# A Novel Yeast Silencer: The 2μ Origin of *Saccharomyces cerevisiae* Has *HST3*-, *MIG1*- and *SIR*-Dependent Silencing Activity

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ABSTRACT

Silencing in *Saccharomyces cerevisiae* is found at the mating-type loci *HMR* and *HML*, in subtelomeric regions, and at the rDNA locus. Repressed chromatin is built up by the recruitment of the Sir proteins via their interaction with DNA-binding proteins that bind to silencers. Here, we have performed a genetic screen for novel sequence elements within the yeast genome that display silencing activity. We isolated as a novel silencer element the origin of replication from the endogenous 2μ plasmid (2μARS). 2μARS-mediated silencing was dependent upon the Sir proteins, the origin recognition complex (ORC), and Hst3, a Sir2 histone deacetylase homolog, suggesting that it constituted a novel class of silencing in yeast. Moreover, 2μARS carried a binding site for Mig1, a transcriptional repressor of glucose-regulated genes. Both the Mig1-binding site and the *MIG1* gene were necessary for full silencing activity of 2μARS. Furthermore, Hst3 was physically present at 2μARS in a silencing context as well as at the endogenous 2μ plasmid. Also, Hst3 regulated the repression of the flipase gene, although this was likely an indirect effect of *HST3* on *FLP1* expression.

HETEROCHROMATINIZATION is a mechanism of gene regulation that is widespread in eukaryotes and contributes to global chromosomal architecture as well as to the establishment of distinct transcriptional domains within the genome. For instance, centromeric sequences in Drosophila are condensed in the chromocenter, and the translocation of genes into this region causes stochastic inactivation in some cell clones, resulting in a mosaic pattern of gene silencing termed position-effect variegation (PEV; Weiler and Wakimoto 1995). Interestingly, the replication initiator complex origin recognition complex (ORC) is involved in silencing in Drosophila. *Dm*ORC interacts with heterochromatin protein 1 (HP-1) and localizes to the chromocenter, and mutations in *Dm*ORC cause a loss of PEV, showing that ORC is required for gene silencing in Drosophila (Pak *et al.* 1997).

Gene silencing in the yeast *S. cerevisiae* also requires ORC. In yeast, three classes of silencing are known to date: repression of the silent mating-type loci *HML* and *HMR*, which is required for the maintenance of haploid cell types (Loo and Rine 1995), and telomeric and rDNA silencing, which likely are the result of the protection against recombination at these loci (Aparicio *et al.* 1991; Smith and Boeke 1997). Silencing is best studied at the *HM* loci, where a heterochromatin-like structure is found that contains hypoacetylated histones (Braunstein *et al.* 1993) and is refractory to many types of protein-DNA interactions (Singh and Klar 1992; Loo and Rine 1994). Repression is achieved by the combined action of flanking sequence elements termed silencers and several regulatory proteins. The silencers contain binding sites for Rap1, Abf1, and ORC (Loo and Rine 1995). Some of the silencers are also chromosomal origins of replication, but replication initiation *per se* is not required for silencing (Ehrenhofer-Murray *et al.* 1995; Fox *et al.* 1997). One model for ORC's role in silencing is that it recruits Sir1 to the silencer via an interaction between Orc1 and Sir1 (Triolo and Sternglanz 1996), which in turn attracts other factors, for instance Sir2, Sir3, and Sir4, to form repressed chromatin at the *HM* loci (Fox *et al.* 1997; Gardner *et al.* 1999). Sir2 has NAD-dependent histone deacetylase activity and hence may be responsible for the lack of histone acetylation in the silenced regions (Imai *et al.* 2000; Tanny and Moazed 2001), whereas the other Sir proteins likely are heterochromatin components comparable to HP-1 in Drosophila (Nielsen *et al.* 2001).

ORC binding to the silencers is necessary but not sufficient for silencing, because not all origins of replication or ORC-binding sites in the genome are capable of conferring silencing. For instance, replacement of *HMR*-E by ARS1 does not induce *HM* silencing (Fox *et al.* 1995). However, many origins contain binding sites for Rap1 or Abf1 (Rao *et al.* 1994), but to date, no other origin has been found to have silencer activity, and it is currently not clear what distinguishes origins that are solely replicators from silencer origins.

Of the Sir proteins, only Sir2 is required for silencing at all known silent loci in yeast. Sir2 is the only Sir

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protein with homologs in larger eukaryotes, and there are also homologs of Sir2 (Hst proteins) in yeast itself (Brachmann *et al.* 1995), some of which have demonstrated histone deacetylase activity (Smith *et al.* 2000). The Hst protein family can be divided into three subfamilies, depending on the length and sequence of their amino and carboxy termini (Sherman *et al.* 1999). Sir2 and Hst1 are in one subfamily with Sir2 homologs from *Kluyveromyces lactis* and *Candida albicans*. Overexpression of *HST1* in part restores silencing at *HMR* in a *sir2*Δ mutant, showing that Hst1 can partially substitute for Sir2 at the *HM* loci (Brachmann *et al.* 1995). Additionally, Hst1 functions together with Sum1 in the repression of middle sporulation-specific genes during mitosis (Xie *et al.* 1999) and is required for *HMR* silencing that is mediated by Sum1-1 (Rusche and Rine 2001; Sutton *et al.* 2001). The second subfamily of Hst proteins contains Hst2, the human homolog of Sir2, hSIR2A, and other Hst homologs from various organisms. Hst2, a cytoplasmic enzyme, accounts for the majority of detectable NAD-dependent deacetylase activity in yeast (Landry *et al.* 2000). Overexpression of Hst2 disrupts telomeric silencing while improving repression in the rDNA (Perrod *et al.* 2001). The third group of Sir2 homologs contains the *S. cerevisiae* proteins Hst3 and Hst4. An *hst3 hst4* double mutant is defective in telomeric but not *HM* silencing and shows a modest UV sensitivity, a cell cycle delay, and chromosomal instability (Brachmann *et al.* 1995), whereas single mutants have no discernible phenotype. However, little is known about the cellular function of these Hst proteins.

In this study, we have conducted a search for novel silencing activities in the yeast genome and have identified the origin of replication of the endogenous 2μ plasmid (2μARS) as a novel DNA element that conferred transcriptional repression to two reporter genes. The 2μ plasmid found in most *S. cerevisiae* strains contains a unique autonomous replicative sequence (ARS) that serves as the sole *in vivo* origin of replication within the plasmid (Huberman *et al.* 1987) and at which replication is initiated once during each cell cycle (Zakian *et al.* 1979). Other functional elements in the 2μ plasmid are the five 1/2 tandem repeats of a 62-bp sequence in the stability locus (STB) that is required for plasmid segregation; the genes *REP1*, *REP2*, and *RAF1* that encode proteins required for gene expression and plasmid segregation; and the two flipase recognition targets (FRTs), which are binding sites for the flipase Flp1 (for review see Broach and Volkert 1991). Flp1, which itself is encoded on the 2μ plasmid, induces a double-strand break and catalyzes the site-specific recombination between inverted repeats on the plasmid, resulting in the interconversion of two inversion isomers of 2μ.

Interestingly, silencing by 2μARS was dependent on the Sir proteins and also required the proteins Hst3 and Mig1 for full repression. Mig1 is a repressor/activator protein that recruits the Ssn6-Tup1 complex to glucose-repressed gene promoters (Treitel and Carlson 1995), thus leading to deacetylation of histones (Bone and Roth 2001). This is the first demonstration of a class of silencing that was dependent on *HST3* and *MIG1*. It suggests novel roles for transcription factors in repression and furthers our knowledge of alternative mechanisms of gene silencing in yeast.

## MATERIALS AND METHODS

**Plasmid constructions:** The silencing reporter plasmid pAE370 was constructed as follows: A 1.85-kb fragment containing the **a**1 gene and the I silencer of *HMR* was PCR amplified with flanking *Sac*II sites and ligated into the *Sac*II site of a version of pRS315 (Sikorski and Hieter 1989) in which part of the polylinker had previously been removed by digestion with *Xho*I/*Sal*I and religation. Subsequently, the *URA3* gene was PCR amplified from pRS316 (Sikorski and Hieter 1989) with flanking *Not*I sites and ligated into the *Not*I sites of the previous construct, resulting in pAE370. Derivatives of pAE370 carrying silencers were constructed as follows: pAE374 was constructed by PCR amplification of *HMR*-E with flanking *Bam*HI sites and cloning it into the *Bam*HI site of pAE370. The synthetic *HMR*-E silencer (SS; McNally and Rine 1991) was cloned into pAE370 by digesting pDR60 with *Bam*HI and *Sac*II and isolating a 130-bp SS fragment. Overhangs were blunt-ended by treatment with Klenow polymerase and T4 DNA polymerase and ligated into the *Sma*I site of pAE370. This resulted in pAE411, in which SS is in the forward orientation (ORC-Rap1-Abf1), and in pAE412 with SS in a reversed orientation (Abf1-Rap1-ORC). Deletion of the *HMR*-I silencer in the silencing cassette plasmids was achieved by digesting with *Bcl*I and *Xho*I, filling in the ends with Klenow polymerase, and religation.

Plasmid pAE528, which contains the 1.56-kb 2μ fragment, was isolated from a library of genomic *Sau*3AI fragments in pAE370 (see below). *Sna*BI and *Bam*HI digestion, Klenow treatment, and religation of pAE528 resulted in pAE536 that contains the STB of the 2μ fragment with part of the *in vivo*-mapped origin of replication (Huberman *et al.* 1987). A *Hin*dIII and *Sna*BI digestion, Klenow treatment, and religation of pAE528 resulted in pAE572, which contained part of the 2μ origin of replication and the flipase recognition target. A total of 300 bp of the 2μ origin of replication surrounding the ARS consensus sequence (ACS) were PCR amplified with *Bam*HI linkers and cloned into the *Bam*HI site of pAE370. With this strategy, we obtained a series of plasmids containing the 2μARS in one to three copies (pAE480 with 1x-2μARS reverse, pAE481 with 1x-2μARS forward, pAE482 with 3x-2μARS forward, pAE483 with 2x-2μARS forward, and pAE484 with 2x-2μARS reverse).

Plasmids pAE805, -806, and -807 were derivatives from pAE481, -483, and -482, respectively, with the *URA3* gene in the reverse orientation. To this end, *URA3* was amplified from pRS316 with flanking *Not*I and *Xba*I sites and ligated into the *NotI/ XbaI* sites of pAE481, -483, and -482.

The ARS1 origin sequence was isolated from pARS1/WTA (Marahrens and Stillman 1992) by *Eco*RI and *Bam*HI digestion, and the end-repaired 210-bp ARS1 fragment was ligated into the *Sma*I site of pAE370, thus producing pAE503 with ARS1. This plasmid was digested with *Bam*HI and treated with Klenow polymerase, and ARS1 was inserted as above, resulting in pAE515, which carried two copies of ARS1. The plasmid pAE518 that contains three copies of ARS1 in the forward orientation was generated by ligation of the blunt-ended *Eco*RI/*Bam*HI ARS1 fragment into the *Hin*dIII site of pAE515.

Plasmid pAE808, which contains 3x-2μARS without the Mig1-binding sites, and pAE859, which contains 2x-ARS1 with additional Mig1-binding sites, were constructed by PCR amplification and sequential cloning of the resulting fragments for ARS1 into the *Sma*I, *Bam*HI, and *Hin*dIII sites of pAE370, as described above.

**Construction of genomic libraries:** Genomic libraries were prepared by partially digesting yeast genomic DNA with either *Sau*3AI or *Hae*III and ligating size-fractionated fragments into the *Bam*HI and *Sma*I sites of pAE370, respectively. Plasmid DNA was prepared from 14,000 pooled *Escherichia coli* transformants and used to transform the yeast strain AEY565 to leucine prototrophy at a density of ~200 colonies per plate. These colonies were replica plated onto selective fluoroorotic acid (FOA) plates, and FOA-resistant ($FOA^R$) colonies were then replica plated onto YM plates lacking uracil. Colonies with an $FOA^R/Ura^+$ phenotype were analyzed in a serial dilution assay to confirm silencing activity. Genomic DNA prepared from the candidates was used to transform the *E. coli* strain DH5α to ampicillin resistance, and plasmid DNA was prepared. Plasmid inserts were partially sequenced and the sequence was compared to the *Saccharomyces* Genome Database (SGD). Furthermore, plasmids were retransformed into AEY565 and a second serial dilution assay was performed. Only candidates that retained the $FOA^R/Ura^+$ phenotype were used for further investigations.

**Yeast methods:** The strains used in this study are listed in Table 1. Media for the growth of *S. cerevisiae* were as described in Sherman (1991); YM + 5-FOA medium contained the drug 5-fluoroorotic acid (Boeke *et al.* 1984) at 1 mg/ml and 2% glucose. All cultures were grown at 30° except strain AEY2243 that was grown at 23°. Yeast transformations were performed by the lithium acetate procedure (Ito *et al.* 1983). Strain AEY1365 was constructed by inducing a mating-type switch in AEY565 using the plasmid YCp50-Gal1-10-HO. Construction of the different gene disruptions in AEY565 was performed by replacing the respective genes with *kanMX* using the PCR knockout strategy according to the guidelines for EUROFAN (Wach *et al.* 1994). Knockout strains were verified by PCR analysis.

Serial dilution assays were performed as described in Aparicio and Gottschling (1994). For quantitative FOA assays, cells were plated at a density of ~200 colonies on supplemented YM to determine the rate of survival. Cell density was 10- to 1000-fold higher on FOA plates than on YM plates to allow optimal determination of $FOA^R$ colonies. The ratio of $FOA^R$ colonies per viable colonies was determined. Patch mating assays were performed as described in Ehrenhofer-Murray *et al.* (1995).

**Chromatin immunoprecipitations:** Chromatin immunoprecipitation (ChIP) analyses were performed as previously described (Hecht *et al.* 1996). PCR reactions were performed at 94° (1 min), 54° (30 sec), and 72° (40 sec). Cycle numbers were 30 or 35, depending on the experiment. PCR products were analyzed on 1% agarose gels.

**Other:** Site-directed mutagenesis was performed with the QuickChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). Northern blot analysis was performed as described in Sambrook *et al.* (1989). Sequences of all primers used in this study are available upon request from the author.

## RESULTS

**A functional screen for silencers in *Saccharomyces cerevisiae*:** Silencers are sequence elements that confer repression to genes located in their vicinity. The silencers known to date in yeast are the *HM* silencers (Loo and Rine 1994) and the subtelomeric core X element (Fourel *et al.* 1999; Pryde and Louis 1999), but it is unclear whether other silencers exist in yeast that might mediate gene repression by yet unknown mechanisms. Identifying such silencers has the potential of yielding

**TABLE 1**

**Yeast strains used in this study**

| Strain | Genotype | Source[a] |
|---|---|---|
| AEY1 | *MAT*α *ade2-1 leu2-3 can1-100 trp1-1 ura3-1 his3-11, 15* (W303) | J. Rine |
| AEY264 | *MAT***a** *his4* | J. Rine |
| AEY265 | *MAT*α *his4* | J. Rine |
| AEY565 | *MAT***a** *ade2-1 leu2-1 trp1-1 ura3-52 his3-11 lys2-8 ppr1::HIS3* | Aparicio *et al.* (1991) |
| AEY579 | *MAT***a** *leu2-3 can1-100 trp1-1 his4-519 ura3-52* | J. Rine |
| AEY1365 | AEY565, but *MAT*α | |
| AEY1486 | AEY565; *sir2*Δ*::kanMX* | |
| AEY1488 | AEY565; *sir3*Δ*::kanMX* | |
| AEY1490 | AEY565; *sir4*Δ*::kanMX* | |
| AEY1509 | AEY565; *hst1*Δ*::kanMX* | |
| AEY1511 | AEY565; *hst2*Δ*::kanMX* | |
| AEY1513 | AEY565; *hst3*Δ*::kanMX* | |
| AEY1515 | AEY565; *hst4*Δ*::kanMX* | |
| AEY1517 | AEY565; *hdf1*Δ*::kanMX* | |
| AEY2009 | AEY565; *sir1*Δ*::kanMX* | |
| AEY2243 | AEY565; *orc2-1* | |
| AEY2458 | AEY1365; *HST3-HA::K.l.TRP1* | |
| AEY2530 | AEY565; *mig1*Δ*::kanMX* | |
| AEY2724 | AEY565; *hda1*Δ*::kanMX* | |
| AEY2726 | AEY565; *sfp1*Δ*::kanMX* | |

[a] All strains except the ones indicated were constructed during the course of this study.

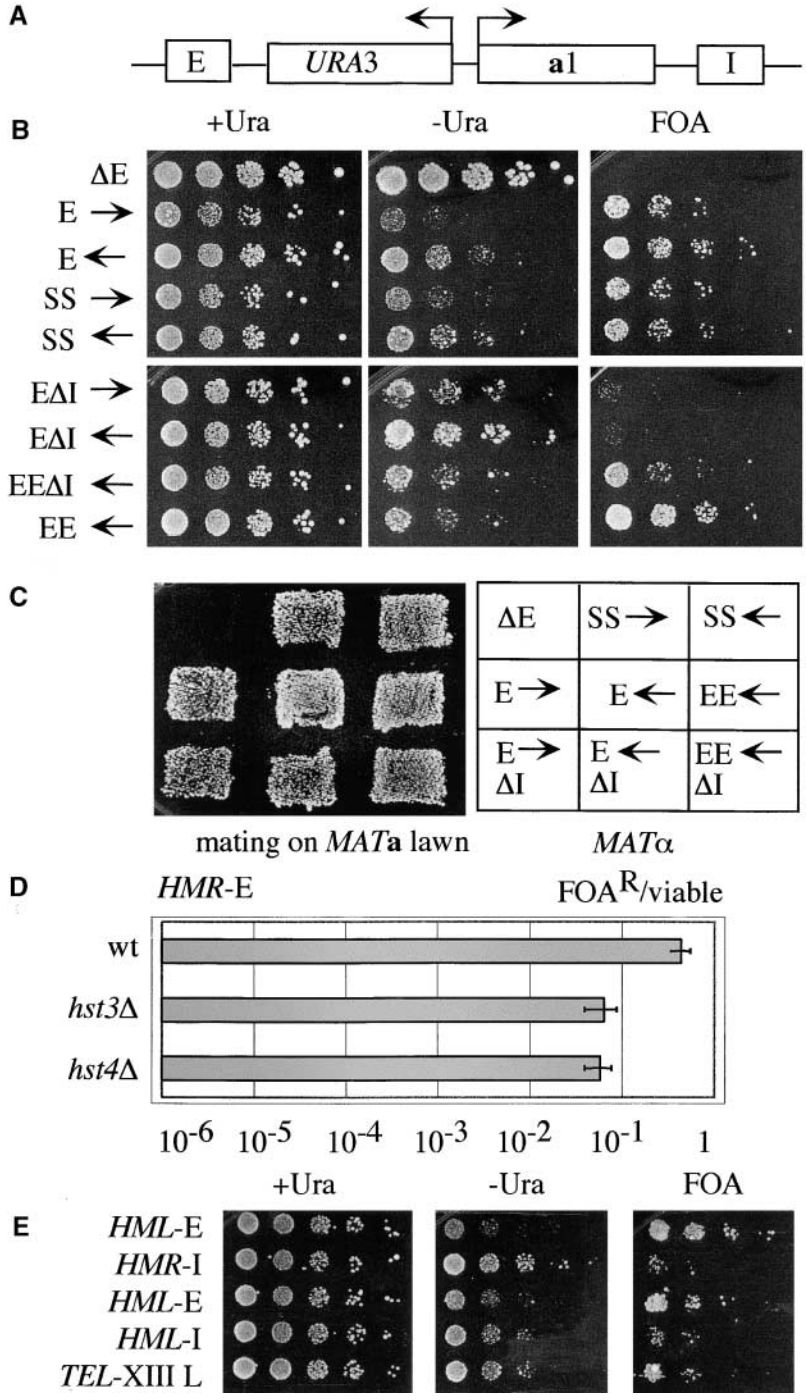


FIGURE 1.—Characterization of the reporter cassette (silencer trap) for measuring silencing activity. (A) Principle of the silencer trap is as follows: a cassette was constructed to contain the *URA3* and the **a**1 genes flanked by the *HMR*-E and -I silencers. Arrows indicate the direction of transcription. For the screen, small genomic DNA fragments were cloned into the reporter plasmid in place of *HMR*-E. (B) A serial dilution assay was performed to characterize the reporter assay. Insertion of *HM* silencers resulted in *URA3* repression and, hence, $FOA^R$. The effect of the wild-type (E) and the synthetic *HMR*-E silencer (SS) in the presence or absence of *HMR*-I on *URA3* gene repression is shown. Arrows indicate the orientation of the silencer with respect to *URA3*. (C) Repression of the **a**1 gene in the silencing cassette, as measured in a mating assay. (D) Effect of *hst3*Δ and *hst4*Δ on silencing activity of *HMR*-E in the reporter cassette. A quantitative FOA assay was performed. The mean values of at least three independent experiments are shown. (E) Effect of known silencers isolated in the silencer trap screen on expression of *URA3* in the silencing cassette.

information on novel silencing mechanisms and may help locate other silenced regions in the yeast genome.

In this study, we sought to find other, potentially novel silencing activities by searching for sequence elements capable of mediating silencing. Our approach was to construct a plasmid carrying a "silencing cassette" with two divergently transcribed reporter genes and to build libraries of yeast genomic sequences inserted in front of the cassette. These libraries were transformed into an appropriate yeast strain, and the transformants were then screened for clones in which both reporter genes were repressed ("silencer trap"). The reporter cassette consists of two components: (1) the *URA3* gene, whose expression can be monitored both on uracil-lacking medium and on medium containing the drug 5-FOA, and (2) the mating-type gene **a**1, which, when expressed, leads to a nonmating phenotype in a *MAT*α strain and hence can be measured in a mating assay. The effect of silencing on *URA3* was sensitized by measuring expression in strains lacking the *trans*-activator of *URA3*, Ppr1 (ROY *et al.* 1990). The silencing cassette was flanked on one side by the cryptic *HMR*-I element (Figure 1A),

which on its own did not confer silencing. *HMR*-I is capable of supporting silencing by weak silencers and thus was expected to improve the likelihood of isolating DNA elements with weak silencing activity.

In a first set of experiments, we investigated the properties of the silencing cassette. In the absence of a silencer (ΔE), the cells were completely $Ura^+$ and FOA sensitive, indicating full expression of *URA3* despite the presence of *HMR*-I. Furthermore, a *MAT*α strain carrying the silencing cassette lacking *HMR*-E (ΔE) was a nonmater, indicating full expression of **a**1. Conversely, expression of both reporter genes was efficiently repressed by the wild-type *HMR*-E silencer as well as by a synthetic version of *HMR*-E (McNally and Rine 1991) independently of the orientation of the silencer (Figure 1, B and C), as indicated by the strong FOA resistance ($FOA^R$) and improved mating ability of the respective strains. At the same time, the strains were also $Ura^+$, indicating that *URA3* was still expressed in a portion of the cells. Furthermore, deletion of the *HMR*-I silencer reduced the silencing activity, showing that *HMR*-I alone did not provide silencing, but supported repression mediated by *HMR*-E. These characteristics of *URA3* repression were reminiscent of subtelomeric insertions of *URA3* that cause strains to be both $Ura^+$ and $FOA^R$ due to telomeric repression of the reporter gene in a portion of the cells (Gottschling *et al.* 1990).

Silencing of the reporter cassette was further characterized by determining whether it displayed some of the known characteristics of *HM* silencing. As for *HM* silencing, repression was dependent on *SIR1*, *SIR2*, *SIR3*, and *SIR4* (data not shown). Furthermore, we investigated the effect of deletions of the Sir2 homologs *HST1* to *HST4* on repression of the silencing cassette by *HMR*-E. Interestingly, silencing was independent of *HST1* and *HST2*, but the deletion of *HST3* (*hst3*Δ) or *HST4* (*hst4*Δ) led to a slight reduction of the silencing capacity of *HMR*-E by ~10-fold (Figure 1D). In addition, the *hst3*Δ and *hst4*Δ colonies grew slower on FOA plates than did the isogenic wild-type strain. These results suggested that *hst3*Δ and *hst4*Δ caused slight derepression at *HMR*-E on the silencing cassette. This was in contrast to the observations made on chromosomal *HMR*, which is not affected by *hst3*Δ and *hst4*Δ (Brachmann *et al.* 1995; data not shown). We also tested the effect of *hst3*Δ or *hst4*Δ on repression by the sensitized synthetic *HMR*-E silencer. However, since neither single nor double deletion caused derepression (data not shown), this suggested that silencing of the reporter cassette was more sensitive to *trans*-acting factors than was chromosomal silencing.

To identify silencing elements in the yeast genome, libraries were constructed with fragments of yeast genomic DNA inserted in front of the reporter cassette lacking *HMR*-E (*i.e.*, downstream of *URA3*). The libraries were transformed into the yeast reporter strain, and the transformants were screened for clones that were $FOA^R$ as well as $Ura^+$. $Ura^+$ clones were selected because $FOA^R$ clones that were completely $Ura^-$ in all cases proved to have lost or mutated the *URA3* gene (data not shown). In screening 25,000 transformants, we isolated 14 $FOA^R$/$Ura^+$ candidates whose phenotype could be reconfirmed (see materials and methods). As expected, we were able to recover the known silencers with this screen: four clones contained *HML*-E, three clones carried *HML*-I, and one clone carried *HMR*-I. Furthermore, we isolated three independent clones of a subtelomeric region of the left arm of chromosome XIII (Figure 1E). Silencing activity in this case could be narrowed down to a subtelomeric core X element and an internal $C_{1-3}$-A repeat (data not shown). Interestingly, we isolated one novel fragment with silencing activity from the endogenous 2μ plasmid of *S. cerevisiae*. An involvement of 2μ DNA in silencing is unprecedented and thus was further analyzed.

**Silencing activity by the 2μ origin of replication:** The 1.56-kb fragment of 2μ plasmid DNA isolated in the screen for silencers contains three functional sequences that play important roles in replication and stability of the extrachromosomal 2μ DNA (Broach and Volkert 1991): the 2μ origin of replication (2μARS), the STB, and the FRT (Figure 2A). In a first experiment, we asked whether $FOA^R$ colonies from the 2μ fragment were truly epigenetically silenced by determining whether they were able to switch to $Ura^+$. About 1/100 of the cells were $FOA^R$, but were inhomogenous in size upon growth on FOA. Also, only 1–5% of the $FOA^R$ colonies were able to grow when replicated onto medium lacking uracil. They hence had a functional *URA3* gene and were capable of epigenetic switching. Further analysis showed that the other ~95% of $FOA^R$ colonies had lost or mutated *URA3* (data not shown). Thus, $\sim 1/10^4$ colonies carrying the 2μ fragment displayed silencing. This was in contrast to silencing by the *HMR*-E silencer, in which all $FOA^R$ colonies were also $Ura^+$ (data not shown).

To narrow down the silencing activity, we divided the 2μ fragment into a 870-bp fragment containing the STB as well as a part of the 2μARS origin of replication and a 697-bp fragment containing the other part of the 2μ fragment including the ACS and the FRT site. Neither construct showed silencing activity in a serial dilution assay as measured by the appearance of $FOA^R$ colonies that were also $Ura^+$ (Figure 2A). Interestingly, the fragment that contained the FRT site gave $FOA^R$ colonies that were all $Ura^-$. These $FOA^R$ colonies carried deletions in or around the *URA3* gene (data not shown), which could be the consequence of an Flp1-induced recombination event at FRT. However, a 300-bp fragment surrounding the 2μ origin of replication (2μARS) alone gave $FOA^R$ colonies at a frequency of $1/10^5$. Notably, all these $FOA^R$ colonies were $Ura^+$, suggesting that they displayed epigenetic repression. Also, introducing two or three tandem repeats of the 2μARS (2x-2μARS, 3x-2μARS) into the reporter cassette further increased

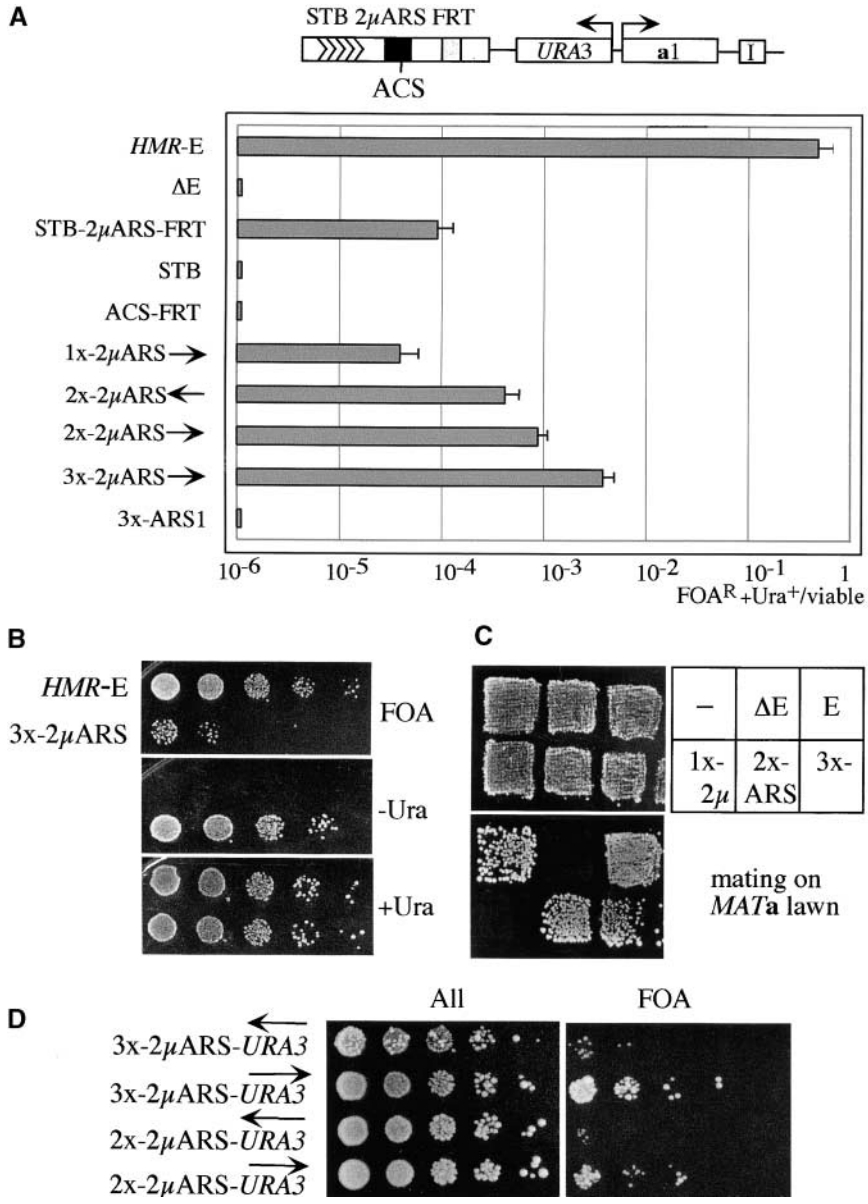


FIGURE 2.—The 2μ origin of replication was a silencer. (A) Schematic representation of the 1.56-kb 2μ fragment isolated in a screen for silencers and quantitative measurement of the frequency of $FOA^R$/$Ura^+$ colonies obtained with derivatives of the 2μ fragment cloned in front of the reporter cassette. $FOA^R$ colonies with a $Ura^+$ phenotype per viable colonies on a logarithmic scale are shown. Arrows indicate the orientation of 2μARS with respect to *URA3*. The graph shows the mean values of at least three independent experiments. STB, stability locus; 2μARS, 2μ origin of replication; FRT, flipase recognition target. (B) *URA3* silencing of the 3x-2μARS construct compared to the *HMR*-E construct is shown in a serial dilution assay. (C) Repression of the **a**1 gene by the 3x- and 2x-2μARS constructs compared to *HMR*-E in a mating assay. Dash indicates no silencing reporter; ΔE, silencing reporter lacking *HMR*-E. (D) Distance-dependent silencing of the *URA3* gene by the 3x- and 2x-2μARS constructs is shown in a serial dilution assay. Arrows indicate the direction of *URA3* transcription.

the number of $FOA^R$/$Ura^+$ colonies (Figure 2, A and B). Mating assays of *MAT*α strains carrying these constructs also showed strong silencing of the **a**1 gene, suggesting that the silencing activity of the 2μARS was gene independent (Figure 2C).

We also measured the effect of 2μARS on mRNA levels of **a**1 and *URA3* by Northern blotting. Whereas **a**1 message was undetectable in strains carrying the silencing reporter plasmid with *HMR*-E, no difference was found in strains carrying one, two, or three copies of 2μARS, compared to strains with no silencer. This may be due to the fact that silencing by 3x-2μARS is approximately two orders of magnitude lower than that by *HMR*-E. Furthermore, *URA3* levels were undistinguishable even in the presence of *HMR*-E due to expression of *URA3* from the endogenous *ura3-52* allele (data not shown).

Silencing induced by tandem repeats is not unprecedented and might simply be a consequence of the repeats themselves rather than the particular sequence involved. Therefore, we tested whether three tandem repeats of another origin of replication, ARS1, could also promote silencing. However, no silencing was observed with this construct (Figure 2A; see also Figure 4B), showing that the silencing activity of the repetitive 2μARS was specific to this origin of replication.

In our silencing cassette, the putative silencer repressing the *URA3* promoter is located downstream of *URA3* at a distance of ~1 kb. To test the effect of promoter distance on silencing by 3x-2μARS, plasmids were constructed in which the *URA3* promoter was proximal to 3x-2μARS. Interestingly, silencing activity by 2x-2μARS and 3x-2μARS in these constructs increased compared to the 2μARS constructs that contained the *URA3* pro-

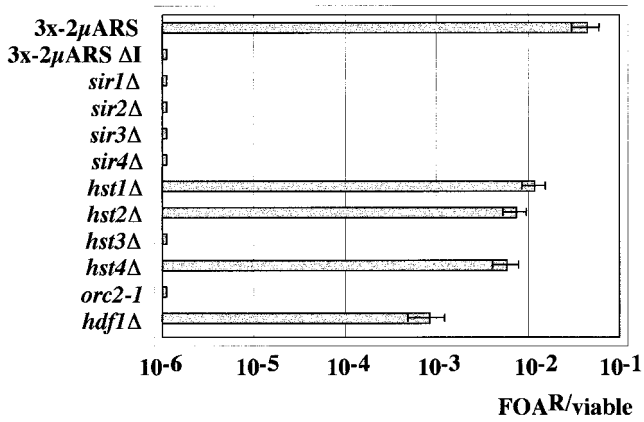


FIGURE 3.—Silencing by 2μARS was *SIR*, *ORC2*, and *HST3* dependent. The effect of the deletion of the *SIR* genes, *HST* genes, and *HDF1* (yKu70) as well as the *orc2-1* mutation on 3x-2μARS silencing activity was determined. The frequency of $FOA^R/Ura^+$ colonies was quantitated and is represented as in Figure 2A.

moter in a silencer distal orientation, demonstrating a distance-dependent silencing effect of 2μARS (Figure 2D).

Silencing activity of 3x-2μARS was so far measured in the presence of the *HMR*-I silencer. We next determined whether the 2μ origin could independently provide silencing. The deletion of *HMR*-I caused a complete loss of $FOA^R$ colonies (Figure 3), showing that 3x-2μARS required *HMR*-I for silencing. Thus, it can be classified as a proto-silencer, which is a silencer that acts only in conjunction with another (proto-)silencer.

**2μARS silencing was *SIR*, *ORC2*, and *HST3* dependent:** To further characterize the silencing capacity of the 2μ origin of replication, we tested the effect of deletions or mutations in genes encoding silencing proteins on 2μ-mediated silencing. 3x-2μARS silencing was dependent upon all four *SIR* genes and *ORC2* and thus displayed similarity to *HM* silencing. Conversely, the deletion of the *SIR2* homologs *HST1*, *HST2*, or *HST4* and deletion of *HDF1* caused no or minor derepression of 3x-2μARS-mediated silencing. However, the disruption of *HST3* caused complete derepression, which was as strong as the loss of silencing by the *SIR* deletions (Figure 3). Notably, the *hst3*Δ-dependent derepression was stronger than that observed for *HMR*-E when inserted into the silencer cassette. Hst3 has been shown by two-hybrid assay to interact with the split finger protein Sfp1 (UETZ *et al.* 2000). However, 3x-2μARS-mediated silencing was unaffected by the deletion of *SFP1* (data not shown), showing that this interaction was not necessary for Hst3 function in this context.

Taken together, these results showed that 2μARS silencing had some similarities, but also some differences to *HM* silencing and thus constituted a novel class of silencer in yeast. Furthermore, these observations showed that the 2μ origin of replication had silencing potential that distinguished it from other origins. Interestingly, the known *HM* silencers also possess ARS activity, although only the *HMR* silencers are chromosomal origins of replication (RIVIER *et al.* 1999). However, not every origin or ORC-binding site confers silencing and it remains to be determined what features unite the 2μ origin and the *HM* silencers.

**The transcriptional repressor Mig1 was necessary for 2μARS-mediated silencing:** One common theme of silencing at the *HM* loci as well as at subtelomeric core X elements is the presence of binding sites for ORC (ACS), Rap1, and Abf1, and all these elements are required for repression. The 2μARS also contains an ACS, and ORC was required for 2μARS-mediated silencing (see above). We next asked whether 2μARS contained binding sites for known silencing proteins or whether other DNA-binding proteins might be present. A sequence analysis of the 2μARS origin of replication using the TransFac Database (QUANDT *et al.* 1995) revealed no binding sites for Rap1 or Abf1. However, 2μARS contained a DNA consensus binding site for the transcriptional repressor Mig1 that was located 218 bp away from the ACS. The ACS is located at positions 3702–3712 of the 2μ GenBank sequence. Furthermore, TransFac identified a potential binding site for Fkh1 in the 2μARS, at a distance of 77 bp from the ACS (Figure 4A). We therefore asked whether these sites were required for 2μARS-mediated silencing. Mutation of all three Fkh1-binding sites in the 3x-2μARS construct did not diminish silencing (data not shown), suggesting that forkhead proteins were not involved in this form of silencing. Interestingly, a deletion of the Mig1 consensus binding sites in 3x-2μARS led to a reduction of both *URA3* and **a**1 silencing (Figure 4, B and C). This demonstrated that this *cis*-element was important for full silencing activity and suggested an involvement of the Mig1 protein in 2μARS-mediated silencing. We next determined whether the deletion of the *MIG1* gene influenced silencing. Significantly, silencing by 3x-2μARS was reduced in a *mig1*Δ strain (Figure 4D), and the contribution of *mig1*Δ was comparable to that of the deletion of the Mig1-binding site. Taken together, these results suggested that 2μARS-mediated silencing depended upon Mig1 for full repression.

Since 2μARS has silencing activity that distinguished it from, for instance, the ARS1 origin and since ARS1 has no Mig1 site in its vicinity, we hypothesized that the Mig1 site might be able to convert a nonsilencer origin into a silencer. To test this possibility, a version of ARS1 was constructed that carried a Mig1-binding site 85 bp away from the ACS. However, this construct displayed no silencing activity (Figure 4B), suggesting that the combination of a Mig1-binding site with other unknown features of an origin were necessary for 2μARS-mediated silencing.

Mig1 functions in glucose repression through the recruitment of Ssn6/Tup1 and the histone deactylase Hda1 (OSTLING and RONNE 1998; WU *et al.* 2001). We

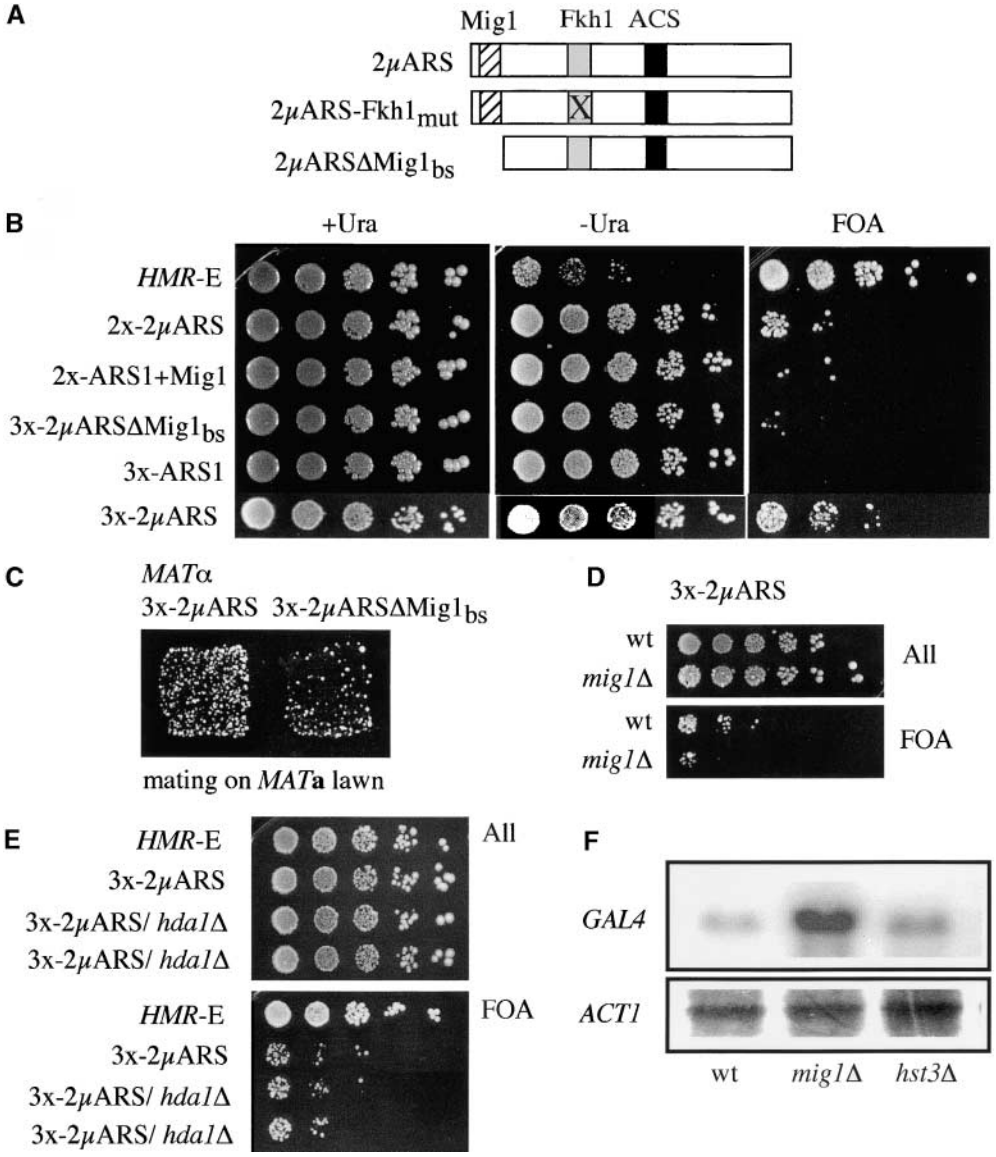


FIGURE 4.—Silencing by 2μARS depended on Mig1. (A) Positions of the putative Mig1- and Fkh1-binding sites relative to the ACS in 2μARS. X, mutation of the Fkh1-binding sites; $\Delta Mig1_{bs}$, deletion of the Mig1-binding site. (B) The Mig1-binding site was required for 2μARS silencing activity. A fusion of the Mig1-binding site to ARS1 did not induce silencing. (C) Effect of the deletion of the Mig1-binding sites in 3x-2μARS on **a**1 repression, as measured in a mating assay. (D) The effect of the deletion of the *MIG1* gene on 3x-2μARS silencing activity. (E) 3x-2μARS silencing was unaffected by deletion of *HDA1*. (F) Repression of *GAL4* was independent of Hst3, as shown by a Northern analysis with a *GAL4*-specific probe. *GAL4* was induced in a *mig1*Δ, but not in an *hst3*Δ strain. *ACT1* was used as a loading control.

therefore tested whether 2μARS-mediated silencing depended upon *HDA1*. However, silencing by 3x-2μARS was unaffected by *hda1*Δ (Figure 4E), suggesting that the role of Mig1 in silencing was distinct from its role in glucose repression.

The observation of an involvement of Mig1 in Hst3-mediated 2μARS silencing prompted us to test whether Hst3 interacted with Mig1. However, we were unable to detect co-immunoprecipitation of the two proteins (data not shown). Furthermore, we tested whether Hst3 was required for Mig1-mediated repression of glucose-regulated genes. However, the expression of *GAL4*, a Mig1-repressed gene, was unaffected by the deletion of *HST3* (Figure 4F), suggesting that Hst3 was not required for glucose repression.

**Hst3 was physically present at the 2μARS origin of replication:** The involvement of the histone deacetylase homolog Hst3 in 2μARS-mediated silencing raised the question whether the influence of Hst3 was indirect, for instance by changing global histone acetylation levels in the cell, or whether Hst3 might be directly recruited to 2μARS and might locally deacetylate histones, much like Sir2 at the *HM* locus (IMAI *et al.* 2000). To distinguish between these possibilities, we asked whether Hst3 was physically present at 2μARS in the silencer cassette. For this purpose, ChIPs were performed with epitope-tagged Hst3, and the precipitates were tested for the presence of sequences from the silencing reporter plasmid. The genomically tagged Hst3-HA was functional in that it was fully capable of silencing 3x-2μARS (data not shown). Significantly, a fragment close to 3x-2μARS was amplified in the precipitates, and the amplification depended upon the presence of the antibody as well as upon tagged Hst3 (fragment P1, Figure 5B). PCR analysis of serial dilutions showed that the amplifications were within the linear range (Figure 5C). In contrast

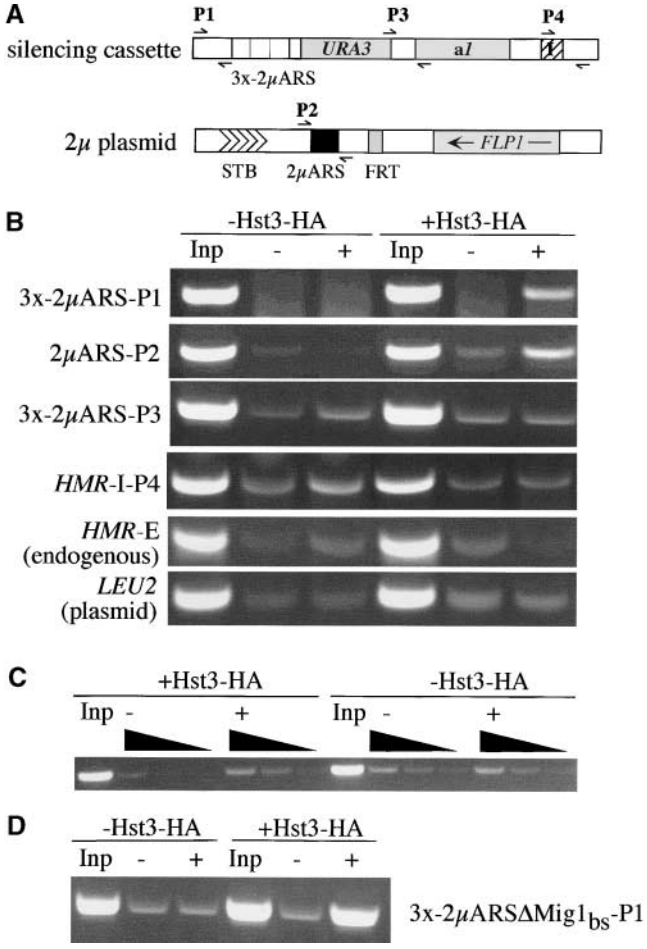


FIGURE 5.—Hst3 was associated with 2μARS sequences in the silencing reporter and on the endogenous 2μ plasmid. (A) Positions of the primers P1, P2, P3, and P4 at 3x-2μARS in the silencing reporter and at the endogenous 2μ plasmid are indicated. (B) ChIP analysis of Hst3-HA with 2μARS. Primers against plasmid-borne *LEU2* are used as a negative control. Inp, input; dash indicates without antibody; +, with HA antibody. (C) PCR analysis with 2.5-fold serial dilutions of ChIPs indicates that the amplifications were within the linear range. (D) Hst3 was associated at 3x-2μARS when the Mig1-binding site was deleted.

to 3x-2μARS, a fragment within the silenced region at the junction of *URA3* and **a**1 (fragment P3), a fragment comprising *HMR*-I (fragment P4), and *LEU2* on the silencing reporter plasmid were not enriched, suggesting that Hst3 did not spread across the silenced region and that unrelated sequences were not enriched in the precipitates (Figure 5B). Taken together, these experiments suggested that Hst3 was directly bound at the silencer. However, 3x-2μARS sequences were still enriched when the Mig1-binding site was deleted (Figure 5D), suggesting that Hst3 was brought to the silencer independently of Mig1.

Since Hst3 was capable of binding 2μARS on the silencing reporter plasmid, we also determined whether it was present at the 2μARS of the endogenous 2μ plasmid. A specific amplification was found using primers

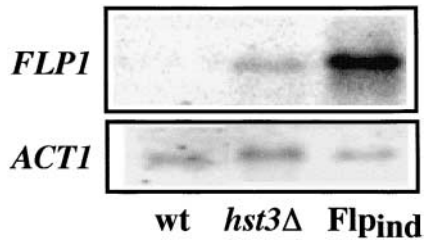


FIGURE 6.—Repression of *FLP1* depends on Hst3. *FLP1* expression was induced in an *hst3*Δ strain compared to wild type as shown in a Northern blot using a probe against *FLP1*. $Flp_{ind}$, galactose-induced overexpression of *FLP1* from a Gal1/10 promoter. An *ACT1* probe was used as a loading control.

that recognized the endogenous 2μARS only (fragment P2, Figure 5, A and B), thus suggesting that Hst3 was also bound at the 2μ plasmid origin. We further tested the possibility that Hst3 was physically present at the endogenous *HMR* locus. However, *HMR*-E sequences were not enriched in the precipitates (Figure 5B).

***FLP1* mRNA was upregulated upon deletion of *HST3*:** The observation that 2μARS displayed silencing activity on our silencing reporter plasmid and that Hst3 was bound to the 2μARS of the 2μ plasmid raised the question whether the 2μARS and Hst3 had a role in regulating 2μ-encoded genes. To test this possibility, we analyzed the expression of the *FLP1* recombinase mRNA in wild-type *vs. hst3*Δ strains. Interestingly, *FLP1* RNA was detectable in a *hst3*Δ strain, whereas it was undetectable in wild-type strains (Figure 6), thus suggesting that Hst3 was directly or indirectly required for the regulation of the amount of *FLP1* mRNA.

Increased amounts of Flp1 have previously been shown to induce 2μ recombination and increase 2μ copy number (SOM *et al.* 1988). However, the copy number of 2μ in *hst3*Δ strains was indistinguishable from that of isogenic wild-type strains (data not shown). Thus, although Hst3 was involved in *FLP1* regulation, it did not influence 2μ copy number.

## DISCUSSION

The chromosomes of multicellular eukaryotes are subdivided into active euchromatin and transcriptionally inactive heterochromatin. Repressed genomic regions that display some of the features of heterochromatin in larger eukaryotes are also found in *S. cerevisiae*. For instance, they replicate late in S phase (REYNOLDS *et al.* 1989), localize to the nuclear periphery (ANDRULIS *et al.* 1998), and are packaged in hypoacetylated histones (BRAUNSTEIN *et al.* 1993). So far, three classes of silencing are recognized in *S. cerevisiae*: the *HM* loci, the telomeres, and the rDNA locus. However, little is known about silencing in other parts of the yeast genome, but indications suggest the existence of other silenced regions in yeast (WYRICK *et al.* 1999). Although Sir2 has long been known to be required for silencing, the cellu-

lar function of the Sir2 homologs (Hst proteins) is still unclear. One hypothesis is that they silence particular genomic regions, like Sir2 represses some classes of silenced loci in yeast (Brachmann *et al.* 1995).

In this study, we undertook a search for novel silencing activities. We developed a silencer trap reporter cassette and screened for genomic sequences that displayed silencing activity. With this approach, we have isolated a novel silencer, the 2μ origin of replication. Gene silencing by 2μARS displayed the classical features of silencing: it was independent of the orientation of the silencer and gene independent. Furthermore, 2μARS-mediated silencing depended upon all four Sir proteins and Orc2, suggesting that a heterochromatin-like structure similar to that at the *HM* loci and the telomeres was established. However, in contrast to known silencing phenomena, 2μARS-mediated silencing depended upon Hst3 and Mig1. Thus, the 2μARS silencing constitutes a novel class of silencing that shares some features with *HM* silencing, but is different in some important aspects.

Origins of replication have been implicated in silencing before. For instance, the *HMR* silencers are chromosomal origins of replication (Rivier and Rine 1992), and the *HML* silencers contain ORC-binding sites, although they do not normally initiate replication (Dubey *et al.* 1991). One model for ORC's role in silencing is that it recruits Sir1 through an interaction between Orc1 and Sir1 (Fox *et al.* 1997). Interestingly, we found 2μARS silencing to be Sir1 and Orc2 dependent, suggesting that an ORC-dependent Sir1 recruitment also takes place at this origin.

How does 2μARS promote silencing? The 2μARS displaying silencing activity contains an ORC-binding site, but no recognizable Rap1- or Abf1-binding site, arguing against an involvement of Rap1 and Abf1 in this class of silencing. Using the TransFac program, we found a binding site for Mig1 in the 2μARS sequence. Mig1 is a DNA-binding protein responsible for glucose repression of several genes such as *GAL1*, *GAL4*, and *SUC2* that contain binding sites for Mig1 in their promoter (Nehlin *et al.* 1991). Repression of these genes is thought to be mediated by recruiting Ssn6 and Tup1, which are general repressors in *S. cerevisiae* (Treitel and Carlson 1995). Our finding that Mig1 was also necessary for 2μARS-mediated silencing suggested a novel role for Mig1 in building silenced chromatin with similarities to *HM* silencing. The mechanism by which Mig1 and the Orc proteins worked together to provide silencing is still unclear, but may be comparable to the role of Rap1 and Abf1 of the *HMR*-E silencer. Perhaps the cooperation of Mig1 with ORC serves to recruit Hst3, whereas Rap1/Abf1 in combination with ORC as at *HMR*-E or *HMR*-I recruit the Sir proteins. Interestingly, 3x-2μARS-mediated silencing depended upon *HMR*-I and the two histone deacetylases Hst3 and Sir2. One hypothesis for the requirement of both deacetylases is that Hst3 is recruited by Mig1/ORC at 2μARS and that Sir2, Sir3, and Sir4 are recruited by Abf1/ORC at *HMR*-I. In principle, this model could be tested by making silencing of the reporter genes solely dependent upon 2μARS, for instance by replacing *HMR*-I by 3x-2μARS. However, recombination events excising the *URA3* reporter in such constructs prevented us from measuring silencing in this context (A. Grünweller, unpublished data). Interestingly, silencing mechanisms in *S. cerevisiae* exist that depend on the recruitment of the Hst proteins. An alternative silencing mechanism has been proposed for the Sum1-1-dependent silencing at *HMR*. Binding of Sum1-1 to ORC in this case leads to the deacetylation of histones by Hst1 and the repression of neighboring genes (Rusche and Rine 2001). Interestingly, Hst proteins contain highly conserved zinc finger and leucine zipper motifs, and they thus may be able to form complexes with other proteins or bind directly to DNA (Brachmann *et al.* 1995).

Interestingly, 2μARS-mediated silencing also required Sir1. Since Sir1 interacts with ORC, it can conceivably be recruited via 2μARS or via *HMR*-I. However, ORC at 2μARS was distinct from that at the *HM* loci in that it led to the participation of Hst3 in 2μARS-mediated silencing. Hence, the context of a particular ORC-binding site is crucial for its ability to function as a silencer and for the class of silencing that it establishes, and a previously unrecognized context is found at 2μARS. Accordingly, we found that another ORC-binding site within the ARS1 origin was unable to provide silencing, even when present in three tandem repeats. Interestingly, ARS1 contains an Abf1-binding site like *HMR*-I, but is unable to act as a proto-silencer, supporting the notion that ORC-binding sites are qualitatively distinct. In this context, it is interesting to note that the 2μARS sequence showed the best homology to a region of the *HMR* locus spanning the ACS and 192 bp of centromere-proximal sequences (60% identity). This was the only ARS-containing DNA region in the SGD with homology to 2μARS. Maybe the region around the ACS is important for silencing relevant features of ORC binding at the ACS. For instance, the affinity of ORC binding to an ACS could be modulated by neighboring sequences and may thus influence the silencing capacity of an origin.

How does replication initiation at 2μARS affect silencing? The native 2μ origin is a strong origin of replication that initiates early in S phase (Zakian *et al.* 1979). The chromosomal context of origins influences their initiation ability and timepoint of replication (Friedman *et al.* 1996). Due to replicator dominance mechanisms, origins can be inactivated by origins close by, likely because replication forks originating from the origin that initiates first inactivate the prereplication complexes formed at the second origin (Palacios DeBeer and Fox 1999). Also, heterochromatinization, for instance at the telomeres, can lead to late initiation or to the prevention of initiation (Stevenson and Gottschling

1999). Our 2μARS silencing reporter plasmid carries three potential origins: (1) ARSH4, which initiates earlier than ARS1 in the chromosomal context (A. Ehrenhofer-Murray, unpublished observation); (2) 2μARS; and (3) *HMR*-I, a late-replicating origin (Rivier *et al.* 1999). Perhaps 2μARS inactivation by replication forks from ARSH4 leads to silencing in these cells, so that in this scenario, initiation at 2μARS is detrimental to silencing. Alternatively, silencing may occur only in those clones in which replication initiates at 2μARS, which may be infrequent because ARSH4 generally initiates earlier than 2μARS. A third possibility is that silencing on the plasmid occurs only in those rare cases in which neither 2μARS nor ARSH4 initiates within one particular cell cycle, so that *HMR*-I now is the plasmid replicator origin. In principle, this question could be resolved by measuring origin initiation on the plasmid by two-dimensional origin mapping gels (Fangman and Brewer 1991). However, this method is unable to distinguish between initiation events that lead to silencing and those that are simple initiation events, so that the question of which origin initiates in the silenced clones cannot currently be resolved.

Another aspect of the role of ORC and replication initiation in silencing concerns replication timing. Silenced genomic regions in yeast as well as other eukaryotes are generally replicated late in S phase (Reynolds *et al.* 1989), but it is not clear whether late replication is a cause or a consequence of silencing. Initiation by an origin late in S phase is achieved by the chromosomal context of the origin, and removing it from this context results in early replication (Friedman *et al.* 1996). Our 2μARS silencing plasmid carries two early and one late-replicating origin, and it thus seems likely that the plasmid is replicated early in the majority of clones. However, it is conceivable that a few clones would escape this pattern and could become late replicating, for instance, in the event that neither 2μARS nor ARSH4 initiates on the plasmid, so that *HMR*-I now takes over initiation late in S phase; perhaps it is in these clones that silencing occurs. Again, replication timing of the plasmid could easily be measured (Friedman *et al.* 1995), but would yield information only on replication timing of the bulk of plasmids, whereas silencing, and thus perhaps late replication, may occur only in a fraction of cells.

A singular role for Hst3 in silencing mechanisms has not been demonstrated so far. Here, we show that Hst3 was necessary for the formation of silenced chromatin induced by the 2μARS origin of replication, because a deletion of Hst3 completely abolished 2μARS-mediated silencing. Moreover, we found a direct association of Hst3 with 2μARS sequences by ChIP analysis. Thus we propose that Hst3 is transiently or permanently associated with the silent chromatin and deacetylates lysines, either on histones or on histone-associated silencing proteins. Perhaps a particular combination of DNA-binding proteins in conjunction with ORC determines the recruitment of a particular deacetylase to specific genomic regions, for instance the recruitment of Hst3 by Mig1 and ORC (see above). However, Hst3 is likely not recruited at other Mig1-binding sites, because the repression of Mig1-regulated genes did not require Hst3. Remarkably, though, *FLP1* was derepressed in *hst3*Δ strains, and Hst3 was localized at the endogenous 2μARS in ChIP experiments. However, it is questionable whether 2μARS plays a role in this repression because (1) 2μARS lies downstream of the *FLP1* gene, ~1.5 kb away from the *FLP1* promoter; (2) 2μARS was a protosilencer in our silencing assay (*i.e.*, it provided repression only in combination with *HMR*-I); and (3) *FLP1* sequences could not be amplified in Hst3 ChIPs (A. Grünweller, unpublished results), suggesting that Hst3 was not bound near the *FLP1* promoter. Thus, we favor the hypothesis that Hst3 indirectly caused *FLP1* derepression, and the role of Hst3 at 2μARS remains to be determined.

In summary, our data provide evidence for a novel alternative silencing mechanism that depended on Hst3 and Mig1. Identifying further determinants of 2μARS silencing will be important for understanding the silencing mechanisms at play and may lead to the discovery of further silencing elements in the yeast genome, thus contributing to our understanding of genomic organization in yeast and other organisms.

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