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

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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/Sir3/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 *sum1*Δ. 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 α (*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).

HM 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

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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-I 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 SIR binding on the nucleosome and thus may prevent the propagation of SIR complexes along the chromatin fibre (28).

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 *HMR*-E that become activated when the ACS of *HMR*-E is mutated, but do not display silencing activity [so-called non-silencer replicators (31)]. Only by the removal of this redundancy has it been possible to genetically identify some of the silencer binding factors, for instance ORC (6) and Abf1 (32).

The demonstration that the ORC, Rap1 and Abf1-binding sites are the only essential silencer elements in *HMR*-E comes from a classical study that constructed a synthetic silencer consisting of these three elements alone (*HMR*-SS) (33). This silencer mediates strong SIR-dependent silencing at *HMR*, and it lacks the functional redundancy of natural *HMR*-E, because the mutation of any single site in this silencer causes strong derepression.

In this study, we sought to determine whether the hitherto characterized silencer elements of *HML*-E were necessary and sufficient for *HML* silencing. To this end, we designed and characterized a synthetic version of the *HML*-E silencer that consisted of the Rap1 and ORC-binding sites and the D2 element alone. This silencer provided strong repressing activity, showing that these elements are sufficient for *HML* silencing. Furthermore, it was sensitized towards mutations *in cis* as well as *in trans*. Therefore, this simplified version of *HML*-E will provide an important genetic tool to identify novel factors involved in *HML* silencing that so far have evaded detection due to the functional redundancy of natural *HML*-E.

MATERIAL AND METHODS

Strains and plasmids

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 *MAT*a *hmlΔ::URA3* strain (AEY3387) by homologous recombination of 3.8-kb *Apa*LI/HindIII fragments from

Table 1. *Saccharomyces cerevisiae* strains used in this study

| Strain	Genotype
AEY2	*MAT*a *can1-100 ade2-1 his3-11,15 leu2-3, 112 trp1-1 ura3-1* (W303-1A)
AEY3	*MAT*α *can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1* W303-1B, but *lys2Δ ADE2*)
AEY3387	AEY2, but *hmlΔ::URA3*
AEY4404	AEY2, but wt *HML*-E (79 bp) ΔI
AEY4406	AEY2, but *HML-SS* ΔI
AEY4408	AEY2, but *HML-SS rap⁻* ΔI
AEY4010	AEY2, but *HML-SS acs⁻* 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 *sir3Δ::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 acs⁻* 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 oligo-nucleotides 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 *Bam*HI/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 *Afl*II restriction site (see Figure 1; cloning details are available from the authors upon request). *Afl*II-linearized pAE1378 was then used for YOGRT and co-transformed into yeast with three oligo-nucleotides 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
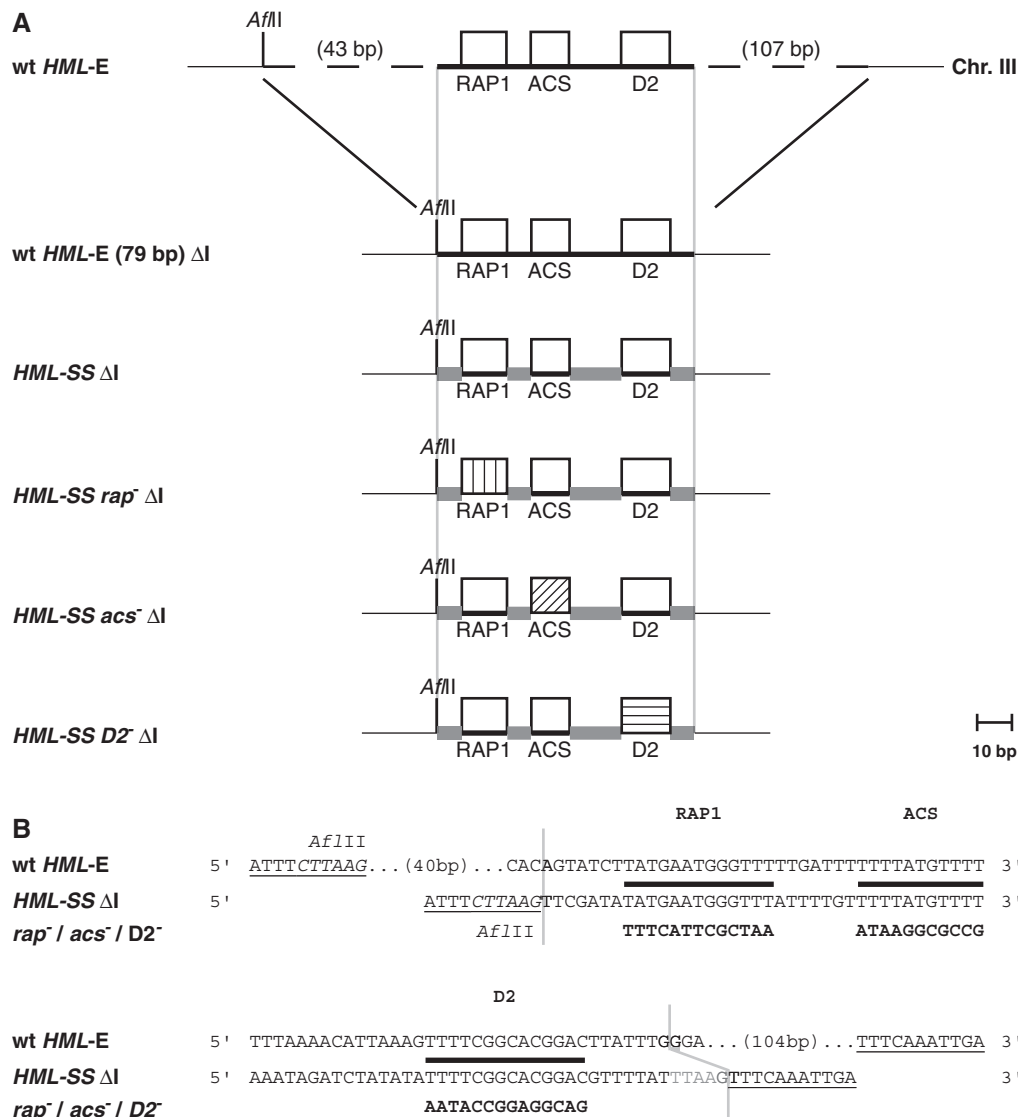
Figure 1. Design of a synthetic *HML*-E silencer. (**A**) The wt *HML*-E locus and five truncated constructs are shown. In the synthetic *HML*-E silencer (*HML-SS* ΔI), the nucleotide order between the silencer elements was partially scrambled while retaining the base pair composition (grey boxes). Mutations affecting binding sites for Rap1 (*rap1⁻*, vertical lines), the ORC complex (*acs⁻*, diagonal lines) and the D2 element (*D2⁻*, 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 *Afl*II site that are only present in the synthetic constructs. Italics indicate the *Afl*II 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 α1 and α2 genes at the *HML* locus were replaced in a *HML-SS* ΔI *sir3*Δ::*HisMX* strain (AEY4873) by the *URA3* gene amplified from pRS406 (37), thus resulting in an *HML-SS*::*URA3 sir3*Δ::*HisMX* strain (AEY4950). *HML–SS*::*URA3* ΔI *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 *MAT***a** strains with a *MAT*α *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.
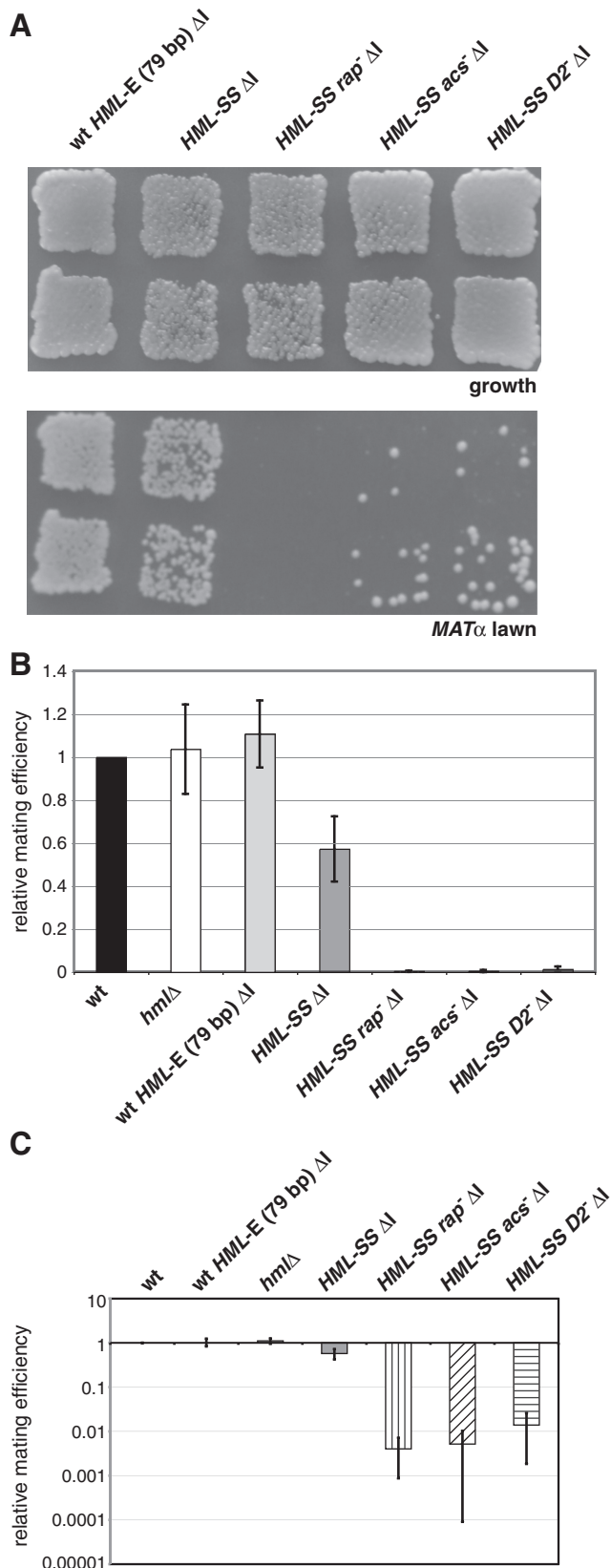
RESULTS

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

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 GAT TTT TTT ATG TTT TTT TAA AAC ATT AAA GTT TTC GGC ACG GAC TTA TTT G
2	ss_HML-E_fw	TTA AGT TCG ATA TAT GAA TGG GTT TAT TTT GTT TTT ATG TTT TAA ATA GAT CTA TAT ATT TTC GGC ACG GAC GTT 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 TTC GGC ACG GAC GTT TTA T
4	ss_HML-E_ACSmut_fw	TTA AGT TCG ATA TAT GAA TGG GTT TAT TTT GTA TAA GGC GCC GAA ATA GAT CTA TAT ATT TTC GGC ACG GAC GTT TTA T
5	ss_HML-E_D2mut_fw	TTA AGT TCG ATA TAT GAA TGG GTT TAT TTT GTT TTT ATG TTT TAA ATA GAT CTA TAT AAA TAC CGG AGG CAG GTT TTA T
6	YOGRT wt HML-E.up	AAT CAA AAA CCC ATT CAT AAG ATA CTC TTA AGA AAT TAC ATT CCA TTG CGA TAC ACC
7	YOGRT wt HML-E.down	GGT GTT TGA ATC AAT TTG AAA CTT AAC AAA TAA GTC CGT GCC GAA AAC TTT AAT G
8	YOGRT ssHML-E.up	CAA AAT AAA CCC ATT CAT ATA TCG AAC TTA AGA AAT TAC ATT CCA TTG CGA TAC AC
9	YOGRT ssHML-E.down	GGT GTT TGA ATC AAT TTG AAA CTT AAA TAA AAC GTC CGT GCC GAA AAT ATA TAG
10	YOGRT RAP1mut.up	CAA AAT TTA GCG AAT GAA ATA TCG AAC TTA AGA AAT TAC ATT CCA TTG CGA TAC ACC
11	YOGRT D2mut.down	GGT GTT TGA ATC AAT TTG AAA CTT AAA TAA AAC CTG CCT CCG GTAT TTA TAT AG

Silencing of *HML* was determined by measuring the mating ability of *MAT*a strains carrying the *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 *HML* in a *MAT*a strain causes an a/α cell type, thus resulting in the loss of a-mating ability of the strain (39). In these assays, wt *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 *HML*-E silencer, we next generated a minimal *HML*-E silencer, which we termed 'synthetic silencer' (*HML-SS* ΔI), 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 *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 *MAT*a strain with this synthetic *HML-SS* ΔI variant showed a strong mating ability indicative of substantial *HML* silencing. However, it was somewhat reduced as compared to the strain with wt *HML*-E (79 bp) ΔI (Figure 2A). The quantitative analysis showed that *HML–SS* ΔI retained ~60% silencing ability of wild-type *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 *HML* silencing. Of note, the silencing provided by synthetic *HML*-E was stronger than that by the synthetic *HMR*-E silencer, which retained ~15% silencing ability compared to wild-type *HMR*-E (38). The difference between *HML-SS* and wt *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 *HML-SS* ΔI allele as the minimal *HML* silencer.

The Rap1 and ORC-binding sites and the D2 element were essential for *HML* silencing

The deletion of individual silencer domains in the natural *HML*-E silencer does not cause *HML* derepression (8), indicating that there is functional redundancy in natural *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 *HML-SS* ΔI. To address this, we constructed three *HML*-E variants in which the sequence of one of these three elements was mutated (Figure 1B). For the *HML–SS rap⁻* ΔI construct,

Figure 2. Characterization of synthetic *HML*-E silencer constructs. Mutation of individual *HML*-E elements in the synthetic silencer caused *HML* derepression. (**A**) Mutations of the Rap1 and ORC-binding sites and the D2 element caused a loss of *HML* silencing as measured by a loss in mating ability. Patch mating assay of *MAT*a yeast strains with genomically integrated *HML*-E alleles. YPD served as growth control. (**B**) Mating efficiency of *MAT*a strains carrying the indicated *HML* alleles was measured in a quantitative mating assay and normalized to the mating efficiency of a wt *HML* strain. Error bars represent the standard deviations of three individual experiments. (**C**) Mating efficiencies as in (B), but tabulated on a logarithmic scale.

every other nucleotide of the Rap1-binding site was changed by a transitional mutation. The mutation of the ACS in the *HML–SS acs⁻* ΔI allele was designed analogous to that in the synthetic *HMR*-E silencer (33). Furthermore, the mutation of the D2 element in the *HML–SS D2⁻* 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 *MAT***a** 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 *rap⁻*, *acs⁻* and *D2⁻* strains compared to the *HML-SS* ΔI strain with wild-type silencer elements (Figure 2B and C). This showed that all three elements within the truncated 79-bp *HML*-E silencer were not only sufficient, but also essential for *HML* silencing.

We further asked how the mutations in the individual subdomains of the synthetic *HML*-E silencer affected silencing. One possibility is that the combination of silencer binding sites serves to recruit the SIR complex, which then spreads over the silenced region (2). Thus, mutations in the *HML*-E domains might be expected to abrogate SIR recruitment. To test this, we measured Sir3 occupancy at *HML* by ChIP in strains carrying the different silencer variants. As expected, we observed Sir3 enrichment in the vicinity of the synthetic *HML*-E, but not in control regions upstream and downstream of *HML* (Supplementary Figure S1). However, Sir3 binding was abolished when the Rap1 or the D2 site were mutated, showing that the recruitment of the SIR complex was abrogated by mutations *in cis*. Notably, there was no Sir3 enrichment downstream of the α1 gene, suggesting that SIR spreading was inefficient in this context.
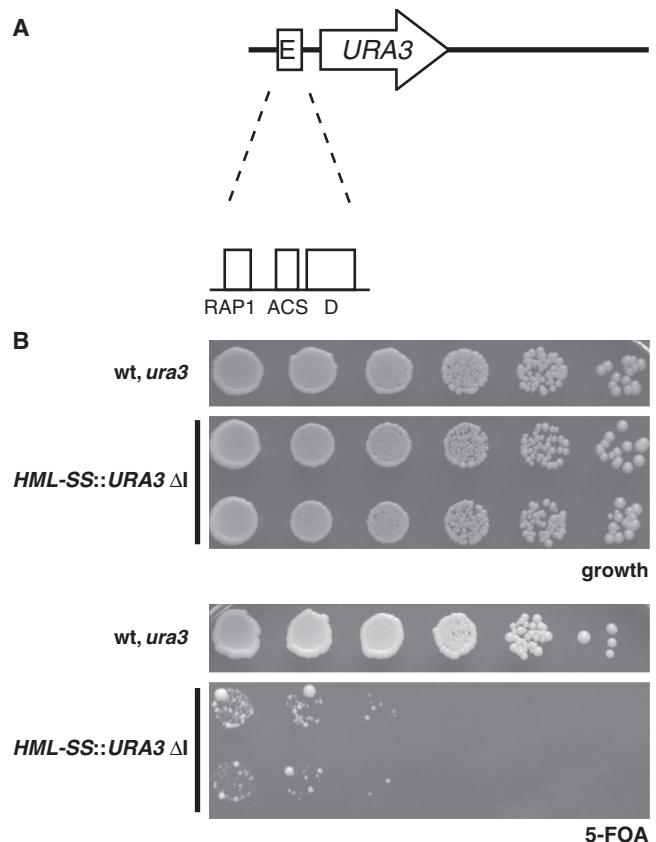
Silencing of *URA3* by the synthetic *HML*-E silencer

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 *MAT***a** 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).

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–SS acs⁻* 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 *acs⁻* 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*

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 *hml*α1/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.
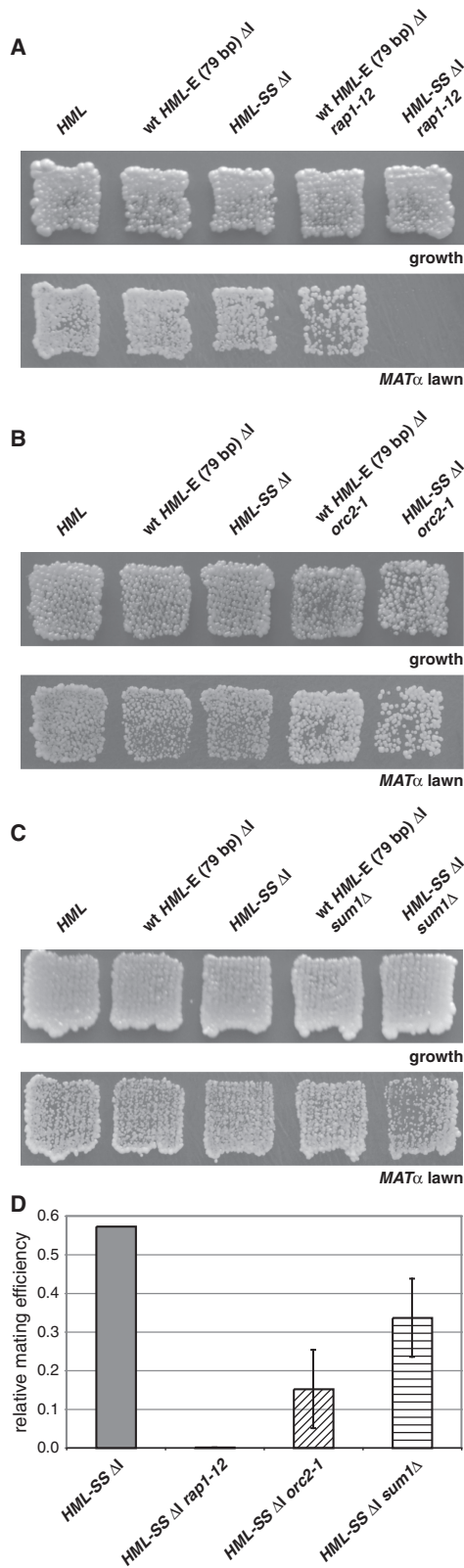
Figure 4. Synthetic *HML*-E was sensitized for mutations in *RAP1* and ORC and for the deletion of *SUM1*. (**A–C**) The mating ability of *MAT***a** *HML-SS* ΔI strains with *rap1-12* (A), *orc2-1* (B) or with *sum1*Δ (C) was compared to that of corresponding wild-type strains with the indicated *HML* alleles. (**D**) Quantitative mating efficiencies of *MAT***a** strains with the indicated genotypes. Error bars represent the standard deviations of three individual experiments.

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 *MAT***a** *HML-SS* ΔI *sum1*Δ 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*, *sir1*Δ strains still show substantial silencing at both loci (44,45). However, *sir1*Δ causes complete derepression of *HMR* when controlled by synthetic *HMR*-E (18). Also, *dot1*Δ only causes derepression of *HML* when silencing is previously compromised by *sir1*Δ (46). Since synthetic *HML*-E constitutes a sensitized silencer, this suggested that *sir1*Δ and *dot1*Δ on their own might be able to derepress *HML-SS* ΔI. Therefore, we investigated whether *sir1*Δ or *dot1*Δ were capable of disrupting silencing in strains with the synthetic *HML-SS* ΔI allele. Significantly, both *sir1*Δ and *dot1*Δ 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
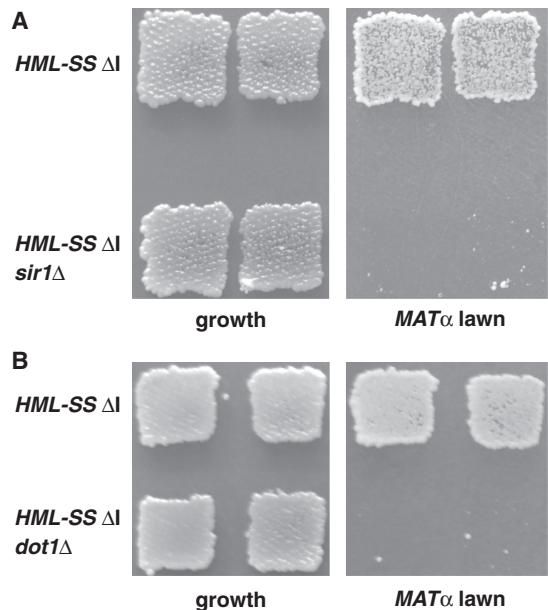
Figure 5. Sir1 and Dot1 were essential for silencing of *HML-SS* ΔI. *MAT***a** *HML-SS* ΔI *sir1*Δ (**A**) and *MAT***a** *HML-SS* ΔI *dot1*Δ (**B**) cells were tested for their ability to mate with a *MAT*α tester strain in a patch mating assay. YPD served as growth control.

*sir1*Δ background (38,47–49). However, *sas2*Δ and *asf1*Δ 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 *hir1*Δ leads to a loss of *HML* silencing in a triple mutant strain with *sir1*Δ and *cac1*Δ (48). However *hir1*Δ 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. *HM* silencing is buffered towards mutations *in cis* and *in trans* that affect the *HM* 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 minimal silencer for the other silent mating-type locus, *HMR*, was generated (33). Similarly to that silencer, our minimal *HML*-E version consisting of a Rap1-binding site, an ACS and the D2 element alone showed a silencing ability comparable to that of wt *HML*-E, thus establishing for the first time that these three domains alone are sufficient for *HML* silencing. Additionally, we were able to show that the minimal silencer was also capable of silencing a heterologous gene, *URA3*, although this silencer was relatively inefficient, suggesting that this construct works better in the context of its natural target genes than with a heterologous gene. Furthermore, this silencer was sensitive to mutations in any one of the three silencer elements, thus also showing that they were necessary for silencing, and that the functional redundancy of natural *HML*-E has been eliminated in this construct. Also, this silencer was able to recruit Sir3 to *HML*, and mutations within the silencer elements caused a loss of Sir3 recruitment. However, we made some unexpected observations concerning the *trans* requirements for silencing of *HML-SS* ΔI.

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 α2 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 *HML*α, 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 *HM* 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 *HML–SS* ΔI silencer developed here is a minimal silencer that provides a sensitized background to identify novel regulators of *HML* silencing. Notably, as for synthetic *HMR*-E, our silencer was fully sensitive to the deletion of *SIR1*. In addition, *dot1*Δ, which derepresses natural *HML* only in a *sir1*Δ background (46), caused complete derepression of *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 *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, *HML-SS* ΔI was sensitive to some, but not other silencing factors. For instance, its silencing was refractory to *asf1*Δ and *sas2*Δ, although both cause derepression of natural *HML* in *sir1*Δ 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 *HML*-E silencer is more efficient at retaining the SIR complex in *asf1*Δ and *sas2*Δ than natural *HML*-E in the absence of Sir1. Apparently, the absence of a Sas2-mediated chromatin boundary at *HML* has less dramatic effects on the redistribution of SIR proteins than the absence of Dot1. This further indicates that the sensitization by *HML-SS* ΔI is distinct from that of the absence of Sir1, and that synthetic *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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