequency. Therefore, taken together, these data indicated that replication initiation at *HMR* under the control of intact *HMR*-E was relatively inefficient, occurring in an estimated 20–30% of all cell divisions.

In two-dimensional origin-mapping experiments, removal of the silencer ACS appeared to enhance replication initiation frequency, as judged by the ratio of bubble intermediates to small forks (Figures 3A and 4B), suggesting that in the presence of the silencer, ACS initiation within *HMR* was suppressed. A second independent measure of replication initiation frequency within an *HMR* locus that lacked the silencer ACS [*HMR*-E(acs-)] was provided by a fork-migration analysis experiment (Figure 7B and C). At the *HMR*-E(acs-) mutant version of *HMR*, the non-silencer replicators were responsible for replication initiation within the *HMR* locus.

A fork-migration experiment of this mutant *HMR* locus indicated that initiation frequency at *HMR* under the control of the non-silencer replicators was enhanced relative to the initiation frequency that occurred in the presence of the silencer ACS (Figure 7B and C). Analysis of the fragment centromere-proximal to *HMR*-E indicated that *HMR* was replicated by a fork emanating from a neighboring centromere-proximal origin in only a very small fraction of cell divisions; a horizontally displaced replication fork was barely detectable in a fork-migration experiment, in contrast to what was observed for *HMR* under the control of intact natural *HMR*-E (Figure 7B, compare panel 1 with panel 2). Analysis of the telomere-proximal fragment in this mutant strain was complicated by an incomplete digest prior to separation of replication intermediates by second dimension agarose electrophoresis. However, the signal

from the horizontally displaced fork, which was due to an origin emanating from the direction of the silencer, was enhanced slightly relative to the vertically displaced fork, in contrast to what was observed for the replication of this fragment in the presence of the intact silencer (Figure 7C, compare panel 1 with panel 2). Taken together, these data indicated that the replication initiation frequency within *HMR* was enhanced by removal of the silencer ACS, consistent with the results observed for two-dimensional origin-mapping experiments.

Discussion

This work concerned the hypothesis that the DNA region surrounding the natural *HMR*-E silencer contributes to both replication initiation and silencing at *HMR*. This hypothesis could explain why the natural *HMR*-E-associated origin was insensitive to the same defects in ORC that reduced replication initiation at other replication origins and silencing and initiation at an *HMR* locus controlled by the synthetic silencer (Fox *et al*., 1995; Liang *et al*., 1995). One prediction of this hypothesis was that the ACS within the natural *HMR*-E silencer would be dispensable for both the origin and silencing functions of natural *HMR*-E.

Sequences within HMR required for ORC's silencing and replication functions were separable and contributed to both positive and negative roles for ORC in silencing

The results presented here indicate that the sequences controlling the silencing and replication functions of ORC at natural *HMR*-E are distinct. Thus ORC's behavior at natural *HMR*-E is different from its behavior at the synthetic silencer; the synthetic silencer ACS is critical for both the silencing and replication functions of ORC (Rivier and Rine, 1992; Fox *et al*., 1995; Loo *et al*., 1995a). In contrast, the natural *HMR*-E silencer ACS

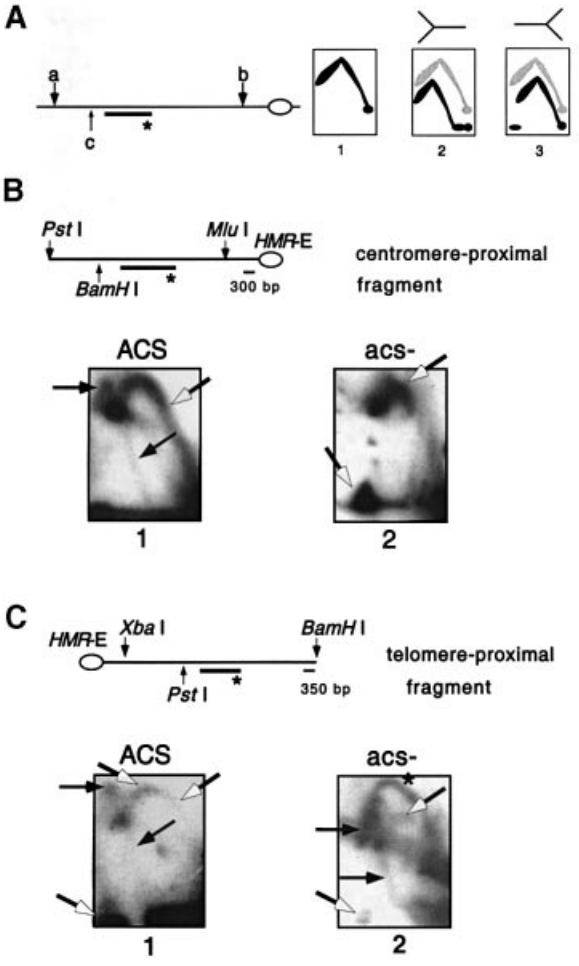

Fig. 7. Replication initiation at *HMR* controlled by natural *HMR*-E occurred in a small fraction of the cell divisions and was enhanced by mutation of the silencer ACS. (**A**) Representation of a two-dimensional fork-migration experiment and how it is used to determine the direction of replication fork-migration through a fragment of interest (Fangman and Brewer, 1991). Panel 1: The fragment adjacent to a replication origin released by restriction enzymes a and b will result in a simple pattern of replication forks when visualized after two-dimensional gel electrophoresis and DNA blot hybridization with the probe indicated by an asterisk. To determine the direction of fork-migration through this fragment, a second digest must be performed with restriction enzyme c in the gel after gel electrophoresis in the first dimension, but prior to electrophoresis in the second dimension. Panel 2: If the fragment defined by restriction enzymes a and b is replicated by a fork emanating from an origin outside a then, after an in-gel digest with restriction enzyme c, a new pattern of forks like those shown black in will be observed after DNA blot hybridization. Panel 3: If, however, the fragment is replicated by a fork emanating from the origin shown next to restriction enzyme site b then, after an in-gel digest with restriction enzyme c, a different pattern of forks will be observed after DNA blot hybridization. In panels 2 and 3 the gray replication forks represent the simple fork pattern observed in the absence of the second in-gel digest as shown in panel 1. A mixed pattern could result if the fragment is replicated in only a fraction of cell cycles by a fork emanating from the origin indicated adjacent to restriction enzyme site b. Fork-migration patterns from each fragment adjacent to an origin of interest can be used to determine the fraction of cell cycles in which that origin initiates. (**B**) Diagram of the *Pst*I–*Mlu*I fragment centromere-proximal to *HMR*-E examined by fork-migration experiments. The *Bam*HI restriction enzyme was used for the in-gel digest after first dimension electrophoresis and prior to second dimension electrophoresis. The probe used is indicated by the bold line marked with an asterisk and was the same probe used to detect replication origin intermediates in Figure 1. Panels 1 and 2: The results from fork-migration analysis of the fragment centromere proximal to the silencer in isogenic strains containing *HMR* under the control of either the wild-type natural silencer or the natural *HMR*-E silencer with a mutant ACS [*HMR*-E(acs-)] (CFY37, CFY108). The black arrowheads point to the signal generated by a fork coming from an origin on the centromere (left) side of the *Pst*I restriction site. The white arrowheads point to the signal generated by a fork coming from the direction of *HMR*-E. (**C**) Diagram of the *Xba*I–*Bam*HI fragment telomere-proximal to *HMR*-E examined by fork migration experiments. The *Pst*I restriction enzyme was used for the in-gel digest after first dimension electrophoresis and prior to second dimension electrophoresis. The probe used is indicated by the bold line marked with an asterisk. The DNA fragment used for the probe was generated by PCR using the following primers to *HMR*: GACATTCAGTGCGTCACG and GCTTACTCCCAAGAGTGC. Panels 1 and 2: The results from fork migration analysis of the fragment telomere-proximal to the silencer in isogenic strains containing *HMR* under the control of either the wild-type natural silencer or the natural *HMR*-E silencer with a mutant ACS [*HMR*-E(acs-)] (CFY37, CFY108). The black arrowheads point to the signal generated by a fork coming from the direction of *HMR*-E. The white arrowheads point to the signal generated by a fork coming from an origin on the telomere (right) side of the *Bam*HI restriction site. *The original fork which was not completely digested in the gel by *Pst*I prior to second-dimension gel electrophoresis.

was required for ORC silencing function, but not ORC replication function, at an *HMR* locus controlled by natural *HMR*-E. This discovery was somewhat unexpected for two reasons. First, previous studies indicate that the ACS within the natural *HMR*-E silencer is not critical for silencing an otherwise intact *HMR* locus (Brand *et al*., 1987). However, these studies were performed in the presence of the *HMR*-I silencer. Thus, in the absence of *HMR*-I, the ACS within the natural *HMR*-E silencer was critical for silencer function. Secondly, even in the absence of *HMR*-I, the functions of natural *HMR*-E are not reduced in an *orc2-1* mutant (Fox *et al*., 1995). Why did removal of a site that bound ORC, the ACS, cause a phenotype at *HMR* that was different from a defect in ORC itself? The observation that ORC contributed both positively and negatively to silencing at natural *HMR* helps answer this question. ORC's positive role is mediated through its binding to the ACS within the silencer itself. This role presumably includes ORC's ability to recruit the Sir1 protein to *HMR* (Triolo and Sternglanz, 1996; Gardner *et al*., 1999). ORC's negative role in silencing is mediated by its function in the 181 bp region immediately adjacent and telomere-proximal to *HMR*-E. Sequences within this region were compatible with ORC's replication function, as judged by the replication initiation controlled by this non-silencer replicator, but were incompatible with ORCs silencing function, as judged by the region's *ORC2*-dependent inhibition of silencing. Therefore, removal of the natural *HMR*-E silencer ACS removes ORC's positive role in silencing without reducing, and perhaps enhancing, its negative role in silencing. However, in an *orc2-1* mutant, both ORC's positive and negative roles will be similarly reduced, such that there is no net effect on silencing. Furthermore, within the context of natural *HMR*-E, ORC's function at both non-silencer replicators may be more severely compromised than its function at the silencer ACS in an *orc2-1* mutant. Consistent with this possibility, replication initiation by the non-silencer replicators was severely reduced by the *orc2-1* mutation. If the presence of the non-silencer replicators at *HMR* contributes to an overall inhibition of replication initiation at this locus, then this explanation could account for the enhanced initiation frequency at natural *HMR*-E in an *orc2-1* mutant. Regardless of the exact mechanism, the data presented here indicate that the modulation of ORC function in this region of *HMR* is complex and involves both positive and negative regulation of ORC's silencing and replication functions. Thus, the redundancy at natural *HMR*-E that causes this silencer to be insensitive to defects in ORC is not due simply to a number of equivalent ORC-binding sites at *HMR*.

The silencing and replication functions of ORC can be separated by mutants in the ORC itself, suggesting that ORC has a region that is required for silencing but dispensable for replication. For example, certain alleles of *ORC5* are functional for replication initiation but defective for silencing by the synthetic silencer (Fox *et al*., 1995; Dillin and Rine, 1997). In addition, ORC's ability to function in replication initiation does not appear to be required for its silencing function (Ehrenhofer-Murray *et al*., 1995; Dillin and Rine, 1997; Fox *et al*., 1997). However, since most replication origins are not silencers, the separation of ORC's silencing and replication functions by mutation within *ORC* genes cannot explain how the function of wild-type ORC is modulated at *HMR*, such that both its replication and silencing roles are operative at this locus. One simple view is that ORC binds within *HMR* in such a way that its interaction with silencing factors is favored relative to an ORC molecule bound to a non-silencer replicator such as *ARS1*. The data presented here indicate that differences in ORC-binding sites within the confines of *HMR*-E and its flanking sequences are critical for ORC's silencing function at *HMR*. For example, ORC molecules within the region containing the telomere-proximal non-silencer replicator were not compatible with the assembly of silent chromatin. Furthermore, the silencer ACS could suppress the function of this telomere-proximal negative region, whereas the region centromere-proximal to *HMR*-E, although compatible with silent chromatin assembly and replicator function, failed to prevent the ORC-dependent inhibition of silencing caused by the region telomere-proximal to *HMR*-E. Taken together, these data suggest that ORC function within *HMR* can be significantly influenced by both DNA context and neighboring ORC molecules. It will be interesting to determine whether these different functions in ORC are reflected in measurable differences in ORC binding within *HMR*.

Replicator dominance as a factor in silencing

A possible role for replicator dominance at the silent mating-type cassettes is provided by an analysis of the work presented here. Previous studies established that two closely spaced replication origins could interfere with each other's initiation (Brewer and Fangman, 1993; Dubey *et al*., 1994; Marahrens and Stillman, 1994). Depending upon the sequences that flank each competing origin, initiation from each origin can be reduced equivalently, or initiation from one origin can be substantially more reduced than initiation from the other. In the latter case, this uneven competition between two closely spaced origins is referred to as replicator dominance to emphasize the ability of one origin to completely dominate replication on a given region of a chromosome (Marahrens and Stillman, 1994). Since the function of the telomere-proximal non-silencer replicator correlated with an ORC-dependent inhibition of silencing, it is possible that in a wild-type cell in which silencing is efficient, this replicator's function is normally suppressed.

Two mechanisms were proposed for replicator dominance (Marahrens and Stillman, 1994). In the first mechanism, initiation occurs from one origin and the DNA containing the second origin is replicated by a replication fork emanating from the first origin before the second origin has time to initiate. Once replicated, the second origin does not initiate because of the block to re-replication of DNA within a given cell cycle. In the second proposed mechanism, some feature of the origin actively inhibits replication initiation when two or more origins are closely spaced on a chromosome. Since the replicators surrounding and including *HMR*-E are so close together, it is not yet possible to favor one mechanism over another. However, the efficiency of initiation within *HMR* under the control of the intact silencer was low and was enhanced somewhat in the absence of the silencer's ACS, suggesting that the presence of ORC at the silencer

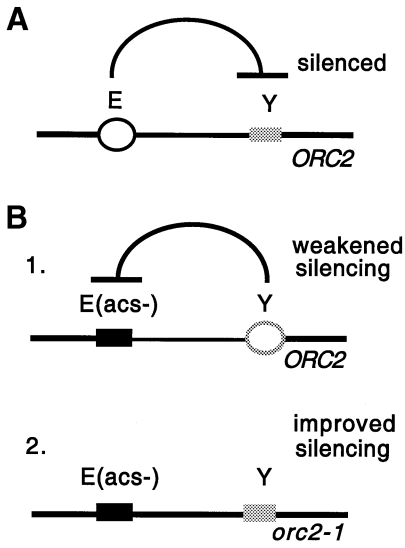

Fig. 8. A simple model for ORC dynamics at *HMR*. The data presented in this report are consistent with at least two replicators existing in close proximity: the *HMR*-E (E) silencer and the non-silencer replicator (Y). (**A**) In the presence of intact *HMR*-E, the activity of the non-silencer replicator is suppressed and silencing is efficient. (**B**) (1) Mutation of the ACS within *HMR*-E inactivates the silencer replicator and allows for the function of the non-silencer replicator. The function of the non-silencer replicator inhibits the function of the remaining elements at the *HMR*-E silencer and silencing is reduced. (2) Defects in ORC caused by the *orc2-1* mutation reduce the function of the non-silencer replicator and prevent inhibition of silencing at *HMR*.

ACS suppresses initiation by the non-silencer replicators. Regardless of the exact mechanism, this study provides evidence for two functions of the ORC bound to the *HMR*-E silencer ACS: one function is to facilitate recruitment of the Sir1 protein to *HMR*, and one is to suppress the activity of neighboring ORC molecules, which could otherwise inhibit silent chromatin assembly.

A replicator-dominance mechanism provides an explanation for the inability to detect chromosomal replication initiation at *HML*, even though this locus contains silencers that provide ORC-dependent ARS activity on plasmids and requires ORC for silencing (Dubey *et al*., 1991; Loo *et al*., 1995a). Perhaps *HML* is more efficient at suppressing origin function through a replicator-dominance mechanism than *HMR*. In this view, one prediction would be that removal of an ORC-binding site, or sites, within *HML* might lead to detectable replication initiation at this locus. However, it is worth noting that removal of one active replicator does not insure enhanced activation of a neighboring replicator (Greenfeder and Newlon, 1992).

A model for ORC dynamics at HMR

In the view of *HMR* presented in Figure 8, and consistent with the data discussed here, at least two potential replicators exist in close proximity: the silencer repicator (E), whose function is compatible with the assembly of silent chromatin; and the non-silencer replicator (Y), whose function is incompatible with the assembly of silent chromatin. In the presence of an intact silencer, initiation by the non-silencer replicator is suppressed (Figure 8A). Removal of the silencer ACS relieves this suppression, and the activity of the non-silencer replicator, or an element closely associated with it, inhibits the function of the remaining silencer elements, a Rap1p- and Abf1p-binding site, in assembling silent chromatin (Figure 8B, 1). This inhibition may be due to a specific structure formed by ORC when bound to the non-silencer replicator, or it may be due to a change in the timing of replication of this chromosomal region. Regardless, inactivation of the non-silencer replicator caused by the *orc2-1* mutation abolishes this inhibition and enhances silencing by the remaining silencer elements (Figure 8B, 2).

The model presented in Figure 8 indicates the presence of two distinct origins, or unwinding sites, each controlled by a different replicator. However, the data presented here are also consistent with a single origin, or unwinding site, that can be controlled by any one of several replicators. A replicator is a genetic element that controls origins (Jacob *et al*., 1963; Stillman, 1993), and in budding yeast origins and their replicators appear to have a relatively simple relationship, with the site of origin unwinding existing in relatively close proximity to the replicator (Newlon, 1996; Bielinsky and Gerbi, 1998; DePamphilis, 1999). However, it is probable that replication initiation in fission yeast and multicellular eukaryotes is controlled by replicators and origins with more complex relationships (Dubey *et al*., 1994; DePamphilis, 1999), and it is also possible that *HMR* contains a replicator/origin control region more similar to those found in these organisms. Further analysis of *HMR* will reveal the precise relationships between origins and replicators in this region of the yeast genome and should provide insights into the relationships between chromosome replication and expression relevant to all eukaryotes.

Materials and methods

General

Yeast-rich medium (YPD), minimal medium (YMD), amino acid and base supplements, and standard yeast genetics methods were as described previously (Guthrie and Fink, 1991). Recombinant DNA methods were as described by Sambrook *et al*. (1989). The strains used in this report are presented in Table I and were isogenic to W303-1A.

The deletion of *HMR*-I has been described recently (Rivier *et al*., 1999), and all strains used in this study contained this deletion.

The minimal *HMR*-E silencer was constructed using a high-fidelity PCR to amplify the natural silencer from a pUC19 plasmid containing a *Eco*RI–*Hin*dIII fragment that included *HMR* (pCF47). The sequences of the primers used were: CCGCTGCCGGCGTAGA and CCGCTCGAGGCTTTCAAATATTTTTATG. The fragment generated by PCR with these primers was 198 bp long and contained the entire natural *HMR*-E silencer flanked by *Xho*I sites that were engineered into each primer. The fragment was cleaved with *Xho*I and cloned into the *Xho*I site that marks a deletion of *HMR*-E and its flanking sequences (p8ΔE; McNally and Rine, 1991). The minimal *HMR* fragment was therefore identical to natural *HMR* except that it lacked 491 bp centromere-proximal to *HMR*-E and 181 bp telomere-proximal to *HMR*-E, and contained two additional *Xho*I sites flanking the silencer fragment.

To construct the identical mutation of the ACS in both natural *HMR*-E and the minimal *HMR*-E silencers, site-directed mutagenesis was used to change the ACS within the silencer from TAAATATAAAA to TCGGATCCGAA. This change substituted a *Bam*HI site and some additional nucleotides for the ACS within the silencer.

All versions of *HMR* used in this study were integrated at the *HMR* locus on chromosome III and analyzed in that location for their effects on silencing and chromosomal replication initiation.

Two-dimensional origin-mapping and fork-migration analyses

The isolation and analysis of replication intermediates were performed essentially as described previously (Brewer and Fangman, 1987), except that all cultures were grown at 23°C. The probes used to detect specific

Table I. Strains used in this study

Strain	Genotype	Reference[a]
CFY616	*MAT***a** *his4 leu2 trp1 ura3*	
JRY2334	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100* (W303-1A)	Thomas and Rothstein (1989)
JRY3009	*MAT*α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100* (W303-1B)	
CFY145	JRY2334 *ADE2 lys2*Δ	Herman and Rine (1997)
CFY36	JRY3009 *HMR-SS*ΔI	Fox *et al*. (1995)
CFY285	JRY3009 *HMR-SS*ΔI *orc2-1*	Fox *et al*. (1995)
CFY37	JRY3009 *HMR*ΔI	Fox *et al*. (1995)
CFY290	JRY3009 *HMR*ΔI *orc2-1*	Fox *et al*. (1995)
CFY393	JRY3009 *HMR*ΔI *sir2*Δ*::LEU2*	
CFY391	JRY3009 *HMR*ΔI *sir2*Δ*::LEU2 orc2-1*	
CFY108	JRY3009 *HMR*(acs-)ΔI	
CFY143	JRY3009 *HMR*(acs-)ΔI *orc2-1*	
CFY201	JRY3009 *HMR*(acs-)ΔI *orc2-1 lys2*Δ	
CFY3	JRY3009 minimal *HMR*ΔI	
CFY140	JRY3009 minimal *HMR*(acs-)ΔI	
CFY244	JRY3009 minimal *HMR*(acs-)ΔI *orc2-1 lys2*Δ	
CFY692	JRY3009 *HMR*(acs-)Δ181ΔI	
CFY693	JRY3009 *HMR*(acs-)Δ491ΔI	

[a]Unless noted otherwise, the strains listed were part of the laboratory collection or were constructed for the experiments in this paper.

fragments differed from those used previously (Rivier and Rine, 1992) and are described in the figures and in the figure legends. High specific-activity radiolabeled probes were generated by either multiprime labeling the appropriate DNA fragments or amplifying the appropriate DNA fragment using radioactive dCTP in the PCR. For fork-migration analysis, the enzymes used for in-gel digestion were from New England Biolabs, and the procedure followed was essentially as described by Fangman and Brewer (1991). For hybridizations of the DNA blots, Hybond N+ membrane (Pharmacia-Amersham) was used following the manufacturer's instructions.

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Note added in proof

A recent independent study also demonstrates the presence of origins flanking the silencer (Hurst and Rivier, 1999).