Position Effect on the Directionality of Silencer Function in
Saccharomyces cerevisiae

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ABSTRACT

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

I N the yeast *Saccharomyces cerevisiae*, the *HML* and *HMR* loci and regions near the telomeres are transcriptionally silenced via the formation of a repressive chromatin structure that is akin to metazoan heterochromatin (MOAZED 2001a; GREWAL and MOAZED 2003). Silencing at the *HM* loci is initiated by small specialized flanking sequences called the *HML-E* and *-I* and *HMR-E* and *-I* silencers that are composed of various combinations of two or three of the binding sites for Abf1p, Rap1p, and origin recognition complex for DNA replication (ORC) (Figure 1A) (RUSCHE *et al.* 2003). The silencer-binding proteins initiate silencing by recruiting the Sir silencing complex consisting of Sir2p–Sir4p through physical interactions. Sir2p is an NAD-dependent histone deacetylase that is believed to be responsible for the characteristic histone hypoacetylation associated with silent chromatin (MOAZED 2001b; RUSCHE *et al.* 2003). Once recruited to the silencer, the Sir complex is thought to deacetylate nearby nucleosomes. As the Sir complex self-interacts and preferentially binds hypoacetylated histones (CARMEN *et al.* 2001; LIOU *et al.* 2005), additional Sir complexes are recruited to the newly deacetylated nucleosomes. The nucleosome-bound Sir complex then starts another round of nucleosome deacetylation and Sir complex binding. In this manner, the Sir complex is proposed to promote autonomous stepwise propagation along an array of nucleosomes, leading to the formation of transcriptionally silent chromatin across the *HM* loci (MOAZED 2001a; GREWAL and MOAZED 2003; RUSCHE *et al.* 2003). The telomeric repeats contain multiple binding sites for Rap1p that can recruit Sir proteins, and propagation of the Sir proteins inward along the chromosome leads to the formation of silent chromatin in regions near the telomeres (RUSCHE *et al.* 2003).

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

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collectively contribute to the difference in the potency of silencing among silencers.

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

Two silencers separated by a distance of up to 4 kb can cooperate to bring about silencing in the region they bracket that is stronger than that promoted by either silencer alone (FELDMAN *et al.* 1984; BOSCHERON *et al.* 1996; RIVIER *et al.* 1999; SEKINGER and GROSS 1999). Moreover, a single binding site for any of the silencer-binding proteins can enhance the activity of a distant silencer without acting as a silencer on its own and is therefore referred to as a protosilencer (BOSCHERON *et al.* 1996). In known cases of silencer–protosilencer interactions, silencing of genes located in the region flanked by the silencer and protosilencer was found to be increased (BOSCHERON *et al.* 1996; CHENG and GARTENBERG 2000; LEBRUN *et al.* 2001). Silencers and protosilencers are collectively called silencing elements. How two silencing elements collaborate to induce stronger silencing has not been resolved.

The current model for the establishment of silencing implies that initiation of silencing does not discriminate against either direction. This is consistent with the finding that the *HMR-E* silencer silences the a1 gene at *HMR* in an orientation-independent manner (BRAND *et al.* 1985). However, this notion is challenged by our discovery that the *HML-I* silencer acts only in one direction despite its close similarity to *HMR-E* in structure (Figure 1A) (BI *et al.* 1999). Moreover, *HMR-E* ectopically inserted near the *MAT* locus preferentially functions in one direction (SHEI and BROACH 1995). What then determines the directionality of a silencer? It is possible that inherent structural characteristics of a silencer determine its directionality in silencing. Every silencer is clearly asymmetric with an order of ORC–Rap1p–Abf1p sites for both *HML-I* and *HMR-E* and Rap1p–ORC sites for *HML-E* (Figure 1A). We have recently shown that such a structural asymmetry leads to an asymmetric organization of nucleosomes around the silencer (ZOU *et al.* 2006). Consistent with the notion that a continuous array of nucleosomes is required for the spreading of silent chromatin (RUSCHE *et al.*

2003; BI *et al.* 2004), we found that the different patterns of nucleosome distribution on the two sides of a silencer coincide with unequal potentials for silencing (ZOU *et al.* 2006). These results suggest that the two sides of a silencer are inherently associated with unequal potentials for transcriptional silencing.

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

MATERIALS AND METHODS

Plasmids and strains: Plasmid pUC26 was made by inserting the *Bam*HI–*HML*–*Bam*HI fragment (coordinates 9666–16,263 of chromosome III) into pUC19. Plasmid pRS416-HMR was made by inserting the *Hin*dIII–*HMR*–*Hin*dIII sequence (289,227–294,210) into pRS416. A 1.1-kb *URA3* sequence (BI *et al.* 1999) was inserted at the *Eco*RV site (15,411) of pUC26 to make p101. The *HML-I* sequence (14,561–14,838) in pUC26 was replaced by a *Hin*dIII site to make pYC61. *URA3* was inserted at the *Eco*RV site of pYC61 to make p303. *HML-I* in the opposite direction was inserted at *Hin*dIII of p303, resulting in p102. The *HMR-E* sequence (291,276–291,539) in either orientation was inserted at the *Hin*dIII site of p303, resulting in p103 and p104, respectively. The *HML-E* sequence (11,172–11,295) in either orientation was inserted at the *Hin*dIII site of p303, resulting in p105 and p106, respectively. *URA3* was inserted at the *Bsu*36I site (10,979) of pUC26, resulting in p111. The *Afl*II–*HML-E*–*Eco*RI fragment of p111 was inverted to make p112 and replaced by *HML-I* or *HMR-E* in either direction to make 107–110, respectively. Plasmid p113 was made by inserting *URA3* at the *Spe*I site (291,110) of pRS416-HMR. The *HMR-E* sequence in p113 was replaced by a *Bam*HI site, resulting in p114m. An inverted *HMR-E* was inserted at the *Bam*HI site of p114m, making p114. The *Pst*I–*HML*–*URA3*–*Pst*I fragment in p101 or p102 was inverted, making p115 or p116, respectively.

Plasmid p201 was derived from pUC26 by inserting *URA3* at its *Pvu*II site (14,441). The *HML-I* sequence of p210 was replaced by a *Hin*dIII site, resulting in p203. An inverted *HML-I* was inserted at the *Hin*dIII site of p203, making p202. The *HML-E* sequence in p201 and -202 was replaced by an *Afl*II site, making p204 and -205, respectively. The *HMR-E* sequence in

either direction was inserted at the HindIII site of p203, making p210 and p211, respectively. The HML-I silencer in p204 was replaced by HMR-E in either direction, making p212 and 213, respectively. The UASα sequence (12,985–13,279) of p212 and p213 was replaced by a HindIII site, making p214 and p215, respectively.

The HML-E sequence in p101–p104 was replaced by an AflII site, making p301, p302, p304, and p305, respectively. The HML-I sequence in p101 was replaced by a HindIII site, making p303. The HML-I sequence in p109 or p110 was replaced by a HindIII site, making p306 and p307, respectively. UASα was deleted from p102, p104, and p305, making p401, p402, and p403, respectively. The Rap1p-binding site R2 (14,538–14,550) was deleted from p101–p104, making p405, p406, p408, and p409, respectively. R2 was deleted from p403, making p404. HML-I plus R2 was inserted at the HindIII site of p303, making p407. The AflII–HML–HindIII sequence in p101 and p102 was inverted, making p410 and 411, respectively. The AflII–HML–HindIII sequence in p101–p104 was replaced by the HMR sequence (291,539–293,577), making p501–p504, respectively. Plasmid pMB21 is an integration plasmid containing SIR3-TRP1 and SUP4-o (BI et al. 1999).

All strains used in this study were derived from DMY2 (MATa ura3-52 leu2-3,112 ade2-1 lys1-1 his5-2 can1-100 sir3::LEU2) (MAHONEY and BROACH 1989). Strains 101s–112s, 115s, 116s, 201s–205s, 210s–215s, 301s–307s, 401s–411s, and 501s–504s were constructed by transforming strain DMY2 to Ura$^+$ with BamHI-digested plasmids p101–p112, p115, p116, p201–p205, p210–215, p301–p307, p401–p411, and p501–p504, respectively. Strains 113s, 114s, and 206s–209s were constructed by transforming DMY2 to Ura$^+$ with HindIII-digested plasmids p113, p114, and p206–p209, respectively. These strains were rendered SIR3$^+$ by integrating pMB21 at TRP1 in the genome, resulting in strains 101–116, 201–215, 301–307, 401–411, and 501–504, respectively, as illustrated in Figures 1–7. Strains 101r–104r and 303r–305r were derived from 101–104 and 303–305, respectively, by replacing the coding sequence of PPR1 with kanMX. The relevant genotypes of all strains were confirmed by Southern blotting.

RT–PCR: Cells were grown at 30° to log phase in SC medium. Total RNA was isolated from ∼5×10^7 cells and 0.4 μg of RNA was used for multiplex RT–PCR with a SuperScript III one-step RT–PCR system with Platinum Taq DNA Polymerase (Invitrogen, San Diego). The proper concentration of RNA template for RT–PCR had been determined to be in the linear range by serial dilutions. Oligo(dT)$_{(20)}$ was used for the cDNA synthesis step and additional dNTPs were added to accommodate multiple PCR products. URA3 and ACT1 ORF primers were used to generate PCR products of 300–550 bp that were fractionated on 2.0% agarose gels.

RESULTS

The apparent directionality of a silencer measured in an experiment is dependent on the sensitivity of the assay used: The URA3 gene is a widely used reporter for studying transcriptional silencing (VAN LEEUWEN and GOTTSCHLING 2002). Ura3p is involved in pyrimidine biosynthesis and can convert 5-fluoroorotic acid (5-FOA) to a toxic metabolite (BOEKE et al. 1987). In the presence of uracil, URA3 is expressed at a low basal level, whereas uracil depletion activates the Ppr1p transactivator, thereby increasing the expression of URA3 to a higher activated level (LOSSON and LACROUTE 1981).

Therefore, while cell growth on 5-FOA-containing medium (which also contains uracil) indicates the repression of the basal level transcription of URA3, lack of growth on medium depleted of uracil (−Ura) reflects the silencing of activated expression of URA3 (VAN LEEUWEN and GOTTSCHLING 2002). In the absence of Ppr1p, URA3 cannot be expressed at the higher activated level even when uracil is depleted from the medium. Therefore, for ppr1$^-$ cells, growth on 5-FOA and lack of growth on −Ura media would both indicate the silencing of basal expression of URA3.

By measuring the repression of basal expression of URA3 inserted near HML-I we showed previously that HML-I initiated silencing in only one direction (Figure 1B, compare growth phenotypes of strains 101 and 102 on 5-FOA) (BI et al. 1999). HMR-E ectopically placed in the position of HML-I also functioned unidirectionally (Figure 1B, growth phenotypes of strains 103 and 104 on 5-FOA) (ZOU et al. 2006). We showed here that HML-E inserted in the position of HML-I only weakly silenced basal URA3 expression in one direction (Figure 1B, 105 and 106 on 5-FOA).

To complement the assay of URA3 silencing by monitoring cell growth on 5-FOA, we also directly measured the level of URA3 mRNA, using RT–PCR in a representative set of strains used in this work. URA3 mRNA was abundant in strains 101, 103, 303, and 304, but was barely detectable in strains 102, 104, and 305 (Figure 2A). These results are consistent with the growth phenotypes of strains 101–104 and 303–305 on 5-FOA (Figure 1B, 101–104 on 5-FOA; Figure 6, 303–305 on 5-FOA). Note that URA3 silencing measured in this work was strictly Sir dependent as deletion of SIR3 completely abolished URA3 silencing (Figure 2A, compare URA3 RNA in 102, 104, and 305 to that in their sir3$^-$ derivatives 102s, 104s, and 305s, respectively; Figure 2B, lack of growth of the sir3$^-$ strains on 5-FOA; and data not shown).

We also examined the directionality of silencing of silencers, using activated expression of URA3 as the reporter. Interestingly, none of the silencers in either direction (in the context of HML-I) was able to silence the activated expression of URA3 as indicated by the robust growth of all strains 101–106 on −Ura (Figure 1B). Since the activated expression of URA3 is mediated by the trans-activator Ppr1p, it should not occur if PPR1 is deleted. Therefore, silencing of URA3 under non-inducing conditions could be better examined in the absence of PPR1. We deleted PPR1 from the aforementioned set of seven representative strains (Figure 2A), resulting in strains 101r–104r and 303r–305r (Figure 3, left). URA3 silencing in these strains was measured by monitoring their growth phenotypes on 5-FOA and −Ura and comparing them to that in their PPR1$^+$ parents (Figure 3). As shown in Figure 3, unlike strains 102 and 104, their ppr1$^-$ derivatives 102r and 104r were not able to grow on −Ura, which confirmed that
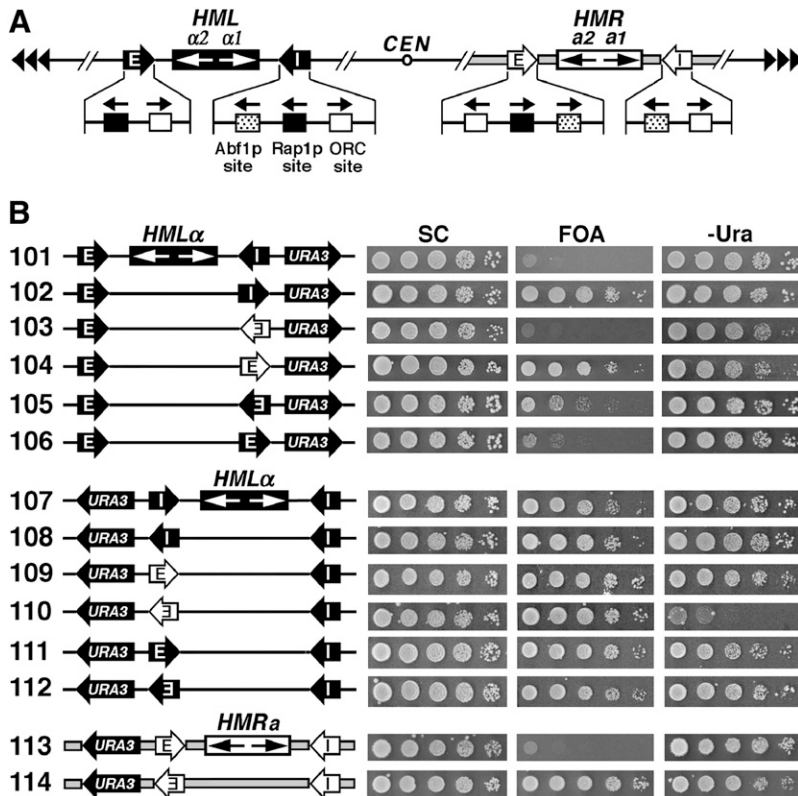
FIGURE 1.—The apparent directionality of a silencer observed in a specific experiment depends on the genomic location of the silencer as well as the sensitivity of the silencing assay. (A) Schematics of the *HML* and *HMR* loci on chromosome III in *S. cerevisiae*. The *HML-E* and *HML-I* silencers are shown as solid arrows with white letters, and the *HMR-E* and *HMR-I* silencers are shown as open arrows with black letters. The direction of each silencer sequence is drawn as pointing toward the *HMLα* or *HMRa* genes, but is not necessarily the functional direction. The binding sites for Abf1p, Rap1p, and ORC in the silencers as well as their $5' \to 3'$ directions are indicated. In *HML-E*, 16 bp centromere-proximal to the ORC site is a putative Sum1p-binding site whose deletion has been shown to reduce the activity of a weakened *HML-E* silencer (IRLBACHER *et al.* 2005). The α1 and α2 genes at the *HML* locus and the a1 and a2 genes at *HMR* are also indicated. *CEN*, centromere. Tandem arrowheads, telomeric repeats. The regions spanning the *HMR* locus and flanking sequences are indicated by thick shaded lines. (B) Effects of the genomic contexts of *HML-I* and *HML-E* on the directionality of silencers. Left, the modified *HML* or *HMR* loci in strains 101–114 (see MATERIALS AND METHODS for their construction). The silencers, *HM* genes, and the *URA3* reporter gene are indicated. Cells of each strain were grown to late log phase and serial 10-fold dilutions were spotted on test plates and allowed to grow for 3 days. SC, synthetic complete medium. 5-FOA, SC supplemented with 1 mg/ml 5-fluoroorotic acid (5-FOA). −Ura, SC depleted of uracil. Growth phenotypes of each strain are shown on the right.

Ppr1p-independent basal expression, but not Ppr1p-dependent activated expression of *URA3*, could be silenced by *HML-I* or *HMR-E* orientated toward the *URA3* gene.

Results from the above experiments suggest that the outcome of a silencing experiment depends on the sensitivity of the reporter gene to the silencing machinery. Throughout this report, both the basal and the activated expressions of *URA3* were used as silencing reporters to examine the potency and directionality of silencers.

The apparent potency and directionality of a silencer are dependent on its genomic context: To investigate whether the directionality of a silencer is influenced by its genomic context, we compared the behaviors of a silencer in the contexts of the *HML-I*, *HML-E*, and *HMR-E* silencers (Figure 1B). The basal expression of *URA3* inserted to the left (telomere-proximal) of *HML* was strongly silenced by *HML-E* in either orientation (Figure 1B, strains 111 and 112 on 5-FOA). This was in contrast to the weak and unidirectional silencing by *HML-E* transplaced in the position of *HML-I* (Figure 1B, 105 and 106 on 5-FOA). *HML-I* and *HMR-E* in the context of *HML-E* also efficiently silenced *URA3* in an orientation-independent manner (Figure 1B, 107–110 on 5-FOA). Therefore, the context of *HML-E*, opposite to that of *HML-I*, allows/facilitates silencers to function robustly and bidirectionally. Notably, whereas *HML-E*

or *HML-I* in either direction was not able to silence the *activated* expression of *URA3* (Figure 1B, growth of 107, 108, 111, and 112 on −Ura), *HMR-E* was able to do so in one orientation (toward *URA3*) but not the other (Figure 1B, compare 109 and 110 on −Ura). Therefore, the directional nature of *HMR-E* silencing was revealed only when activated expression of *URA3* was used as the reporter. These results suggest that, compared to the context of *HML-I*, the context of *HML-E* increases that efficiency of silencing on both sides of a silencer so that basal *URA3* expression could be silenced independent of the orientation of the silencer. However, the strengths of silencing on the two sides of the silencer were likely still unequal. This was manifested by the fact that *HMR-E* silenced activated *URA3* expression in only one direction. We also examined the function of *HMR-E* in its native position at *HMR* using *URA3* as the reporter. *URA3* inserted to the left of *HMR-E* was not silenced (Figure 1B, lack of growth of strain 113 on 5-FOA). However, *URA3* was silenced when *HMR-E* was inverted to face *URA3* (Figure 1B, 114 on 5-FOA). On the other hand, *HMR-E* in either direction was not able to silence activated expression of *URA3* (Figure 1B, 113 and 114 on −Ura).

In summary, the above results suggest that each silencer is inherently unidirectional (presumably due to its asymmetric structural features), but may promote bidirectional silencing if placed in a context that can
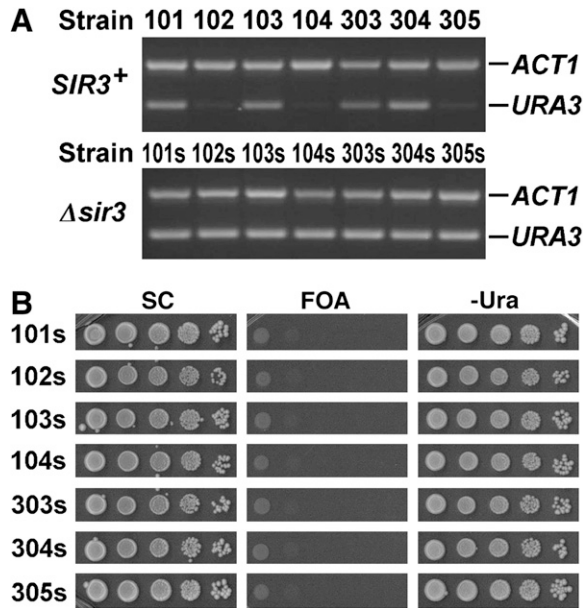
FIGURE 2.—Sir-dependent *URA3* silencing in representative strains. (A) The abundance of *URA3* mRNA in strains 101–104 and 303–305 and their *sir3⁻* derivatives 101s–103s and 303s–305s grown to log phase in SC medium was measured by RT–PCR. The abundance of *ACT1* mRNA in each strain was simultaneously measured as an internal control. (B) Shown are growth phenotypes of the *sir3⁻* strains 101s–103s and 303s–305s on SC, 5-FOA, and −Ura.

increase the efficiency of silencing on the "disfavored" side of the silencer to a level comparable to that on the other side. They also show that the apparent directionality of a silencer measured in a particular experiment depends on the sensitivity/resolution of the assay employed.

High activity of a silencer in the context of *HML-E* is correlated with its relative proximity to the telomere: Results presented in Figure 1B demonstrated that the context of *HML-E* is clearly more permissive to silencer function than those of *HML-I* and *HMR-E* (*e.g.*, compare 103 and 113 to 109 on 5-FOA). It was possible that

the flanking sequences of *HML-E* acted to enhance silencing by *HML-E* or any other silencer ectopically inserted there, whereas the flanking sequences of *HML-I* or *HMR-E* failed to do so. In an attempt to test this hypothesis, we inverted the sequence encompassing the entire *HML* locus plus ∼1 kb flanking sequence on each side (Figure 4, zigzag and thick lines), together with the *URA3* gene inserted to the right of *HML-I* in strains 101 and 102 (Figure 4, large shaded arrow), resulting in strains 115 and 116 (Figure 4). Interestingly, although this inversion did not change the local context of *HML-I*, or the position and orientation of *URA3* relative to *HML-I* (Figure 4, left, compare 115 and 116 to 101 and 102, respectively), it greatly improved *URA3* silencing by *HML-I* in either orientation (Figure 4, compare 115 to 101 on 5-FOA, as well as 115 and 116 to 101 and 102, respectively, on −Ura). Because the telomere-proximal flanking sequence of the resident *HML-E* (Figure 4, zigzag line) was not in the vicinity of *HML-I* in strains 115 or 116, it was probably not involved in the enhancement of the activity of *HML-I*.

Inversion of *HML* and its flanking sequences (indicated by the large shaded arrows in Figure 4) shortened the distance between *HML-I* and the left telomere of chromosome III (*TEL III-L*) by ∼3.3 kb. It is possible that *TEL III-L* enhances silencer function at *HML* by directly interacting with the silencer (Figure 4, bottom), which is in line with the evidence for physical contact between *HML* and *TEL III-L* (LEBRUN *et al.* 2003). However, if such a looping model were correct, then it would be hard to imagine why *TEL III-L* enhanced the function of the endogenous *HML-E* but not *HML-I*, despite that the two silencers were only 3.3 kb apart. On the other hand, it is also possible that a signal that could positively regulate silencing (*e.g.*, the Sir complex) propagates from *TEL III-L* toward *HML* (Figure 4, bottom, large arrow). Because this putative signal may gradually lose strength along its path, it may have a stronger effect on *HML-E* (or any silencer ectopically placed there) than on *HML-I*.
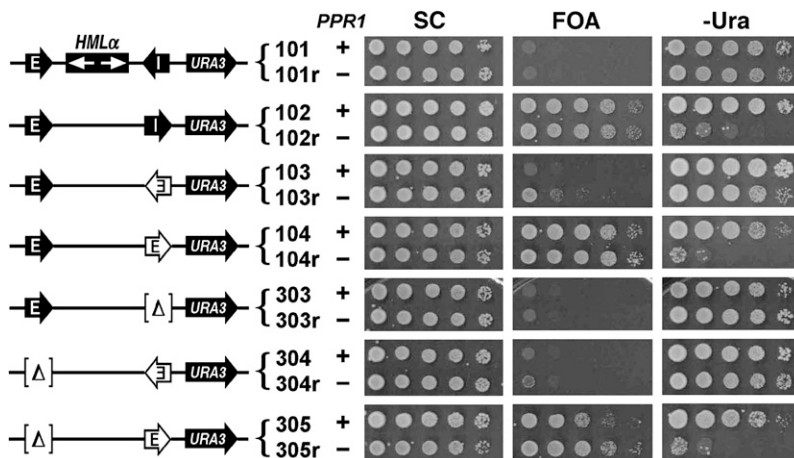

FIGURE 3.—Examination of silencer function by measuring basal *URA3* silencing in the absence of *PPR1*. The growth phenotypes of strains 101–104 and 303–305 and their *ppr1⁻* derivatives 101r–104r and 303r–305r on SC, 5-FOA, and −Ura are shown.
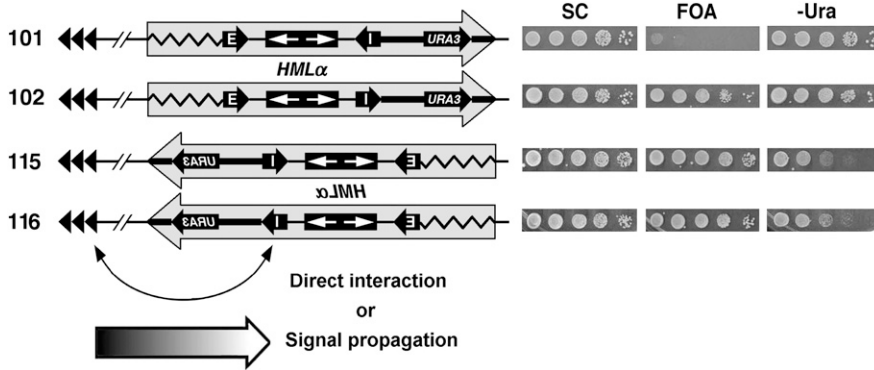
FIGURE 4.—Effect of inverting *HML* and flanking sequences on the activity of *HML-I*. A sequence (large shaded arrow) encompassing *HML* and ∼1 kb of left (zigzag line) and right (thick line) flanking sequences, as well as *URA3* inserted 600 bp from *HML-I*, in strains 101 and 102 was inverted to make 115 and 116. Growth phenotypes of strains 101, 102, 115, and 116 on SC, 5-FOA, and −Ura media are shown on the right. Possible means of interaction between the left telomere of chromosome III (*TEL III-L*) and silencers at *HML* are shown at the bottom. Curved arrow, direct interaction. Large arrow at the bottom, propagation of a signal along the chromosome.

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

As shown in Figure 5A, the basal expression of *URA3* inserted between the α1 gene and *HML-I* was strongly silenced independent of the orientation of *HML-I* (strains 201 and 202 on 5-FOA). The activated expression of *URA3* was strongly silenced when *HML-I* was orientated toward *URA3*, but was only moderately silenced when *HML-I* was in the opposite direction (Figure 5A, 201 and 202 on −Ura). Therefore, *URA3* experienced stronger silencing when located within *HML* than when located to the right of *HML-I*, but the directionality of *HML-I* was not changed (Figure 5A, compare 201 and 202 to 101 and 102, respectively, on −Ura). To test whether *URA3* silencing in strains 201 and 202 resulted from combined actions of *HML-E* and *-I*, we examined the effect of deleting either silencer on silencing. Deletion of *HML-I* abolished *URA3* silencing, demonstrating that *HML-E* alone was not sufficient to silence *URA3* (Figure 5A, 203). In the absence of *HML-E*, *HML-I* in its natural direction (toward α1) silenced basal *URA3* expression, but failed to do so in the opposite direction (Figure 5A, strains 204 and 205). *HML-I* in either direction was not able to silence activated expression of *URA3* in the absence of *HML-E* (Figure 3A, 204 and 205 on −Ura). These results indicate that the inability of *HML-I* to silence *URA3* on its ORC

side (Figure 5A, 205) could be overcome with the assistance of *HML-E* (Figure 3A, 202). As *HML-E* alone was not able to silence *URA3* (Figure 5A, 203), silencing of *URA3* in strain 202 reflected a synergistic interaction between *HML-E* and *HML-I*. These results confirmed that *HML-I* was a unidirectional silencer (Figure 5A, compare 204 and 205 on 5-FOA) and also demonstrated that *HML-E* could alter the apparent directionality of *HML-I* (when the basal expression of *URA3* was used as the silencing reporter) (Figure 5A, compare 201 and 202 to 204 and 205 on 5-FOA, respectively).

We next examined whether the apparent directionality of *HMR-E* was affected by *HMR-I* at the *HMR* locus. The basal expression of *URA3* inserted between *HMR-E* and the a2 gene was strongly silenced (Figure 5B, robust growth of 206 on 5-FOA). This was independent of the orientation of *HMR-E* (Figure 5B, compare 207 and 206 on 5-FOA). However, when *HMR-I* was deleted, *HMR-E* silenced *URA3* only in one direction (Figure 5B, compare 208 and 209 on 5-FOA). Therefore, *HMR-I* could alter the apparent directionality of *HMR-E* (when the basal expression of *URA3* was used as the silencing reporter). On the other hand, *HMR-E* significantly silenced the activated expression of *URA3* in one direction only when *HMR-I* was present (Figure 5B, compare 206 and 207 to 208 and 209 on −Ura, respectively).

We also examined if the apparent directionality of *HMR-E* ectopically inserted at *HML* was affected by the resident silencing elements. Strains 210 and 211 were derived from 201 by replacing *HML-I* with *HMR-E* in opposite directions (Figure 5C, left). Robust silencing of both the basal and the activated expression of *URA3* was observed in these strains, which was independent of the orientation of *HMR-E* (Figure 5C, compare 210 and 211 on 5-FOA and −Ura, respectively). Deletion of *HML-E* had no effect on the silencing of basal *URA3* expression regardless of the direction of *HMR-E* (Figure 5C, 212 and 213 on 5-FOA). However, it greatly reduced the silencing of activated expression of *URA3* when *HMR-E* was orientated away from it (Figure 5C, compare 212 and 210 on −Ura). As a consequence, *HMR-E*
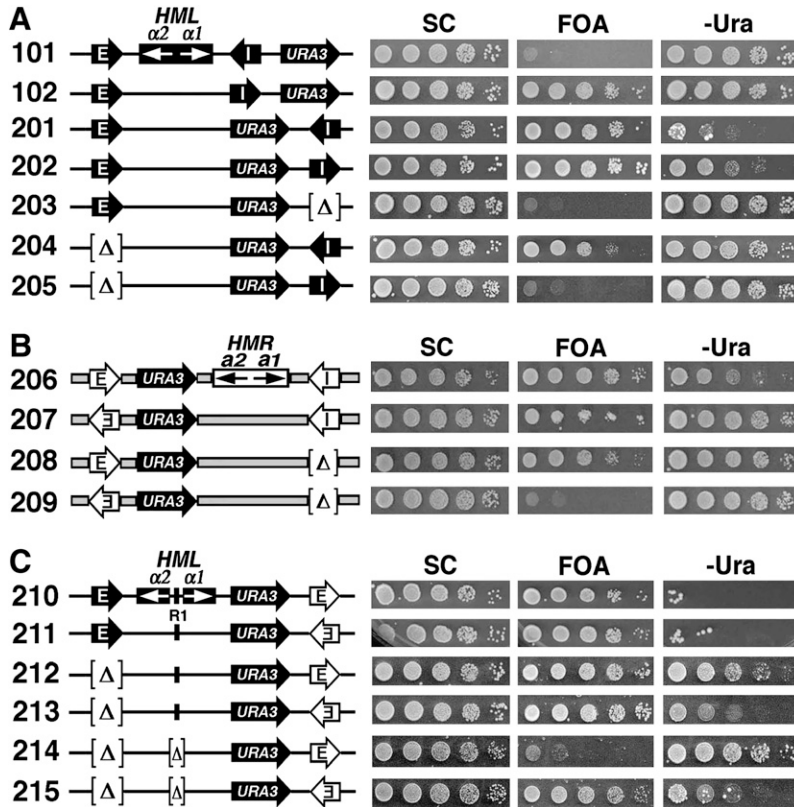
FIGURE 5.—Silencing elements in the genomic context of a silencer regulate its potency and directionality. (A) *HML-E* affects the apparent directionality of *HML-I*. Left, strains used. Right, growth phenotypes of these strains on SC, 5-FOA, and −Ura media. [Δ] denotes the deletion of a sequence. (B) *HMR-I* affects the apparent directionality of *HMR-E*. Left, strains used. Right, growth phenotypes. (C) Effect of UASα on the directionality of *HMR-E* in the context of *HML-I*. The Rap1p-binding site (designated R1) in UASα (upstream activating sequence of the α1 and α2 genes) is indicated by a bar in strains 210–213. Note that R1 is highlighted only in certain relevant strains in this report. UASα was deleted in strains 214 and 215. Growth phenotypes of strains 210–215 are shown on the right.

appeared to act unidirectionally (Figure 5C, compare 212 and 213 on −Ura). Further deletion of UASα containing a Rap1p binding site (designated R1) reduced the efficiency of silencing by *HMR-E* oriented away from *URA3* but not that of *HMR-E* in the opposite orientation (Figure 5C, compare 214 and 215 to 212 and 213, respectively). Therefore, both the apparent potency and the directionality of *HMR-E* were affected by the *HML-E* silencer and the protosilencer R1 at *HML*. We conclude that the apparent potency and directionality of a silencer are regulated by other silencing elements in its context.

Dispersed silencing elements can act in synergy to promote silencing outside the region harboring them: Results from the above experiments revealed collaborative interactions among silencers and protosilencers at *HML* and *HMR*. These and previous examples of such interactions concerned the silencing of a reporter gene located *between* the two participating silencing elements (Figure 5; FELDMAN *et al.* 1984; BOSCHERON *et al.* 1996; RIVIER *et al.* 1999; CHENG and GARTENBERG 2000; LEBRUN *et al.* 2001). Here we describe clear cases of two silencers separated by the 3.3-kb *HML* sequence functioning in synergy to silence a gene located *outside* *HML*. As shown in Figure 4, in strain 102 bearing *HML-E* and the inverted *HML-I*, *URA3* inserted to the right of *HML* was silenced. However, neither *HML-I* nor *HML-E* alone was able to silence *URA3* (Figure 6, 302 and 303 on 5-FOA). Therefore, *URA3* silencing in strain 102 requires the synergistic interaction of *HML-E* and

inverted *HML-I*. On the other hand, *HML-E* and *HML-I* in its native orientation failed to work together to silence *URA3* (Figure 6, strain 101). Robust *URA3* silencing was retained when *HML-I* in strain 102 was replaced by *HMR-E* oriented toward *URA3* (Figure 6, strain 104 on 5-FOA). Deletion of *HML-E* decreased *URA3* silencing (Figure 6, compare 305 to 104 on 5-FOA), indicating that *HML-E* enhanced the silencing activity of *HMR-E* in strain 104. These results demonstrate that a silencer in the position of *HML-I* can collaborate with the *HML-E* silencer at a distance of 3.3 kb to silence a gene located to the right of the *HML* sequence. The *HMR-E* silencer placed in the context of *HML-E* did not require the assistance of *HML-I* to fully silence the basal expression of *URA3* located to the left of *HML* (Figure 6, 5-FOA, compare 306 and 307 to 109 and 110, respectively). However, *HML-I* helped *HMR-E* promote stronger silencing of the activated expression of *URA3* (Figure 6, compare 307 to 110 on −Ura). In summary, these results clearly demonstrated that two silencers separated by the *HML* sequence can cooperate to silence genes outside of *HML* more efficiently.

As the Rap1p site R1 in the middle of *HML* could cooperate with an ectopic *HMR-E* to silence *URA3* within *HML* (Figure 5C, compare 214 to 212 on 5-FOA), we wondered whether R1 could also collaborate with silencers to silence *URA3* outside of *HML*. To address this question, we deleted UASα containing R1 from strain 102, resulting in strain 401 (Figure 7A). The failure of strain 401 to grow on 5-FOA (Figure 7A)
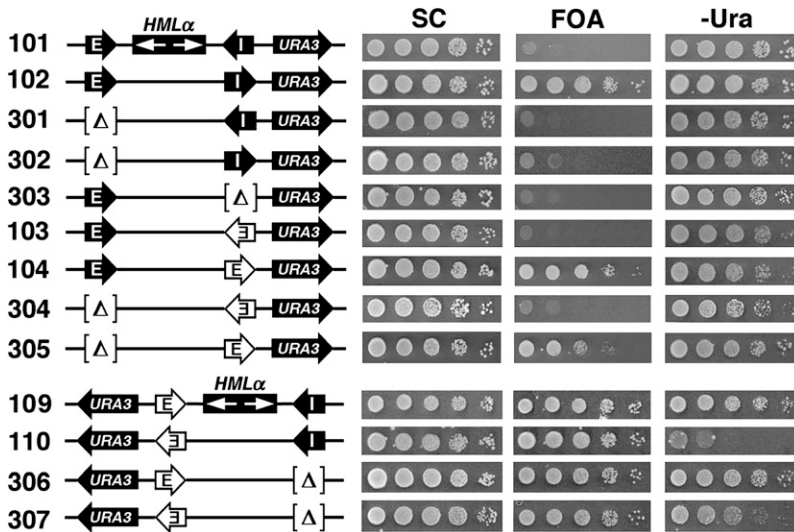
FIGURE 6.—Two silencers separated by a sizable distance can synergize to promote silencing outside the region they bracket. Schematics of the modified *HML* loci in strains 301–307 as well as 101–104, 109, and 110 are shown on the left. Symbols used are as described in Figures 1 and 2. Growth phenotypes of these strains are shown on the right.

indicated that R1 was also required for *URA3* silencing in strain 102. On the other hand, R1 did not seem to be required for *URA3* silencing mediated by *HMR-E* (Figure 7A, compare strains 402 to 104, as well as 403 to 305), which is consistent with the notion that *HMR-E* is stronger than *HML-I*.

The fact that R1 was required for the silencing of *URA3* outside of *HML* in strain 102 raised the question of whether the other known Rap1p site within *HML* was also required. This site (referred to as R2) resides near *HML-I* (Figure 7B) and only weakly binds Rap1p *in vitro* (BOSCHERON *et al.* 1996). R2 is apparently not required for the silencing of *HMLα* by the resident *HML-I* (FELDMAN *et al.* 1984). However, we showed that deletion of R2 abolished the silencing of *URA3* inserted to the right of the inverted *HML-I* silencer (Figure 7B, compare strains 102 and 406 on 5-FOA). Therefore, R2, like R1, is also required for the silencing of *URA3* outside of *HML*. On the other hand, R2 was not required for the unidirectional silencing activity of the *HMR-E* silencer (Figure 7B, compare 408 and 409 to 103 and 104, respectively). In fact, even when all of *HML-E*, R1, and R2 were simultaneously deleted, *HMR-E* in place of *HML-I* was still able to significantly silence *URA3* (Figure 7A, 404 on 5-FOA).

To verify the importance of the *HML* sequence bearing R1 and R2 in aiding the silencing of genes outside *HML*, we replaced the entire 3.3-kb *HML* sequence bracketed by *HML-E* and *HML-I* with the 2.0-kb *HMR* sequence that bears no known protosilencers in strains 101–104, resulting in 501–504, respectively (Figure 7C, left). *URA3* silencing by the inverted *HML-I* silencer was abolished (Figure 7C, 502), which was consistent with the fact that R1 and R2 in *HML* were both necessary for *URA3* silencing in strain 102 (Figure 7, A and B, compare 401 and 406 to 102). On the other hand, *HMR-E* in place of *HML-I* still significantly silenced *URA3* in a unidirectional manner (Figure 7C,

strains 503 and 504), which is in line with the fact that *HMR-E* activity was relatively independent of R1 and R2 (Figure 7, A and B, compare 402 and 409 to 104).

Taken together, the above data demonstrate that dispersed silencing elements can synergize to silence a gene located outside of the region harboring them. Remarkably, in strain 102, at least four silencing elements (*HML-E*, *HML-I*, R1, and R2) are required for the silencing of *URA3* inserted outside *HML* (Figures 6 and 7, compare strains 302, 303, 401, and 406 to 102).

The relative positions of the silencing elements are important for their functional interactions: As at least four silencing elements participated in *URA3* silencing in strain 102, it was possible that these elements all contribute to the putative buildup of the "strength" of silencing (perhaps in the form of the abundance of Sir proteins) over a threshold for silencing to occur. If this were the case, then the relative positions of the auxiliary protosilencers R1 and R2 might not be important for silencing. We tested this by precisely inverting the *HML* sequence in strain 102, making strain 411 (Figure 7B). In strain 411, sequences within *HML* that were previously close to *HML-I* were now distant from it, and vice versa. It was clear that inversion of *HML* abolished *URA3* silencing (Figure 7B, compare 411 to 102 on 5-FOA). One possibility was that R2 had to be physically close to *HML-I* for productive collaborations between the silencing elements. However, relocating R2 to a position near the right side of *HML-I* also abolished *URA3* silencing (Figure 7B, compare strain 407 to 102). Therefore, the mere proximity of R2 to *HML-I* was not sufficient for its role in promoting *URA3* silencing. The above results demonstrated that the relative position of R2 is important for its role in facilitating silencing in strain 102 and suggested that R2 was part of an intricate silencing network consisting of *HML-E*, *HML-I*, R1, and R2 (Figure 7D).
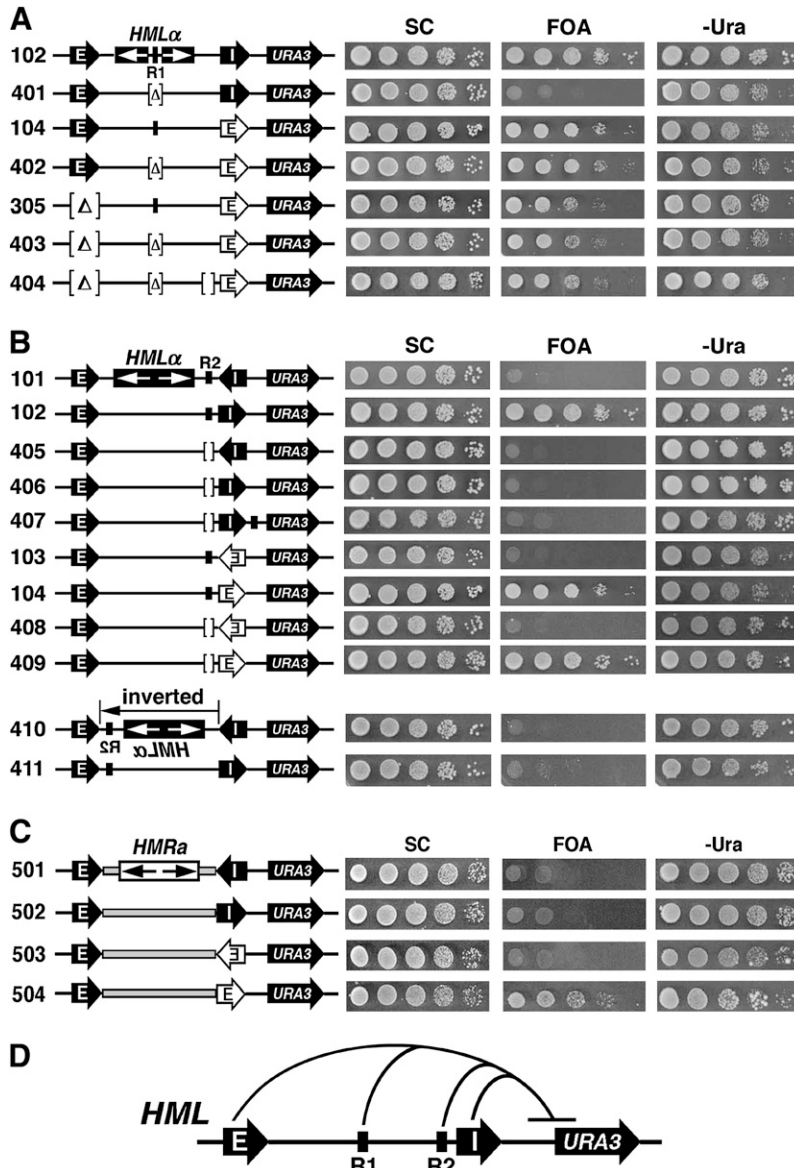
FIGURE 7.—Multiple dispersed silencing elements can cooperate to promote silencing outside the region harboring them. (A) The protosilencer R1 within the *HML* sequence is involved in silencing outside of *HML*. Left, strains used. Right, growth phenotypes. (B) The Rap1p-binding site near *HML-I* in *HML* (designated R2) is involved in silencing outside of *HML*. Left, strains used. Strains 410 and 411 are identical with 101 and 102, respectively, except that the *HML* sequence bracketed by the *HML-E* and *-I* silencers is inverted. Note that R2 is highlighted only in certain relevant strains in this report. Right, growth phenotypes. (C) Effects of replacing the *HML* sequence with the *HMR* sequence on silencing outside of *HML*. The *HMR* sequence excluding the *HMR* silencers (coordinates 291,539–293,532 of chromosome III) indicated by shaded rectangles was used to replace the *HML* sequence excluding the *HML* silencers in strains 101–104, making strains 501–504. (D) Four dispersed silencing elements at *HML* synergize to promote silencing outside *HML*. The *HML-E* and inverted *HML-I* silencers as well as the R1 and R2 protosilencers were all required for the silencing of *URA3* located to the right of *HML*. This is a summary of results concerning strains 102, 302, 303, 401, and 406 described in Figures 1 and 5–7.

DISCUSSION

The principal mechanism for how yeast silencers initiate the formation of transcriptionally silent chromatin has been elucidated (MOAZED 2001a; GREWAL and MOAZED 2003). However, the issue regarding whether and how silencers function in an orientation-dependent or -independent manner has not been completely resolved (BRAND *et al.* 1985; SHEI and BROACH 1995; BI *et al.* 1999). We have recently obtained evidence indicating that a silencer promotes asymmetric positioning of nucleosomes around it, leading to unequal silencing potentials on the two sides (ZOU *et al.* 2006). In other words, silencers are by nature unidirectional. It is therefore puzzling that the same silencer (*e.g.*, *HMR-E*) could act in an orientation-independent fashion as measured in one experiment, but appear unidirectional in another (SHEI and BROACH 1995; BRAND

et al. 1985). Results presented in this work provide two explanations for this conundrum.

The first concerns the distinct sensitivities of silencing assays used to measure silencer activity in different studies. The efficiency of silencing of a particular reporter gene by a silencer depends on the potency of the silencer as well as the strength of the promoter of the reporter gene that is inversely correlated with its sensitivity to the silencing machinery (VAN LEEUWEN and GOTTSCHLING 2002). Using both the basal and the activated expression of *URA3* as reporters, we found that a silencer may (i) silence both the basal and the activated expressions, (ii) silence the basal but not the activated expression, or (iii) silence neither the basal nor the activated expression. As a consequence, the directionality of a silencer examined in a specific experiment in this work depended on whether the basal or the activated expression of *URA3* was used as the

silencing reporter. For example, *HMR-E* inserted in the position of *HML-E* silenced the basal expression of *URA3* on the left of *HML* in an orientation-independent manner, but silenced activated *URA3* expression in only one direction (Figure 1B). Moreover, although *HMR-E* was able to silence the a1 gene at *HMR* independent of its orientation (Brand *et al.* 1985), we found it silenced basal *URA3* expression in one direction, but failed to silence activated *URA3* expression in either direction (Figure 1B). In addition, despite the directional behavior of *HML-I* detected by assaying *URA3* silencing, the endogenous *CHA1* gene located on the disfavored side of *HML-I* is still subject to Sir-dependent repression in native strains (Moreira and Holmberg 1998). In summary, the directional nature of the function of a silencer may be revealed only by using a silencing assay with sufficient resolution, and, regarding the silencing of native genes, the apparent directionality of a native silencer in its native context is not absolute.

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

We have demonstrated in this report that the activity of a silencer concerning both its potency and directionality can be affected by other silencing elements (silencers, protosilencers, and telomeres) that are present in its context. For instance, the *HML-E* silencer was able to increase silencing on the disfavored ORC side of *HMR-E* (in the context of *HML-I*) to a level comparable to that on its Abf1p side (Figure 5C). In other words, *HML-E* served to transform *HMR-E* into a bidirectional silencer. This is one of many examples observed in this work regarding two silencing elements cooperating to promote stronger silencing in the region bracketed by them. Moreover, we also showed that two silencers separated by the 3.3-kb *HML* sequence could synergize to silence a gene located *outside* of the region bordered by them (Figure 6). In addition, we found evidence that being close to the left telomere of chromosome III enhances the efficiency of silencing by a silencer (Figure 4).

The mechanism(s) underlying functional interactions among silencing elements have not been resolved, but several models have been proposed (Boscheron *et al.* 1996; Bi *et al.* 1999; Fourel *et al.* 2002). The fact that two silencers separated by up to several kilobases are able to cooperate to silence a reporter located between them can be explained by assuming that convergent spreading of Sir proteins emanating from the silencers is additive or synergistic so that silent chromatin established between the silencers is stronger than that formed by either silencer alone. In support of this

model, it was shown that silent chromatin formed by two bracketing *HMR-E* silencers had a higher density of positioned nucleosomes and thus a more compact chromatin structure than that formed by a single *HMR-E* (Reimer and Buchman 1997). However, this interpretation does not apply to silencer–protosilencer cooperation since a protosilencer is not able to autonomously initiate silencing. On the other hand, because a protosilencer is actually a binding site for Abf1p, Rap1p, or ORC that can position nucleosomes (Yu and Morse 1999; Lipford and Bell 2001; Bi *et al.* 2004; Yarragudi *et al.* 2004; Zou *et al.* 2006), it is conceivable that a protosilencer helps position nucleosomes in the region between it and the silencer in a configuration that is more favorable for the spread of Sir proteins from the silencer (Boscheron *et al.* 1996). Along this line, we think that the presence of a protosilencer on the disfavored side of a silencer may alter the putatively inhibitory pattern of nucleosome positioning and allow silencing to occur more efficiently on this side, thereby masking the inherent directionality of the silencer.

The two models discussed above cannot readily explain how a silencer cooperates with another silencer or protosilencer to promote stronger silencing in areas *outside* the region containing the silencing elements. Ample evidence suggests that yeast telomeres cluster at the nuclear periphery, creating discrete subnuclear compartments that are rich in Sir proteins (Maillet *et al.* 1996; Taddei and Gasser 2004). Recent evidence indicates that *HML* silencers physically associate with the left telomere of chromosome III and therefore with a Sir-rich compartment (Lebrun *et al.* 2003). Therefore, functional cooperation between two silencers could be the consequence of a mutual enhancement of their tethering to a Sir-rich compartment. We have presented a striking example of four silencing elements (silencers *HML-E* and *HML-I* and protosilencers R1 and R2) working together to silence a distal gene (summarized in Figure 7D). It is possible that all silencing elements act, at least in part, by contributing to the tethering of the locus to a Sir-rich subnuclear compartment.

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