Design of a minimal silencer for the silent mating-type locus HML of Saccharomyces cerevisiae

Jan M. Weber and Ann E. Ehrenhofer-Murray*

Zentrum für Medizinische Biotechnologie, Abteilung Genetik, Universität Duisburg-Essen, 45117 Essen, Germany

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ABSTRACT

The silent mating-type loci HML and HMR of Saccharomyces cerevisiae contain mating-type information that is permanently repressed. This silencing is mediated by flanking sequence elements, the E- and I-silencers. They contain combinations of binding sites for the proteins Rap1, Abf1, and Sum1 as well as for the origin recognition complex (ORC). Together, they recruit other silencing factors, foremost the repressive Sir2/Sir4 complex, to establish heterochromatin-like structures at the HM loci. However, the HM silencers exhibit considerable functional redundancy, which has hampered the identification of further silencing factors. In this study, we constructed a synthetic HML-E silencer (HML-SS ΔI) that lacked this redundancy. It consisted solely of Rap1 and ORC-binding sites and the D2 element, a Sum1-binding site. All three elements were crucial for minimal HML silencing, and mutations in these elements led to a loss of Sir3 recruitment. The silencer was sensitive to a mutation in RAP1, rap1-12, but less sensitive to orc mutations or sum1Δ. Moreover, deletions of SIR1 and DOT1 lead to complete derepression of the HML-SS ΔI silencer. This fully functional, minimal HML-E silencer will therefore be useful to identify novel factors involved in HML silencing.

INTRODUCTION

The eukaryotic genome is organized into regions with open, transcriptionally active euchromatin and regions with condensed, silent heterochromatin. Gene silencing in heterochromatin is not restricted to specific genes, but largely depends on the chromosomal location of a gene, and it involves the establishment of alternative chromatin states that prevent gene expression. Despite events that temporarily affect this compacted state, such as DNA unwinding prior to replication, or DNA repair, silencing is inherited during DNA replication and multiple cell divisions (1).

Studies of silencing in the yeast Saccharomyces cerevisiae have been fundamental in understanding the mechanisms of gene repression. In S. cerevisiae, there are three silenced regions: (i) the two silent mating-type loci HML and HMR, (ii) the telomeres and (iii) the ribosomal DNA (rDNA locus) (2). HML and HMR are located on the left and right arm of chromosome III, respectively, and carry a (HML) and a (HMR) mating-type information that, in contrast to the mating-type information at MAT, is permanently repressed. Silencing is mediated by regulatory sequences known as silencers (3). Both HM loci are flanked by an E- (essential) and an I- (important) silencer that differ in sequence, but contain common silencer elements. While the E-silencer alone can cause silencing of HML and HMR in the absence of the I-silencer, the I-silencer is only sufficient for HML, but not for HMR silencing (4,5).

HML silencing requires multiple cis-acting elements within the silencers that are binding sites for DNA-binding proteins and serve as recruitment sites for heterochromatic proteins (2). Notably, all four silencers contain an ARS consensus sequence (ACS), which is a binding site for the origin recognition complex (ORC) (6,7). The I-silencers both contain an additional Abf1-binding site, and the HMR-E silencer contains an Abf1 and a Rap1-binding site in addition to the ACS (3). HML-E consists of three functional elements, a Rap1-binding site, the ACS and a 93-bp sequence, the D element, which are required for silencing (8). A recent molecular analysis of the D element narrowed it down to a 10-bp core element, termed D2, which is bound by Sum1 (9).

Interestingly, all four known silencer binding factors have functions outside of silencing. ORC functions as the eukaryotic replication initiator and is required for initiation at chromosomal origins throughout the genome (10). Rap1 binds to telomeres and functions in telomeric silencing and telomere length regulation (11,12). It also

*To whom correspondence should be addressed. Tel: +49 201 183 4132; Fax: +49 201 183 4397; Email: ann.ehrenhofer-murray@uni-due.de

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binds to many gene promoters and serves as transcriptional activator (13). Abf1 binds some replication origins, and it also contributes to transcriptional activation by binding to gene promoters (13). Sum1 is part of a histone deacetylase complex that controls the expression of meiotic genes (14) as well as replication initiation of a number of chromosomal origins (9,15). Several origins are known to exhibit a dual role in silencing and replication initiation (16). However, HMR–E (ARS317) but not HML–E (ARS301) functions as a chromosomal origin of replication. HML–E is capable of serving as a replication origin on plasmids, but the chromosomal HML locus is replicated by another origin in the vicinity (17).

In order to establish HM silencing, Orc1 recruits the silent information regulator Sir1 to the silencers (18). This leads to the recruitment of Sir4 via its interactions with Rap1 and Sir1, and finally to binding of Sir2 and Sir3 (19). The NAD−-dependent histone deacetylase Sir2 removes acetyl groups from the N-terminal histone tails of nearby nucleosomes (20) and thus provides new binding sites for the Sir2/Sir3/Sir4 (SIR) complex, which requires deacetylated histones in order to bind to chromatin (21). This process results in a positive feedback loop, which leads to the formation of heterochromatin across the HM loci (19,22).

The spreading of silent chromatin into euchromatic regions is hindered by chromatin boundaries (23). For instance, the histone acetyltransferase complex SAS-1 serves as such a boundary factor in that it antagonizes Sir2 by acetylating H4 K16 (24,25). Among others, histone methylation also restricts heterochromatin spreading. H3 K79 methylation by Dot1 (26,27) inhibits histone methylation also restricts heterochromatin spreading. H3 K79 methylation by Dot1 (26,27) inhibits

The HM silencers exhibit considerable functional redundancy, because the deletion of any single element, for instance in HMR–E (29) or HML–E (8), has no measurable effect on repression. Only the simultaneous mutation of two elements, or the combination of the inactivation in cis of the binding site with a mutation in trans in the gene encoding a second binding factor, causes strong derepression (30). Furthermore, there are several close matches to the ACS around the HM loci (3), and cryptic origins of replication have been described close to strong derepression (30). Several origins are known to exhibit a dual role in silencing and replication initiation (16). However, HMR–E (ARS317) but not HML–E (ARS301) functions as a chromosomal origin of replication. HML–E is capable of serving as a replication origin on plasmids, but the chromosomal HML locus is replicated by another origin in the vicinity (17).

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The yeast strains and plasmids used in this study are listed in Tables 1 and 2, respectively. Unless indicated otherwise, all yeast and Escherichia coli manipulations were carried out according to standard protocols (34). The dot1Δ and sum1Δ gene disruptions were performed using the KanMX or HisMX cassette according to the guidelines of EUROFAN (35) and verified by PCR. The sir1Δ mutant used in this study derived from a genetic cross with a sir1Δ::LEU2 strain from the laboratory collection. Synthetic HML silencer variants were introduced into a MATα hmlα::URA3 strain (AEY3387) by homologous recombination of 3.8-kb ApaI/HindIII fragments from

| Table 1. Saccharomyces cerevisiae strains used in this study |
|---------------|---------------|
| Strain | Genotype |
| AEY2 | MATα can1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 (W303-1A) |
| AEY3 | MATα can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 W303-1B, but lys2Δ ADE2 |
| AEY3387 | AEY2, but hmlα::URA3 |
| AEY4404 | AEY2, but with HML–E (79 bp) ΔI |
| AEY4406 | AEY2, but HML–SS ΔI |
| AEY4408 | AEY2, but with HML–SS rap− ΔI |
| AEY4010 | AEY2, but HML–SS α− ΔI |
| AEY4012 | AEY2, but HML–SS D2− ΔI |
| AEY4428 | AEY4406, but sum1Δ::HisMX |
| AEY4464 | AEY4406, but rap1-12::LEU2 |
| AEY4486 | AEY4406, but orc2-1 |
| AEY4538 | AEY2, but HML–SS ΔI sir1Δ::LEU2 |
| AEY4805 | AEY2, but HML–SS ΔI URA3MX dot1Δ::KanMX |
| AEY4873 | AEY4406, but sir2Δ::HisMX |
| AEY4947 | AEY4408, but sir3Δ::HisMX |
| AEY4949 | AEY4412, but sir3Δ::HisMX |
| AEY4950 | AEY2, but HML–SS::URA3 ΔI sir3Δ::HisMX |
| AEY4960 | AEY2, but HML–SS::URA3 ΔI |

| Table 2. Plasmids used in this study |
|---------------|---------------|
| pAE1378 | YCplac22, HML ΔE ΔI |
| pAE1386 | YCplac22, HML–SS ΔI |
| pAE1388 | YCplac22, HML–SS α− ΔI |
| pAE1390 | YCplac22, HML–SS rap− ΔI |
| pAE1392 | YCplac22, HML–SS D2− ΔI |
| pAE1396 | YCplac22, wt HML–E (79 bp) ΔI |
| pAE1457 | pRS315-SIR3-3xHA::TRP1 |
plasmids carrying HML with synthetic HML-E variants (Table 2) and counterselected on 5–FOA medium. Replacement of hmlΔ::URA3 by the HML constructs was verified by PCR analysis. An URA3MX cassette was introduced into the HML-SS ΔI strain in the direct vicinity of HML-E (Chr. III coordinates 8966–9065) in order to mark the allele for genetic crosses.

**Design of synthetic HML-E silencers**

HML versions containing the synthetic HML-E variants were constructed by introducing complementary oligonucleotides of synthetic HML-E into an HML plasmid (pAE1378) using the oligonucleotide-mediated gap repair technique (YOGRT) (36). pAE1378 is based on YCplac22 (CEN, TRP1) and contains a 4.8-kb BamHI/HindIII HML fragment in which HML-I was deleted (9). Additionally, pAE1378 was constructed to contain a deletion of HML-E (Chr. III coordinates 11 187–11 409) that is marked by an AflII restriction site (see Figure 1; cloning details are available from the authors upon request). AflII-linearized pAE1378 was then used for YOGRT and co-transformed into yeast with three oligonucleotides per silencer construct: A 79-bp oligonucleotide comprising synthetic HML-E (or the mutant versions) and two 55–57-bp oligonucleotides corresponding to the sequences surrounding the 5‘ and the 3‘ junction between

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Design of a synthetic HML-E silencer. (A) The wt HML-E locus and five truncated constructs are shown. In the synthetic HML-E silencer (HML-SS ΔI), the nucleotide order between the silencer elements was partially scrambled while retaining the base pair composition (grey boxes). Mutations affecting binding sites for Rap1 (rap1/C0, vertical lines), the ORC complex (acs/C0, diagonal lines) and the D2 element (D2/C0, horizontal lines) are indicated. Vertical transparent grey lines delineate the mutated region. (B) DNA sequence of wt HML-E (Chr. III, coordinates 11 177–11 420) and synthetic HML-E silencer variants. The Rap1, ACS and D2 elements are indicated by black bars. Corresponding mutations in these elements within the HML-SS ΔI context are shown in bold letters. Transparent grey lines define the 79-bp synthetic HML-E silencer and the corresponding wt sequence. Light grey letters show nucleotides originating from the insertion of an AflII site that are only present in the synthetic constructs. Italics indicate the AflII site.
synthetic HML-E and wild-type HML, respectively (Figure 1; see Table 3 for oligonucleotide sequences and Table 4 for the combinations of oligonucleotides used in YOGRT to construct the silencer variants). Plasmids were isolated from yeast by amplification in E. coli and verified by sequence analysis.

**Insertion of URA3 at HML**

The a1 and a2 genes at the HML locus were replaced in a HML-SS Δ1 sir3Δ::HisMX strain (AEY4873) by the URA3 gene amplified from pRS406 (37), thus resulting in an HML-SS::URA3 Δ1 sir3Δ::HisMX strain (AEY4950). HML-SS::URA3 Δ1 SIR3 cells (AEY4960) were obtained from a genetic cross with a wild-type strain (AEY3). Serial dilutions were pinned on plates containing 5-Fluoro-orotic acid (5-FOA), and URA3 silencing was analysed by documenting the growth after 3 days at 30°C.

## HML silencing assay

HML silencing was measured by determining the mating ability of MATa strains with a MATa his4 tester strain (AEY265). Patch mating assays and quantitative mating analysis in three independent experiments were performed as described previously (38).

### Chromatin immunoprecipitation and quantitative real-time PCR

The mouse anti-HA.11 monoclonal antibody (Covance, Catalog # MMS-101 P Lot # 14943702) was used for chromatin immunoprecipitation (ChIP) analysis of Sir3-HA at HML, and quantitative real-time PCR were performed as described previously (15). Oligonucleotide sequences used for the analysis are available from the authors upon request.

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**RESULTS**

### Three HML-E core elements were sufficient to establish HML silencing

In this study, we sought to determine whether the combination of the three known HML-E silencer domains alone was sufficient to establish HML silencing. To this end, we constructed a synthetic version of HML-E that consisted of the Rap1-binding site, the ACS and the D2 element alone and tested its silencing capacity. In a first step, in order to remove potential binding sites near HML-E, a core version of natural HML-E was constructed in which 43 bp of upstream (telomere-proximal) and 107 bp of downstream sequence were removed, thus retaining 79 bp of natural HML-E (Figure 1A). The upstream deletion was chosen such as not to disturb the function of the neighbouring VBA3 gene, and the downstream deletion removed the intervening sequences between the D2 element and the W region of HML (39).

This HML-E version, termed wt HML-E (79 bp), was introduced into an HML allele lacking the I silencer (9) in order to measure silencing by the E silencer alone.

| Table 4. Combination of oligonucleotides used to generate synthetic HML-E fragments |
|-----------------------------------------------|
| Synthetic HML-E silencer | Oligonucleotide number from Table 3 |
| wt HML-E (79 bp) ΔI | 1, 6, 7 |
| HML-SS ΔI | 2, 8, 9 |
| HML-SS rap− ΔI | 3, 9, 10 |
| HML-SS acs− ΔI | 4, 8, 9 |
| HML-SS D2− ΔI | 5, 8, 11 |

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**Table 3.** Oligonucleotides used for generating synthetic HML-E fragments

| Number | Oligonucleotide | DNA sequence 5’-3’ |
|--------|-----------------|-------------------|
| 1      | wt_HML-E_fw     | TTA AGA GTA TCT TAT GAA TGG GTT TTT TTT ATG TTT TTT TAA AAC ATT AAA GGT TTT GGC GAC GAC TTA TTA G |
| 2      | ss_HML-E_fw     | TTA AGT TCG ATA TAT GAA TGG GTT TTT GTT TTT ATG TTT TAA ATA GAT CTA TAT ATT TCT GGC GAC GAC GGT TTA T |
| 3      | ss_HML-E_RAP1mut_fw | TTA AGT TCG ATA TTT CAT TCG CTA AAT TTT GTT TTT ATG TTT TAA ATA GAT CTA TAT ATT TCT GGC GAC GAC GGT TTA T |
| 4      | ss_HML-E_ACSmut_fw | TTA AGT TCG ATA TAT GAA TGG GTT TTT GTT TTT ATG TTT TAA ATA GAT CTA TAT ATT TCT GGC GAC GAC GGT TTA T |
| 5      | ss_HML-E_D2mut_fw | TTA AGT TCG ATA TAT GAA TGG GTT TTT GTT TTT ATG TTT TAA ATA GAT CTA TAT ATT TCT GGC GAC GAC GGT TTA T |
| 6      | YOGRT wt HML-E.up | AAT CAA AAA CCC ATT CAT AAG ATA CTC TTA AGA AAT TAC ATT CCA TTA CGA TAC ACC |
| 7      | YOGRT wt HML-E.down | GGT GTT TGA ATC AAT TTA GAA CAA ATT GAA TGA TTC CTG GCC GAA AAT TTT AAT G |
| 8      | YOGRT ssHML-E.up | CAA AAT AAA CCC ATT CAT ATA TCG AAG TTA AGA AAT TAC ATT CCA TTA CGA TAC AC |
| 9      | YOGRT ssHML-E.down | GGT GTT TGA ATC AAT TTA GAA CAA ATT GAA TGA TTC CTG GCC GAA AAT ATA TAG |
| 10     | YOGRT RAP1mut.up | CAA AAT TTA GGC AAT GAA ATA TCG AAG TTA AGA AAT TAC ATT CCA TTA CGA TAC AC |
| 11     | YOGRT D2mut.down | GGT GTT TGA ATC AAT TTA GAA CAA ATT GAA TGA TTC CTG GCC GAA AAT TTT AAT G |

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Silencing of \textit{HML} was determined by measuring the mating ability of \textit{MAT}a strains carrying the \textit{HML} allele by a patch mating assay (Figure 2A) as well as by a quantitative mating assay (Figure 2B). These assays are based on the fact that derepression of \textit{HML} in a \textit{MAT}a strain causes an a/\alpha cell type, thus resulting in the loss of a-mating ability of the strain (39). In these assays, \textit{wt} \textit{HML}-E (79 bp) mated as well as a wild-type strain (Figure 2), indicating that this silencer retained full silencing capacity. This suggested that the sequences flanking this core silencer did not significantly contribute to silencer function.

In order to eliminate potential redundant elements within the 79-bp core \textit{HML}-E silencer, we next generated a minimal \textit{HML}-E silencer, which we termed ‘synthetic silencer’ (\textit{HML-SS} \textit{\Delta}1), in which the wild-type Rap1, ACS and D2 elements were retained, whereas the nucleotide order of the short flanking and intervening sequences of the 79-bp \textit{HML}-E truncation was scrambled. The mutations were chosen such that the base composition and the distance between the elements remained unchanged (Figure 1B). A mating test of a \textit{MAT}a strain with this synthetic \textit{HML-SS} \textit{\Delta}1 variant showed a strong mating ability indicative of substantial \textit{HML} silencing. However, it was somewhat reduced as compared to the strain with \textit{wt} \textit{HML}-E (79 bp) \textit{\Delta}1 (Figure 2A). The quantitative analysis showed that \textit{HML-SS} \textit{\Delta}1 retained \textit{\sim}60\% silencing ability of wild-type \textit{HML}-E (Figure 2B). This showed that the combination of binding sites for Rap1 and ORC with the D2 element alone was sufficient to generate strong \textit{HML} silencing. Of note, the silencing provided by synthetic \textit{HML}-E was stronger than that by the synthetic \textit{HMR}-E silencer, which retained \textit{\sim}15\% silencing ability compared to wild-type \textit{HMR}-E (38). The difference between \textit{HML-SS} and \textit{wt} \textit{HML}-E (79 bp) indicated that the sequence scrambling had removed unknown functional sequences that contributed 40\% to silencing. In the further experiments, we used the \textit{HML-SS} \textit{\Delta}1 allele as the minimal \textit{HML} silencer.

The Rap1 and ORC-binding sites and the D2 element were essential for \textit{HML} silencing

The deletion of individual silencer domains in the natural \textit{HML}-E silencer does not cause \textit{HML} derepression (8), indicating that there is functional redundancy in natural \textit{HML}-E. We next asked whether the synthetic silencer eliminated this redundancy by determining whether the binding sites for Rap1 and ORC as well as the D2 element were required for silencing of \textit{HML-SS} \textit{\Delta}1. To address this, we constructed three \textit{HML}-E variants in which the sequence of one of these three elements was mutated (Figure 1B). For the \textit{HML-SS rap} \textit{\Delta}1 construct,
every other nucleotide of the Rap1-binding site was changed by a transitional mutation. The mutation of the ACS in the HML–SSacs− ΔI allele was designed analogously to that in the synthetic HMR-E silencer (33). Furthermore, the mutation of the D2 element in the HML–SSD2− ΔI variant was created by transitional mutation of every other nucleotide, as previously described (9).

Significantly, all three mutations led to strong HML derepression as indicated by a strong loss of mating ability in MATa strains in a patch mating assay (Figure 2A). This was confirmed by quantitative assays, which showed a strong reduction of the relative mating ability of the rap1-12 mutation (40) in combination with HML–SS ΔI caused a complete loss of silencing (Figure 4A). The quantitative analysis showed that the relative mating ability of this strain was comparable to that of the HML–SS rap− ΔI strain (Figure 4D). These data supported an essential role for Rap1 in HML silencing (41).

Surprisingly, the orc2-1 mutation within the ORC complex (6) in combination with the HML–SS ΔI silencer displayed a much weaker silencing defect than the HML–SSacs− ΔI allele (Figure 2A and Figure 4A). The relative mating ability of this strain was ~10% of the HML–SS ΔI control, but several-fold higher than that of the HML–SS ΔI ace− strain (Figure 4B). This was surprising, because silencing by a synthetic silencer at the other HM locus, HMR, is sensitive to orc mutations (42). We also tested the orc5-1 mutation (43) in the HML–SS ΔI strain, but it also did not enhance HML silencing.

Silencing of URA3 by the synthetic HML-E silencer

The analysis above showed that the synthetic HML-E silencer was capable of silencing the native α genes at HML. We next asked whether this silencing was more general by determining whether the minimal silencer was also able to silence a heterologous gene. For this purpose, we constructed a strain in which the HML α1 and α2 genes were replaced by URA3 and tested silencing by measuring the ability of the strain to grow in URA3-counterselective medium containing 5-fluoro-orotic acid (5-FOA). The strain showed a weak, but measurable ability to grow on 5-FOA (Figure 3), indicating that the synthetic HML-E silencer was able to provide a low level of silencing to the URA3 gene and thus had general silencing capacity. However, the URA3 silencing was inefficient as compared to silencing of the mating-type genes. Perhaps this inefficiency is due to the poor SIR spreading as measured by ChIP analysis (Supplementary Figure S1).

Mutations in trans caused a reduction in silencing by the minimal HML-E silencer

Since mutations of the individual silencer domains of HML–SS ΔI silencer caused a loss of HML silencing, we next asked whether mutation or deletion of the genes encoding the respective binding proteins lead to a similar loss of silencing. To this end, strains were constructed which combined HML–SS ΔI with mutations in RAP1, ORC (because the genes are essential) or with the deletion of SUM1, and HML silencing was tested by measuring the mating ability of MATα strains (Figure 4A–C). Importantly, the rap1-12 mutation (40) in combination with HML–SS ΔI caused a complete loss of silencing (Figure 4A). The quantitative analysis showed that the relative mating ability of this strain was comparable to that of the HML–SS rap− ΔI strain (Figure 4D). These data supported an essential role for Rap1 in HML silencing (41).

Figure 3. Silencing of URA3 by the synthetic HML-E silencer. The minimal HML-E silencer was tested for the ability to silence URA3 in HML-SS::URA3 ΔI cells. (A) Schematic representation of the hmlα1/α2 replacement by URA3. (B) Serial dilutions of wild-type (AEY2) and HML-SS::URA3 ΔI (AEY4960) strains plated on 5-FOA medium. Cells were grown for 2 days at 30°C. YPD served as growth control.
derepression (data not shown). This indicated that for unknown reasons (see ‘Discussion’ section), the ACS of HML-SS was not sensitive to the orc2-1 and orc5-1 mutations.

Similarly, the absence of Sum1, which has been shown to bind to the D2 element (9), caused only a slight reduction in silencing of HML-SS ΔI. The mating ability of a MATa HML-SS ΔI sum1Δ strain was reduced to ~50% of that of a strain with the minimal HML silencer alone (Figure 4C), and the effect was much less pronounced than for a D2 element mutation within the HML-SS ΔI variant (Figures 2B and 4D). This showed that Sum1 had some effect on silencing, but further suggested that other factors are involved in silencing via the D2 element.

Sir1 and Dot1 were required for silencing of HML-SS ΔI

Although Sir1 is required for full silencing of both HML and HMR, sir1Δ strains still show substantial silencing at both loci (44,45). However, sir1Δ causes complete derepression of HMR when controlled by synthetic HMR-E (18). Also, dot1Δ only causes derepression of HML when silencing is previously compromised by sir1Δ (46). Since synthetic HML-E constitutes a sensitized silencer, this suggested that sir1Δ and dot1Δ on their own might be able to derepress HML-SS ΔI. Therefore, we investigated whether sir1Δ or dot1Δ were capable of disrupting silencing in strains with the synthetic HML-SS ΔI allele. Significantly, both sir1Δ and dot1Δ caused a complete loss of mating ability, indicating a complete derepression of HML-SS ΔI (Figure 5). This showed that the minimal HML silencer sensitized HML silencing to mutations in SIR1 and DOT1.

Sas2 and Asf11 are other factors that have previously been shown to cause HML derepression upon deletion in a

Figure 4. Synthetic HML-E was sensitized for mutations in RAP1 and ORC and for the deletion of SUM1. (A–C) The mating ability of MATa HML-SS ΔI strains with rap1-12 (A), orc2-1 (B) or with sum1Δ (C) was compared to that of corresponding wild-type strains with the indicated HML alleles. (D) Quantitative mating efficiencies of MATa strains with the indicated genotypes. Error bars represent the standard deviations of three individual experiments.

Figure 5. Sir1 and Dot1 were essential for silencing of HML-SS ΔI. MATa HML-SS ΔI sir1Δ (A) and MATa HML-SS ΔI dot1Δ (B) cells were tested for their ability to mate with a MATa tester strain in a patch mating assay. YPD served as growth control.
sir1Δ background (38,47–49). However, asa2Δ and asf1Δ did not impair silencing of HML-SS ΔI (data not shown). Furthermore, we analysed the effect of a HIR1 deletion in HML-SS ΔI strain, since sir1Δ leads to a loss of HML silencing in a triple mutant strain with sir1Δ and cac1Δ (48). However hir1Δ did not affect the mating ability of an HML-SS ΔI strain. Similarly, the absence of Hir2, another component of the HIR nucleosome assembly complex (50), did not affect HML-SS ΔI (data not shown). Taken together, these results showed that the synthetic HML-E silencer sensitized silencing to some silencing factors, but that the sensitization was different from that caused by the absence of Sir1.

DISCUSSION

Repression of mating-type information at the silent mating-type loci HMR and HML in S. cerevisiae is necessary to maintain cell-type identity in haploid strains. HML silencing is buffered towards mutations in cis and in trans that affect the HML silencers. Here, we have generated a synthetic, minimal HML-E silencer that lacks the functional redundancy of natural HML-E. It therefore will be useful for future studies to identify new factors that are involved in the regulation of HML silencing, but so far escaped identification due to the redundancy of the wild-type HML silencer. This will hopefully allow novel insights into the mechanism of HML silencing.

The construction of a synthetic HML-E silencer conducted here was inspired by a classical study in which a silent mating-type I strain, since sir1Δ leads to a loss of HML silencing in a triple mutant strain with sir1Δ and cac1Δ (48). However hir1Δ did not affect the mating ability of an HML-SS ΔI strain. Similarly, the absence of Hir2, another component of the HIR nucleosome assembly complex (50), did not affect HML-SS ΔI (data not shown). Taken together, these results showed that the synthetic HML-E silencer sensitized silencing to some silencing factors, but that the sensitization was different from that caused by the absence of Sir1.

As expected, the silencing-defective rap1-12 allele (40) caused as strong derepression as mutation of the Rap1-binding site of HML-SS ΔI, which was consistent with its known role in HML silencing. The strong effect of rap1-12 may also be related to the fact that there is an additional Rap1-binding site in the UAS of the n2 gene, which has been shown to serve as a proto-silencer in HML silencing (41).

Surprisingly, while mutation of the putative ORC-binding site (ACS) caused strong HML derepression, two mutant orc alleles, orc2-1 (6) and orc5-1 (43), only caused a mild loss of silencing. These alleles were originally isolated based on their ability to cause derepression of a version of natural HMR-E, and they also derepress synthetic HMR-E (6,42). One could therefore argue that the HML-E ACS for some reason is not sensitive to these particular orc alleles. In fact, a recent genome-wide study of ORC-binding showed that not every chromosomal origin is equally sensitive to orc2-1 (51). However, the ACS of synthetic HML-E is identical to that of synthetic HMR-E. Also, in a highly sensitive silencing assay, natural HMLα, which also contains the same ACS, showed slight derepression by orc2-1 as measured by the α-mating ability of a strain lacking coding information at MAT (43). Therefore, this suggests that the sequences surrounding the ACS at the HML-E silencer determine whether it is sensitive to the orc alleles or not. It is also possible that ‘non-silencer replicator origins’ remain in the synthetic silencer, much as has been described for the natural HMR-E silencer (31) and despite our efforts to remove them in the synthetic HML-E construct. In light of this, there may exist a competition between the silencer ACS at HML-E and other putative ORC-binding sites in the vicinity, which may be responsible for the unexpected insensitivity of silencing to orc2-1. Of note, this would have to be a competition between silencer and non-silencer ORC-binding sites at HML, rather than between silencer and non-silencer origins at HMR, because HML-E is not a chromosomal replication origin, but is passively replicated by a replication fork originating from a nearby origin, ARS305 (17).

Alternatively, in light of a recent study showing that ORC binding spread throughout the HMR silent domain rather than being restricted to the HMR silencers (52), it is also possible that ORC similarly binds HML beyond the silencer, and that this binding, and thus the contribution of ORC to silencing, is not abrogated by orc2-1 and orc5-1.

Furthermore, we observed that the mutation of the D2 site of HML-SS ΔI caused strong derepression, but that the absence of Sum1, which we previously showed to bind to D2, caused only a minor amount of derepression (9). Our earlier genetic evidence for the involvement of Sum1 in HML silencing showed that it caused derepression of natural HML-E that was sensitized by the deletion of the Rap1 or ACS elements, but not the D element. Thus, the difference in sensitivity to Sum1 between natural and synthetic HML-E may lie in the sequence differences between the two silencers. It is also possible that the D2 element binds another protein in addition to Sum1, and that both need to be mutated to cause strong HML derepression. Further work will be required to identify such a factor.

A number of proteins involved in HML silencing have been identified over the years. Among these, one can distinguish between those generally essential for silencing, like the Sir2, Sir3 and Sir4 proteins (45), those that have an important function in silencing like Sir1 (44,45), and factors whose contribution to silencing is only apparent upon mutation or deletion of a second factor (2).
The \textit{HML–SS} ΔI silencer developed here is a minimal silencer that provides a sensitized background to identify novel regulators of \textit{HML} silencing. Notably, as for synthetic \textit{HMR–E}, our silencer was fully sensitive to the deletion of \textit{SIR1}. In addition, \textit{dot1Δ}, which derepresses natural \textit{HML} only in a \textit{sir1Δ} background (46), caused complete derepression of \textit{HML–SS} ΔI, thus providing a first example for a factor whose effect only becomes apparent in the sensitized background. Mechanistically, this may be explained by a less robust binding of the SIR proteins to synthetic \textit{HML–E}, such that they are more easily redistributed to euchromatic sites when genome-wide H3 K79 methylation is lost in the absence of Dot1.

Surprisingly, \textit{HML–SS} ΔI was sensitive to some, but not other silencing factors. For instance, its silencing was refractory to \textit{asf1Δ} and \textit{sas2Δ}, although both cause derepression of natural \textit{HML} in \textit{sir1Δ} cells (47,48). This indicates that there are important mechanistic differences between these factors and, for instance, Dot1, in silencing. It further suggests that the synthetic \textit{HML–E} silencer is more efficient at retaining the SIR complex in \textit{asf1Δ} and \textit{sas2Δ} than natural \textit{HML–E} in the absence of Sir1. Apparently, the absence of a Sas2-mediated chromatin boundary at \textit{HML} has less dramatic effects on the redistribution of SIR proteins than the absence of Dot1. This further indicates that the sensitization by \textit{HML–SS} ΔI is distinct from that of the absence of Sir1, and that synthetic \textit{HML–E} opens up the possibility of identifying novel silencing factors whose effect has so far been masked by genetic redundancy. The simplicity of the synthetic silencer will thus facilitate new insights into the mechanisms of transcriptional silencing.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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