MOLECULAR AND CELLULAR BIOLOGY, Nov. 1991, p. 5648–5659 Vol. 11, No. 11
0270-7306/91/115648-12$02.00/0
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A Synthetic Silencer Mediates *SIR*-Dependent Functions in *Saccharomyces cerevisiae*

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Received 25 April 1991/Accepted 9 August 1991

Copies of the mating-type genes are present at three loci on chromosome III of the yeast *Saccharomyces cerevisiae*. The genes at the *MAT* locus are transcribed, whereas the identical genes at the silent loci, *HML* and *HMR*, are not transcribed. Several genes, including the four *SIR* genes, and two sites, *HMR-E* and *HMR-I*, are required for repression of transcription at the *HMR* locus. Three elements have been implicated in the function of the *HMR-E* silencer: a binding site for the RAP1 protein, a binding site for the ABF1 protein, and an 11-bp consensus sequence common to nearly all autonomously replicating sequence (ARS) elements (putative origins of DNA replication). RAP1 and ABF1 binding sites of different sequence than those found at *HMR-E* were joined with an 11-bp ARS consensus sequence to form a synthetic silencer. The synthetic silencer was able to repress transcription of the *HMRa1* gene, confirming that binding sites for RAP1 and ABF1 and the 11-bp ARS consensus sequence were the functional components of the silencer in vivo. Mutations in the ABF1 binding site or in the ARS consensus sequence of the synthetic silencer caused nearly complete derepression of transcription at *HMR*. The ARS consensus sequence mutation also eliminated the ARS activity of the synthetic silencer. These data suggested that replication initiation at the *HMR-E* silencer was required for establishment of the repressed state at the *HMR* locus.

In the yeast *Saccharomyces cerevisiae*, mating type and sporulation are controlled by the *MAT***a** allele (**a***1* and **a***2* genes) and the *MAT*α allele (α*1* and α*2* genes) of the mating-type locus (*MAT*). Haploid yeast cells with the *MAT*α allele have the α mating type and are able to mate with haploid cells with the *MAT***a** allele, which have the **a** mating type, to form **a**/α diploids. The simultaneous expression of the *MAT***a** and *MAT*α alleles in an **a**/α diploid results in a nonmating phenotype and provides the ability to sporulate (reviewed in reference 18). In addition to *MAT*, mating-type genes are found at two other loci known as *HML* and *HMR*. Only the mating-type genes at the *MAT* locus are transcribed. In most strains, nontranscribed copies of the **a***1* and **a***2* genes are present at the *HMR* locus (*HMR***a**) and nontranscribed copies of the α*1* and α*2* genes are present at the *HML* locus (*HML*α). These silent genes have intact promoters and coding sequences and differ from the transcribed copies at *MAT* only in the sequences that flank the loci (2, 33). Repression of the mating-type genes at *HML*α and *HMR***a** is essential to maintain the mating competence of *MAT***a** and *MAT*α strains, respectively. In addition, repression of both *HML* and *HMR* is required for the orderly pattern of mating-type interconversion in homothallic strains (27).

Although there are many similarities between transcriptional repression at *HML* and transcriptional repression at *HMR*, this study deals only with repression of *HMR***a**. Transcriptional repression of *HMR***a** requires a regulatory site to the left of the locus, known as the *E* site, and a site to the right of the locus, known as the *I* site (1). The *E* site is essential for repression in both dividing cells and cells under starvation conditions, whereas derepression due to *I*-site mutations has been observed only in cells under starvation conditions (1, 22). Repression of *HMR***a** is also dependent on the products of the *SIR1*, *SIR2*, *SIR3*, and *SIR4* genes (37) and on the product of at least one wild-type histone H4 gene (23).

The *HMR-E* site has been termed a silencer because its properties appear to be opposite those of a mammalian enhancer. Specifically, a 530-bp DNA fragment containing the *E* site is able to maintain repression of *HMR***a** in an orientation-independent manner when positioned at a variety of locations near the *HMR***a** locus (6). In addition, a number of different RNA polymerase II and RNA polymerase III-transcribed genes are repressed in a *SIR*-dependent manner when these genes are inserted adjacent to the silencer in the *HMR* locus (6, 42). Whereas mammalian enhancers act as independent elements that can function when moved to heterologous contexts (3), there is limited information as to whether the *HMR-E* silencer can act as an independent element. In the only relevant case, a 490-bp DNA fragment containing the *E* site, when placed upstream of a chimeric promoter on a plasmid, was able to repress transcription in an orientation-independent manner (10). The generality of this observation has not yet been established.

In addition to transcriptional repression, three other properties have been associated with the 490-bp fragment of the *HMR-E* silencer. This fragment, which contains an 11-bp autonomously replicating sequence (ARS) consensus sequence, is able to provide ARS activity to plasmids (1, 25) and is therefore a putative plasmid origin of DNA replication (8). In contrast to transcriptional repression, the ARS activity conferred by this fragment does not require the products of the *SIR* genes (25). The presence of a potential origin of DNA replication at the *HMR-E* silencer is particularly interesting because evidence for an S-phase requirement for establishment of repression at *HML* and *HMR* has been presented. Specifically, cells with a temperature-sensitive mutation in the *SIR3* gene express a high level of **a***1* mRNA

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from the silent loci when incubated at the nonpermissive temperature. Repression of **a***1* transcription from the silent loci is restored after these cells are shifted to the permissive temperature. This establishment of transcriptional repression is blocked by hydroxyurea (inhibitor of DNA synthesis) and by mating pheremone, which causes cells to arrest in the G_1 phase of the cell cycle (31).

These observations raise the question of whether replication initiation at the *HMR-E* silencer is the S-phase requirement for establishment of repression at *HMR*. This model predicts that mutations that prevent replication initiation at the *HMR-E* silencer would cause a depressed phenotype. Such an experiment has thus far been difficult because of the redundant nature of the ARS element at *HMR-E*. Specifically, two nonoverlapping restriction fragments encompassing the left and right halves of *HMR-E*, respectively, are each sufficient to confer ARS activity on plasmids (7). This redundancy of ARS information may be due to multiple near matches to the 11-bp ARS consensus sequence found at *HMR-E*, since multiple near matches to this consensus sequence have been shown to be sufficient to confer ARS activity (26, 51, 53).

A second property of the 490-bp *HMR-E* fragment is the ability to provide a segregation function to plasmids. Conventional ARS plasmids are rapidly lost by most cells in a growing culture as a result of the tendency of mother cells to retain ARS plasmids at mitosis (32). The 490-bp *E* fragment reduces the rate of plasmid loss in mitosis. This segregation function is *SIR* dependent (25).

A third property of the silencer-containing fragment is called centromere antagonism, which refers to the ability of the silencer to reduce the mitotic stability of centromere plasmids. Plasmids containing both the 490-bp *E* fragment and a centromere are more mitotically unstable than are plasmids that contain only one of these elements. Like the segregation property, centromere antagonism is also dependent on the *SIR* genes (25). It is not yet clear whether the segregation function and centromere antagonism are different manifestations of the same mechanism. Moreover, it is not known whether the ARS activity, segregation function, and centromere antagonism of the silencer are functionally related to the mechanism of transcriptional repression.

A combination of biochemical and mutational studies has revealed specific protein binding sites and functional domains within the *HMR-E* silencer. DNase I footprinting experiments have revealed binding sites for two proteins within the *HMR-E* silencer. One protein, known by several names, is referred to here as ABF1, and the other protein, known by several names, is referred to here as RAP1 (9, 13, 14, 44, 46). Binding sites for these two proteins have been found in a wide variety of DNA sequences in the yeast genome (9). ABF1 is an essential protein (14) that binds near the ARS consensus sequence in many but not all ARS elements and is a component of the upstream activation sequence (UAS) of at least one essential gene (12). RAP1 is an essential protein (44) and, at least in vitro, binds to telomere sequences, silencers, and the UASs of a number of genes encoding glycolytic enzymes and ribosomal proteins (9, 11, 21). The in vivo roles for RAP1 in telomere regulation (29) and UAS function have been demonstrated through analysis of temperature-sensitive alleles of the *RAP1* gene (28).

Mutational analysis has defined three elements within the *E* silencer, A, E, and B, which exhibit functional redundancy. The E domain is clearly a binding site for the protein RAP1, since insertion of several different RAP1 binding sites from other locations in the genome restores repression to an *HMR* locus with an E-domain mutation (44). In addition, temperature-sensitive alleles of *RAP1* show phenotypes similar to those of RAP1 site mutations in the silencer (28). The nature of the other two domains is less clear. The position of the B domain coincides with the position of a binding site for the protein ABF1. The position of the A domain coincides with the position of an 11-bp ARS consensus sequence, now known to be a binding site for the protein ACBP (19). Mutations in the A or B domain allow wild-type repression of *HMR***a** transcription. Mutations in the E domain have a weakly derepressed phenotype. In contrast, mutations in any two domains show a completely derepressed phenotype (7). The mutational analysis used to define these domains leaves unresolved the questions of whether the ARS consensus sequence is the functional component of the A domain and whether the ABF1 binding site is the functional component of the B domain.

This paper reports the construction and analysis of a synthetic silencer. This synthetic silencer consisted of an 11-bp ARS consensus sequence, a RAP1 binding site that is found in the *MAT*α UAS, and a synthetic ABF1 binding site. These elements were arranged with the same relative orientation and spacing as found in the *HMR-E* silencer (see Fig. 1). The sequences of the sites themselves are different from those of their counterparts in the *HMR-E* silencer, and all sequences between these sites were randomized relative to the wild-type *HMR-E* silencer. The synthetic silencer should therefore have no cryptic elements that might overlap or lie between the known elements in the *HMR-E* silencer. The synthetic silencer was inserted into an 800-bp deletion of the *HMR-E* silencer and assayed for its ability to repress transcription at *HMR* in a *SIR*-dependent manner. Several questions could be addressed more easily with the synthetic silencer than with the *HMR-E* silencer. Analysis of the synthetic silencer allowed the determination of whether the ABF1 binding site and the ARS consensus sequence are indeed functional elements of the silencer and determination of whether any other elements are required for silencer function besides the RAP1 binding site. More importantly, the synthetic silencer was devoid of the near matches to the ARS consensus sequence found in the wild-type silencer. Thus, the synthetic silencer was more amenable to mutation of the ARS activity and determination of the role of DNA replication initiation in transcriptional repression of *HMR-E*.

MATERIALS AND METHODS

Media and genetic methods. Yeast rich medium (YPD) and yeast minimal medium (YM) were prepared as described previously (43). Solid media contained 2% agar (Difco or BBL). For transformation assays of ARS activity and plasmid loss rates, Difco agar was washed extensively to remove trace amounts of uracil. Sixty grams of agar was suspended in 4 liters of deionized water and allowed to settle. The water was decanted, and the procedure repeated four times in total. Amino acid and base supplements were added at 30 μg/ml as needed. Standard genetic methods were as described previously (43).

Plasmid constructions. The synthetic silencer was constructed from complementary oligonucleotides which were annealed and ligated into the *Sma*I site of pUC18 (52), resulting in plasmid pJR797. The sequence of the insert was determined by the chain termination method (40) and is presented in Fig. 1. The 142-bp *Sal*I fragment of pJR797 was

inserted into the *Xho*I site of p8ΔE. p8ΔE is composed of the *Eco*RI-to-*Hin*dIII region of *HMR* with an 800-bp deletion of *E*, marked by a unique *Xho*I site (Δ534) (24), all in the 2μm-based *URA3* vector pSEY8 (15). This deletion presumably removed all of the *HMR-E* element since only a 246-bp region, located entirely within this deleted region, is required for repression (1). A recombinant plasmid with the silencer in one orientation was named pJR639. A recombinant with two synthetic silencers in tandem in the opposite orientation was named pJR640. The silencer-containing *Eco*RI-*Bgl*II fragment of pJR640 was subcloned into *Eco*RI-*Bam*HI-cut pUC18, yielding pJR966. pJR966 was cut with *Bam*HI, liberating the extra copy of the synthetic silencer, and religated, yielding pJR934 with only one copy of the synthetic silencer. To compare the synthetic silencer with the wild-type silencer, the 138-bp *Alu*I-*Dra*I *HMR-E* fragment (Fig. 1) was isolated from pJR315 (25) and ligated to *Sma*I-cut pUC18. One isolate was sequenced, found to have the *Alu*I site closest to the sequencing primer (5′-TCCCAGT CACGACGT-3′), and named pJR793. The silencer-containing insert of pJR793 was then isolated as a *Bam*H-*Eco*RI fragment and was inserted into the *Xho*I site of p8ΔE after all ends were made blunt with Klenow fragment and deoxynucleoside triphosphates (dNTPs). A recombinant plasmid with the *E* fragment in one orientation was named pJR637, and a recombinant plasmid with the *E* fragment in the opposite orientation was named pJR638.

An oligonucleotide of the sequence 5′-GATCCCCCCTC GAGGATCGGCGCCTTATTGCTGATGCATG-3′ (named ssa, for synthetic silencer A domain) was synthesized to facilitate construction of an ARS consensus mutation in the synthetic silencer. This oligonucleotide had one end capable of annealing with *Sph*I-generated ends and one end capable of annealing with *Bam*HI-generated ends. This oligonucleotide was phosphorylated with T4 polynucleotide kinase and ATP and ligated with *Sph*I-*Bam*HI-cut pJR639. These sites flank the ARS consensus sequence in the synthetic silencer. The resulting partially single-stranded plasmid, pJR927, was repaired by the *Escherichia coli* host after transformation. The silencer-containing *Eco*RI-*Bam*HI fragment of pJR927 was subcloned into *Eco*RI-*Bam*HI-cut pUC18, yielding pJR967, and was sequenced to confirm the expected *ssa* mutation. The mutant synthetic silencer-containing *Sac*II-*Bam*HI fragment of pJR967 was then ligated with *Sac*II-*Bam*HI-cut pJR966, producing pJR932.

The complementary oligonucleotides 5′-CGATAAATAA AATTACCCCATATTTTTGAGCTCGC-3′ and 3′-TATTTA TTTTAATGGGGTATAAAAACTCGAG-5′ were synthesized to facilitate construction of an ABFI binding-site mutation in the synthetic silencer. These oligonucleotides were annealed and named ssb (synthetic silencer B domain). This double-stranded DNA fragment had one end complementary to *Cla*I-generated ends and one end complementary to *Sac*II-generated ends. *Cla*I and *Sac*II sites flanked the ABFI binding site in the synthetic silencer. The *ssb* mutant fragment was ligated with *Cla*I-*Sac*II-cut pJR965 to yield pJR935 and with *Cla*I-*Sac*II-cut pJR966 to yield pJR936. These plasmids were sequenced and found to contain the expected *ssb* mutation in the ABFI binding site of the synthetic silencer. pJR965 was the result of ligating the *Eco*RI-*Bgl*II fragment of pJR639 into *Eco*RI-*Bam*HI-cut pUC18.

A series of plasmids was constructed to test the replication and segregation properties of the synthetic silencer. The synthetic silencer-containing 142-bp *Sal*I fragment of pJR797 was inserted into the *Sal*I site of pJR811, producing plasmid pJR780. pJR811 was a derivative of YIp5 (50) with the *Nsi*I site filled in to aid in subsequent manipulations. The 900-bp *Eco*RV-*Eag*I fragment of pJR780, which contains the synthetic silencer flanked by pBR322 sequences, was inserted into the *Xho*I site of p8ΔE after filling in of all single-stranded overhangs with Klenow fragment and dNTPs, producing plasmid pJR786. The 8-kb *Eco*RI-*Bgl*II fragment and the 800-bp *Eco*RI-*Sac*II fragment of pJR639 were ligated with the 1.8-kb *Sac*II-*Bgl*II fragment of pJR786 to yield pJR922. The 8-kb *Xho*I-*Eco*RI fragment of pJR637 was ligated with the 900-bp *Eco*RI-*Sal*I fragment of pJR786 to yield pJR921. The 8-kb *Eco*RI-*Bgl*II fragment of pJR639 was ligated with the silencer-containing *Sac*II-*Bam*HI fragment of pJR639 and the 900-bp *Eco*RI-*Sac*II fragment of pJR786 to yield pJR923. Plasmid pJR781 was made by substituting the 2.1-kb synthetic silencer-containing *Bam*HI-*Ava*I fragment of pJR780 for the corresponding *Bam*HI-*Ava*I fragment of YRp17 (49). Plasmid pJR784 was made by substituting the 2.1-kb *Bam*HI-*Ava*I fragment of pJR780 for the corresponding *Bam*HI-*Ava*I fragment of YCp50 (35). The result of these manipulations was the insertion of the synthetic silencer into YRp17 (pJR781) and into YCp50 (pJR784) in the same relative position as in pJR780. Note, however, that 300 bp of pBR322 sequences directly adjacent to the synthetic silencer in pJR780 were deleted in pJR781 and pJR784.

A series of plasmids was constructed to test the replication and segregation properties of the 138-bp *Alu*I-*Dra*I fragment of *HMR-E*. The 138-bp *HMR-E* insert of pJR793 was isolated as a *Bam*HI-*Eco*RI fragment and inserted into the *Sal*I site of pJR811 after all ends were made blunt with Klenow fragment and dNTPs to produce pJR782. The 2.3-kb *Bam*HI-*Sma*I fragment of pJR782 was substituted for the corresponding *Bam*HI-*Sma*I fragment of YRp17 to produce pJR783 and for the corresponding fragment of YCp50 to produce pJR785. The result of these manipulations was the insertion of the 138-bp *HMR-E* fragment in the same relative position into YIp5 (pJR782), YRp17 (pJR783), and YCp50 (pJR785). Plasmid pJR787 was constructed by ligating *Eco*RI-*Hin*dIII-cleaved pJR780 with a 1.6-kb *Eco*RI-*Hin*dIII fragment of pJR615 that contained *CEN6* (25). The *CEN6*-containing *Apa*I-*Hin*dIII fragment of pJR787 was ligated with the silencer-containing *Apa*I-*Hin*dIII-containing fragment of pJR783 to produce pJR928. The synthetic silencer-containing *Apa*I-*Hin*dIII fragment of pJR781 was ligated with the *CEN6*-containing *Apa*I-*Hin*dIII fragment of pJR787 to produce pJR929.

A series of plasmids was constructed such that each contained a silencer construct flanked by the *HMR* sequences that surrounded that silencer allele when it was tested for transcriptional repression. These plasmids did not contain the **a***1*/**a***2* promoter region, thus allowing the replication properties of the synthetic silencer to be evaluated without the complication of transcription of the **a***1* or **a***2* gene. An *Fsp*I site 3′ of the start of transcription of the **a***2* gene was used. The silencer-containing *Eco*RI-*Fsp*I fragment of each of the following plasmids was ligated with *Eco*RI-*Sma*I-cut pUC18, yielding the plasmids indicated in parentheses: p8ΔE (pJR946), pJR934 (pJR940), pJR965 (pJR937), pJR923 (pJR944), pJR932 (pJR939), pJR927 (pJR938), pJR936 (pJR942), and pJR935 (pJR941). Isolation of this *Eco*RI-*Fsp*I fragment from pJR786 and pJR922 was complicated by the presence of a second *Fsp*I site. A three-way ligation of an *Eco*RI-*Nhe*I fragment and an *Nhe*I-*Fsp*I fragment from each of these two plasmids with *Eco*RI-*Sma*I-cut pUC18 yielded the analogous subclones of pJR786 (pJR943) and pJR922 (pJR945). The *Eco*RI-*Fsp*I fragment of

each of these plasmids could then be liberated with *Eco*RI and *Hin*dIII (*Hin*dIII is a polylinker site). Three-way ligation of each of these silencer-containing *Eco*RI-*Hin*dIII fragments with a 1.6-kb *CEN6*-containing *Bam*HI-*Hin*dIII fragment from pJR615 and *Eco*RI-*Bam*HI-cut YIp5 yielded the series of plasmids shown in Fig. 7. The YIp5-*CEN6*-containing plasmid derived from each pUC18-based plasmid is listed in parentheses after its parent plasmid here: pJR946 (pJR954), pJR940 (pJR950), pJR937 (pJR947), pJR943 (pJR956), pJR944 (pJR955), pJR945 (pJR953), pJR939 (pJR949), pJR938 (pJR948), pJR942 (pJR952), and pJR941 (pJR951). pJR957 was constructed by substituting the silencer-containing *Eco*RI-*Sst*I fragment pJR637 for the equivalent fragment in pJR952.

A series of centromere-based plasmids was constructed to evaluate the role of *HMR-I* with different *HMR-E* elements. pJR919 was constructed by ligating the *HMR*-containing *Eco*RI-*Hin*dIII fragment of Δ60 (1) with *Eco*RI-*Hin*dIII-cut pSEYC58 (15). pJR920 was constructed by ligating the *HMR*-containing *Eco*RI-*Hin*dIII fragment of pJR640 with *Eco*RI-*Hin*dIII-cut pSEYC58. pJR968 was the source of the *HMR-I* deletion used in subsequent constructions. pJR968, which has a deletion of *HMR-E* and a deletion of *HMR-I* but intact **a***1*/**a***2* genes, was constructed by the following series of manipulations. Δ59 (1) was cut with *Xho*I and incubated with Klenow fragment and dNTPs, after which the Klenow fragment was heat inactivated. This reaction was then digested with *Bgl*II, and the 500-bp *Bgl*II-*Xho*I (blunt) fragment was isolated and ligated with the 2.7-kb *Eco*RI-*Bgl*II fragment from p8ΔE and *Eco*RI-*Sma*I-cut pEMBL19. The resulting plasmid was cut with *Sal*I and *Hin*dIII and was ligated with the 400-bp *Xho*I-*Hin*dIII fragment from Δ296-39 (1), yielding pJR968. pJR925 was generated by a three-way ligation of the Δ*I*-containing *Bgl*II-*Hin*dIII fragment of pJR968 with the silencer-containing *Eco*RI-*Bgl*II fragment of pJR919 and *Eco*RI-*Hin*dIII-cut pRS316 (47). pJR926 resulted from ligation of the silencer-containing *Eco*RI-*Bgl*II fragment of pJR638 with the Δ*I*-containing *Bgl*II-*Hin*dIII fragment of pJR968 and *Eco*RI-*Hin*dIII-cut pRS316. pJR924 resulted from ligation of the silencer-containing *Eco*RI-*Bgl*II fragment of pJR640 with the Δ*I*-containing *Bgl*II-*Hin*dIII fragment of pJR968 and *Eco*RI-*Hin*dIII-cut pRS316. The 800-bp *Eco*RI-*Sph*I fragment of pJR966 was substituted for the corresponding fragment in pJR924 to yield pJR933. The *HMR-I*-containing *Bgl*II-*Hin*dIII fragment of pJR640 was substituted for the corresponding fragment in pJR926 and pJR933 to yield pJR962 and pJR961, respectively.

Gene replacements. Replacement of *HMR* alleles was carried out as described previously (24) except that lithium acetate transformation (43) rather than spheroplast transformation was used. All replacements were carried out in strain JRY1707 (*MATα HMLα HMR***a** *ura3-52 his3 trp1 lys2 cir*^{0} with *LYS2* inserted into *hmre-Δ534*) (24). For some plasmids (those in the vector pUC18), a slight variation was used. The inserts of these plasmids were liberated with appropriate restriction enzymes and transformed into JRY1707 by the lithium acetate procedure. Transformed cells were incubated for 4 h in YPD broth and then plated on α- aminoadipic acid plates to select for loss of the *LYS2* gene. Plasmid pRS229 (41) was then used to disrupt the *SIR4* gene in each of these strains. DNA was isolated from each strain by the method of Holm et al. (20), and the structure of each allele was confirmed by DNA blot hybridization.

S1 analysis. Total nucleic acids were prepared from strains growing exponentially in either YPD broth or supplemented YM broth (for plasmid-containing strains). Uniformly labeled probes complementary to *MAT***a1** and *SIR3* mRNAs were prepared from the M13 clones described in references 30 and 45, respectively. Hybridizations and S1 nuclease digestions were done as described elsewhere (4) except that hybridizations were carried out in 50% formamide (Fluka) at 45°C and hybrids were digested with S1 nuclease (Bethesda Research Laboratories) at 30°C.

Plasmid loss rates. Plasmid loss rates were determined in strains DBY703 (*MATα ura3-52 his3 trp1 cir*^{0}) and JRY1263 (*MATα ura3-52 his3 trp1 sir4::HIS3 cir*^{0}) (24). Four transformants of each plasmid-host strain combination were grown to stationary phase in YM broth lacking uracil. Aliquots were diluted into 50 ml of YPD broth supplemented with additional uracil and tryptophan and grown at 30°C with vigorous shaking. At four time points, aliquots were diluted and plated onto solid YM medium containing uracil and onto solid YM medium lacking uracil. The fraction of cells in the culture that were able to grow without uracil was based on the evaluation of a minimum of 600 colonies per time point. The number of cell divisions elapsed after dilution into nonselective medium was determined from the dilutions plated out onto solid YM medium containing uracil. The log of the fraction of cells able to grow without uracil supplementation (y axis) was plotted against the number of cell divisions in nonselective media (x axis). The slope (m) of the best-fit line through four points was determined by least-squares analysis. The loss rate, which is a measure of the frequency of plasmid loss events per cell division, was determined from the equation $L = 1 - 10^m$, where L is the loss rate. The loss rate is equivalent to the fraction of daughter cells that have received no plasmid during the previous cell division. The loss rate is therefore equivalent to 0.5 × the fraction of cell divisions in which one daughter cell receives no plasmid. For experiments used to determine ARS efficiency, two time points were used in determination of the loss rate.

Quantitative mating. *MAT***a** tester strains were grown for 3 days on solid YPD medium and then resuspended in liquid YPD medium to a cell density of 10^8/ml. *MATα* cells to be tested were grown to mid-log phase in either liquid YPD medium (for strains with chromosomal *HMR* constructs) or liquid YM medium with appropriate supplements (for plasmid-borne *HMR* constructs). Appropriate dilutions of these cultures were mixed with 0.3 ml of the YPD-*MAT***a** cell suspension, and these mixtures were spread onto unsupplemented YM plates. Appropriate dilutions of the *MATα* cultures were also plated on YPD plates to measure cell density. The mating efficiency is the number of colonies formed on the mating plates divided by the number of colonies formed on the YPD plates, adjusting for dilution. For strains with chromosomal *HMR* alleles, the *MAT***a** tester strain was JRY12 (*MAT***a** *SUC1 his4 ura1*), and mating efficiencies were standardized to a value of 1.0 for DBY703, whose unadjusted mating efficiency was 0.78. For plasmid-borne *HMR* alleles in strain JRY2698, the *MAT***a** tester strain was JRY2120 (*MAT***a** *his4 lys2 ura3*), and mating efficiencies were standardized to a value of 1.0 for JRY2891, whose unadjusted mating efficiency was 0.16.

RESULTS

A synthetic silencer fragment bound ABFI and RAP1. The identification of three domains within the *HMR-E* silencer did not exclude the possibility of multiple additional domains that might have eluded biochemical and mutational analysis. An 11-bp ARS consensus sequence and binding sites for

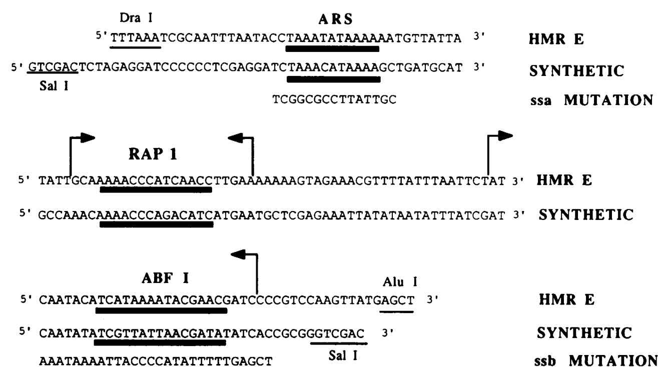

FIG. 1. DNA sequence of the synthetic silencer, aligned with the sequence of the 138-bp *Alu*I-*Dra*I fragment of the wild-type *HMR-E* silencer. Arrows indicate the extent of DNase I footprints of RAP1 and ABF1. Black bars indicate consensus sequences. The sequences of the *ssa* ARS consensus mutation and the *ssb* ABF1 site mutation are shown aligned with the corresponding sequences in the synthetic silencer. Only the 5'-3' strand of each sequence is shown.

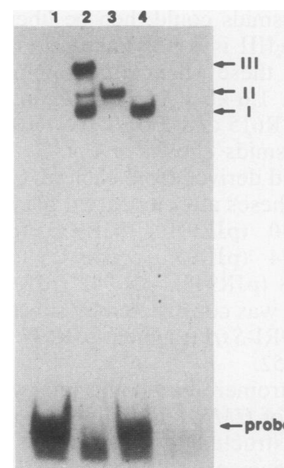

FIG. 2. Band shift assay for binding of ABFI and RAP1 to the 142-bp *Sal*I restriction fragment consisting of the synthetic silencer. The probe was end labeled with Klenow fragment and [α-^{32}P]dCTP, incubated with a cleared glass bead lysate of the *SIR* strain DBY703, subjected to electrophoresis on a native 4% polyacrylamide gel, and visualized by autoradiography. Lanes: 1, no extract; 2, no competitor oligonucleotide; 3, ABFI competitor oligonucleotide EI; 4, RAP1 competitor oligonucleotide EII. Protein-DNA complexes are indicated by roman numerals as follows: I, ABFI; II, RAP1; and III, ABFI plus RAP1.

ABFI and RAP1, the three elements identified in the *HMR-E* silencer, were joined into a single synthetic oligonucleotide (Fig. 1) to determine (i) whether these three elements were sufficient for silencer function and (ii) whether transcriptional repression could be genetically separated from the other properties of the wild-type silencer. The RAP1 binding site used was from the *MAT*α UAS, where it has been implicated in transcriptional activation (17, 48). The ABFI binding site used was a synthetic site somewhat more symmetric than previously identified ABFI binding sites (9). The sequences of these binding sites were quite different from those of the wild-type silencer, with only 14 of 22 nucleotides conserved (in the DNase I-protected region) for the RAP1 binding site and 19 of 29 nucleotides conserved for the ABFI binding site. The spacing between the three elements, as measured between consensus sequences, was identical to that in the corresponding *HMR-E* fragment. However, the nucleotide sequence between the consensus sequences was designed to maintain the sequence composition found in the wild-type silencer yet randomize the sequence of these regions. Thus, the synthetic silencer was only 48% identical in overall sequence to the wild-type silencer. Because of these design features, the synthetic silencer was unlikely to contain cryptic binding sites for unidentified factors or other functional domains that may exist in the wild-type *HMR-E* sequences.

The first step in the analysis of the synthetic silencer was to determine whether it had the same properties in vitro as did the wild-type silencer. A gel mobility shift assay was used to evaluate the ability of ABFI and RAP1 in yeast whole-cell extracts to bind the synthetic silencer. Three complexes, labeled I, II, and III, were observed when a *Sal*I fragment containing the synthetic silencer was incubated with a whole-cell extract of the *SIR* strain, DBY703 (Fig. 2, lane 2). Competition with the ABFI binding site-containing oligonucleotide EI (9) resulted in formation of complex II only (lane 3). Competition with the RAP1 binding site-containing oligonucleotide EII (9) resulted in formation of complex I only (lane 4). These results indicated that ABFI binding formed complex I, RAP1 binding formed complex II, and binding of both factors formed complex III. Similar sequence-specific binding complexes have been observed in experiments with the 490-bp fragment of *HMR-E* (9) and with a 138-bp *Alu*I-*Dra*I subfragment of *HMR-E* (36a).

The synthetic silencer was capable of repressing transcription of *HMR*a*1*. The synthetic silencer and the 138-bp *Alu*I-*Dra*I fragment from the wild-type *HMR-E* element were tested for the ability to repress transcription of *HMR*a*1* following their replacement of the wild-type chromosomal copy of the *HMR-E* silencer (described in Materials and Methods). Specifically, an 800-bp sequence spanning the wild-type *HMR-E* silencer was removed and replaced with each of these two elements in either orientation. Isogenic *sir4* derivatives of the resulting strains were constructed by one-step gene replacement (39) to test the *SIR* dependence of silencer function.

Transcriptional repression of the **a***1* gene due to silencer function was assayed by an S1 nuclease protection experiment. A protected fragment of 200 bp corresponding to the 3' end of the *SIR3* transcript served as an internal control for the amount of RNA loaded in each lane. Because the **a***1* gene has two introns and a uniformly labeled genomic probe was used to measure its expression, several different **a***1*-specific protected fragments were observed as previously reported (30). As shown in Fig. 3, a *SIR* strain with an 800-bp deletion of *E* (lane 3; JRY1707) and a *sir4* strain with a wild-type silencer (lane 2; JRY1263) expressed a high level of **a***1* mRNA, whereas a *SIR* strain with a wild-type silencer (lane 1; JRY1222) expressed no detectable **a***1* mRNA. *SIR* strains with the 138-bp *E* fragment in either orientation expressed no detectable **a***1* mRNA (lanes 4 and 6; JRY2148 and JRY2147). These results confirmed earlier work demonstrating the orientation independence of silencer function (6) and extended these conclusions by establishing that a 138-bp subfragment of the previously studied 530-bp element could functionally replace the entire *HMR-E* silencer region. Similarly, *SIR* strains containing the synthetic silencer in either orientation expressed no detectable **a***1* mRNA (lanes 10 and

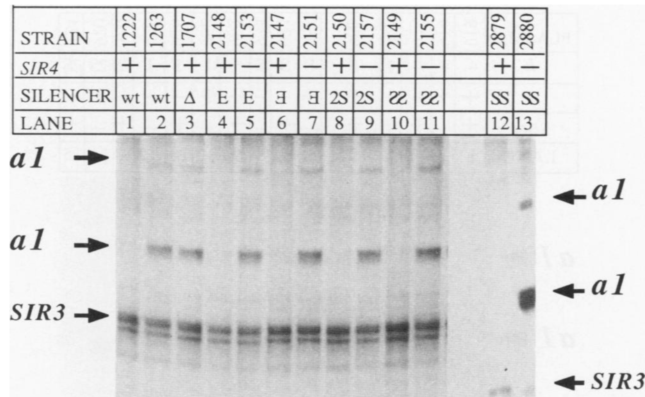

FIG. 3. S1 nuclease protection experiment measuring the amount of **a**1 mRNA relative to *SIR3* mRNA in various strains. The band labeled *SIR3* is due to protection of the *SIR3* probe by the 3′ end of the *SIR3* mRNA. The lower band labeled **a**1 is due to protection of the **a**1 probe by the second exon of the mature **a**1 mRNA. The upper band labeled **a**1 is due to protection of the **a**1 probe by a partially spliced form of the **a**1 mRNA. The strain numbers indicated have the JRY prefix. A plus sign in the *SIR4* column indicates that the strain had a wild-type *SIR4* gene, and a minus sign indicates that the strain had a *sir4*::*HIS3* disruption allele. The notations in the SILENCER column indicate restriction fragments inserted into the same 800-bp deletion of *HMR-E*: Δ, a *LYS2* insertion into the deletion of *HMR-E*; E, the 138-bp *Alu*I-*Dra*I fragment of *HMR-E* in the wild-type (wt) orientation (ABF1 site closest to the **a**2 gene); backward E, the 138-bp *E* fragment in the reverse orientation; 2S, a tandem pair of synthetic silencers in the wild-type orientation; SS, the synthetic silencer in the wild-type orientation; and backward SS, the synthetic silencer in the reverse orientation.

12; JRY2149 and JRY2879). This result indicated that the synthetic silencer was fully capable of repressing transcription of the silent mating-type locus. In contrast, the corresponding *sir4*::*HIS3* strains expressed a high level of **a**1 mRNA (lanes 5, 7, 11, and 13; JRY2153, JRY2151, JRY2155, and JRY2880, respectively). The presence of **a**1 transcripts in RNA from the *sir4* strains demonstrated that all of these silent *HMR* alleles were capable of transcribing the **a**1 gene under conditions in which the silencers were not functional. Furthermore, this result established that transcriptional repression mediated by the synthetic silencer was *SIR4* dependent. Two synthetic silencers in tandem were capable of repressing transcription as well as a single synthetic silencer (lane 8; JRY2150).

A second test for the degree of *HMR***a** expression is provided by the mating phenotype of *MAT*α strains containing various alleles of *HMR-E* (Fig. 4). In every case in which no **a**1 mRNA was detected (e.g., JRY2879), wild-type mating efficiency was observed. The *sir4*::*HIS3* derivatives of each strain, which expressed high levels of **a**1 mRNA (Fig. 3), exhibited a nonmating phenotype (data not shown) due to simultaneous expression of α2 from *MAT* and **a**1 from *HMR*. Disruption of the *SIR2* gene in the synthetic silencer-containing strain JRY2149 (resulting in strain JRY2910) also resulted in a nonmating phenotype (data not shown), indicating that repression by the synthetic silencer was dependent on the *SIR2* gene product as well as the *SIR4* gene product. Thus, the properties of the synthetic silencer depend on the same regulatory proteins as do the properties of the wild-type silencer.

Transcriptional repression by the synthetic silencer required both the ABF1 binding site and the ARS consensus sequence. As described earlier, mutations in neither the ARS consensus sequence nor the ABF1 binding sequence of the natural *HMR-E* silencer lead to derepression. To compare the sequence requirements of the synthetic silencer with those of the natural silencer, a mutation (*ssb*) was made in the synthetic silencer that changed all of the consensus nucleotides in the ABF1 binding site (corresponding to the B domain of the wild-type silencer) but did not affect the spacing or the overall nucleotide composition of the synthetic silencer (Fig. 1). The structures of the *ssb HMR* alleles are shown in Fig. 4. Analysis of **a**1-specific mRNA in yeast strains bearing the *ssb* mutant synthetic silencer in either orientation is shown in Fig. 5. Complete derepression of **a**1 transcription was observed for a strain that had the mutant synthetic silencer in the wild-type orientation, JRY2883 (Fig. 5, lane 13), and this strain had a nonmating phenotype (Fig. 4). Derepression of **a**1 transcription was also observed for a strain that had the mutant synthetic silencer in the reverse orientation, JRY2885 (lane 11). Thus, the ABF1 binding site plays a critical and unique role in the transcriptional repression caused by the synthetic silencer. Surprisingly, there appears to be a remarkable nonlinearity between **a**1 mRNA level (lane 11) and mating efficiency (Fig. 4) for JRY2885. A simple explanation for this would be the existence of a threshold in the amount of **a**1 mRNA required to decrease mating efficiency.

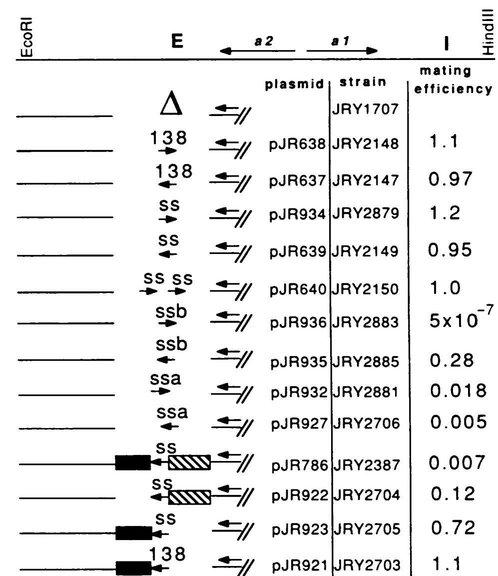

FIG. 4. Schematic representations of the *HMR* alleles used in this study. JRY1707 has the *LYS2* gene inserted into an 800-bp deletion of the *E* site (23). 138 indicates the 138-bp *Alu*I-*Dra*I restriction fragment of *HMR*-E; ss indicates the synthetic silencer; ssb indicates a synthetic silencer with an ABF1 site mutation; ssa indicates a synthetic silencer with an ARS consensus sequence mutation. The arrows below the silencers indicate the orientation (the ABF1 binding site is closest to the arrowhead). The black box and the striped box indicate two different restriction fragments of pBR322 DNA. The plasmid names indicate the plasmids used to gene-replace these alleles; the strain numbers indicate the resulting *SIR4* strain for each allele. The mating efficiencies indicate the fraction of cells in a population that were able to mate with a *MAT***a** tester strain normalized to the efficiency of the wild-type strain DBY703 as 1.0.

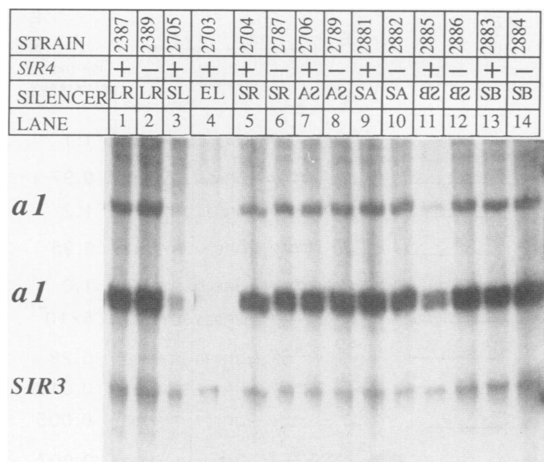

FIG. 5. S1 nuclease protection experiment measuring levels of **a**1 mRNA relative to *SIR3* mRNA in various strains. The notations in the SILENCER column indicate restriction fragments inserted into the same 800-bp deletion of *HMR-E*: SA and backward SA, the forward- and reverse-orientation synthetic silencers with the *ssa* ARS consensus sequence mutation; SB and backward SB, the forward and reverse orientations of the synthetic silencer with the *ssb* ABF1 site mutation; LR, the synthetic silencer with pBR322 restriction fragments flanking its left and right sides (see Fig. 4); SL and EL, the synthetic silencer and the 138-bp *Alu*I-*Dra*I fragment of *HMR-E*, respectively, with a pBR322 restriction fragment flanking the left side; SR, the synthetic silencer with a pBR322 restriction fragment flanking the right side.

A mutation (*ssa*) was made in the synthetic silencer which changed 8 out of 11 bp of the ARS consensus sequence (corresponding to the A domain of the wild-type silencer) without changing the overall size of the synthetic silencer (Fig. 1). The structures of the *ssa HMR* alleles are shown in Fig. 4. Analysis of **a**1-specific mRNA in yeast strains with the *ssa* mutant synthetic silencer in either orientation is shown in Fig. 5. *SIR* strains with the *ssa* mutant synthetic silencer in either orientation appeared to be completely derepressed for **a**1 transcription (Fig. 5, lanes 7 and 9; JRY2706 and JRY2881). Consistent with the observation of high levels of **a**1 mRNA in these strains, the mating efficiency of the *ssa* mutant strains was reduced 200-fold relative to the wild-type level (Fig. 4). These strains, however, retained some ability to mate, indicating that at least a fraction of the cells were repressed at *HMR*. These results indicated that the synthetic silencer was dependent both on the ABF1 binding site and on the 11-bp ARS consensus sequence. In contrast, single mutations in either of these sites in the wild-type *HMR-E* silencer have no phenotype (7, 24). Thus, the functional redundancy evident in the wild-type silencer is missing from the synthetic silencer. Mutations in the RAP1 binding site of the natural silencer lead to partial derepression, indicating that, in contrast to the other two, this domain is not fully redundant (7, 24). Thus, the corresponding mutation was not constructed in the synthetic silencer.

The synthetic silencer could repress transcription on centromere plasmids. Previous comparisons of the requirements for *cis* elements in the silencing of *HMR***a** revealed somewhat different requirements for chromosomal and plasmid-borne *HMR***a** loci (1, 6). To define further the sequences at *HMR* that were sufficient for *SIR*-dependent repression, and to determine whether the synthetic silencer worked

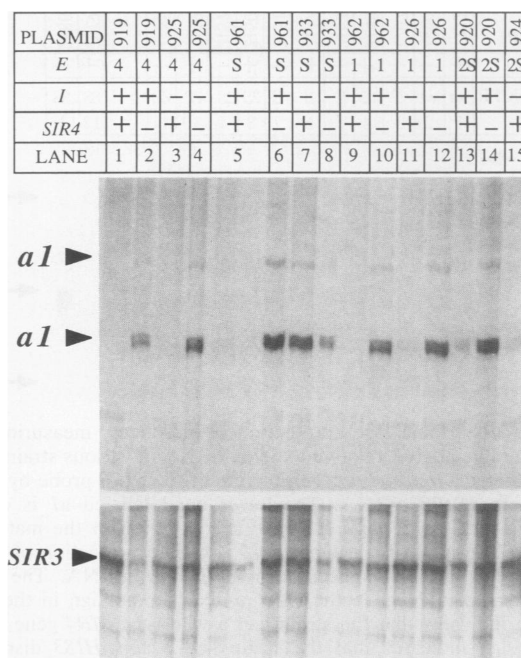

FIG. 6. S1 nuclease protection experiment measuring levels of plasmid-derived **a**1 mRNA relative to *SIR3* mRNA. The *SIR3* band shown in the lower panel is from a longer exposure of the same gel used for the upper panel. Numbers in the plasmid column have the pJR prefix. Each plasmid contains the *Eco*RI-*Hin*dIII region of *HMR***a** inserted into a centromere-based vector. Each plasmid has a different silencer fragment (listed in the *E* row) inserted into an 800-bp deletion of *HMR-E*: 4, the 490-bp *HMR-E* fragment described by Kimmerly and Rine (24); S, the synthetic silencer; 1, the 138-bp *Alu*I-*Dra*I fragment of *HMR-E*; 2S, two copies of the synthetic silencer. A plus sign in the *I* column indicates the presence of the wild-type *HMR-I* element on the plasmid, whereas a minus sign indicates the presence of a deletion of *HMR-I*. Some of the **a**1 mRNA detected in lane 13 was most likely due to lane spillover, since complete repression was observed for a different transformant of the same plasmid into the same strain (data not shown).

equally well in all contexts, the *Eco*RI-*Hin*dIII regions of various *HMR***a** alleles were cloned into either of the centromere-based vectors pSEYC58 (15) and pRS316 (47). Corresponding plasmids were made with and without the *HMR-I* element. The resulting plasmids were transformed into a *SIR* strain (JRY2698) and an isogenic *sir4*::*HIS3* strain (JRY2699). These strains have α information at *HML*, *MAT*, and *HMR*. Therefore, any **a**1 mRNA produced by these strains must be plasmid derived. The S1 analysis presented

TABLE 1. Quantitative mating efficiencies of *MAT*α strains with different plasmid-borne alleles of *HMR***a**

Plasmid	Vector	*HMR***a** allele	Mating efficiency
pJR919	pSEYC58	490-bp *E* fragment, I^+	1.0
pJR925	pRS316	490-bp *E* fragment, ΔI	0.75
pJR962	pRS316	138-bp *E* fragment, I^+	0.56
pJR926	pRS316	138-bp *E* fragment, ΔI	0.42
pJR961	pRS316	Synthetic silencer, I^+	0.31
pJR933	pRS316	Synthetic silencer, ΔI	0.01
pJR920	pSEYC58	2 synthetic silencers, I^+	1.5
pJR924	pRS316	2 synthetic silencers, ΔI	0.36

in Fig. 6 and the mating data presented in Table 1 indicated that the 490-bp wild-type silencer (pJR919 and pJR925; Fig. 6, lanes 1 and 3), the 138-bp wild-type silencer (pJR962 and pJR926; lanes 9 and 11), and two tandem copies of the synthetic silencer (pJR920 and pJR924; lanes 13 and 15) could repress transcription of **a***1* with or without the presence of *HMR-I*. A single synthetic silencer was also able to repress transcription of **a***1* in the presence of *HMR-I* (pJR961; lane 5). In contrast to either of the wild-type silencer fragments or to the pair of synthetic silencers, the single synthetic silencer was unable to exert any significant repression in the absence of *HMR-I* (pJR933; compare lanes 7 and 8). This plasmid, pJR933, was also the only plasmid causing a significant mating defect (Table 1). This result indicated an apparent defect in the synthetic silencer relative to the wild-type silencer. Repression by a pair of synthetic silencers in the absence of *HMR-I* (pJR924; lane 15) indicated that duplication of the synthetic silencer could at least partially compensate for this defect.

The synthetic silencer was dependent on *SIR1* for transcriptional repression. *sir1* strains with a wild-type *HMR***a** locus retain substantial mating ability (38), yet *sir1* strains lacking the *HMR-I* element have greatly reduced mating efficiency (22). Thus, with respect to the *I* element, *sir1* strains mimic the properties of synthetic silencer-containing strains. These observations suggested that *sir1* strains and synthetic silencer-containing strains might share a common defect. This hypothesis was tested by determining whether the phenotype of a strain containing both a disrupted *SIR1* gene and a synthetic silencer was the same as that of strains containing only one of the two alterations, indicating a common defect, or was greater than that of strains with a single alteration, indicating different defects. A *sir1*::*HIS3* gene disruption was created in one step in a strain containing the synthetic silencer at *HMR* (JRY2149), creating strain JRY2785. The *sir1* strain (JRY1705) and the synthetic silencer-containing strain (JRY2149) each mated within twofold of the efficiency of the wild-type control strain (DBY703). In contrast, the *sir1* strain with the synthetic silencer (JRY2785) mated at 10^{-3} the efficiency of the wild-type control strain. Since the synthetic silencer was almost completely nonfunctional in the absence of *SIR1*, *SIR1* function was not the missing ingredient in the synthetic silencer *I*-element deletion experiments described above.

Replication properties of the synthetic silencer are context dependent. DNA replication has been implicated in the mechanism of transcriptional repression by *HMR-E* because the establishment of repression requires passage through the S phase of the cell cycle (31) and because the 138-bp fragment of *HMR-E* allows plasmids to replicate autonomously in yeast cells (1, 7). Therefore, the ability of the synthetic silencer to provide ARS function was compared with that of the 138-bp *E* fragment to determine whether the ability to promote replication had been separated from the ability to promote repression. ARS activity was assayed by transforming an isogenic *SIR4* and *sir4*::*HIS3* pair of strains (DBY703 and JRY1263, respectively) with plasmids containing the selectable marker *URA3*, *CEN6*, and either the 138-bp *E* fragment or the synthetic silencer. The presence of a centromere on these plasmids ensured that the segregation and copy number properties would be constant for these plasmids. Therefore, differences in the maintenance of these plasmids in yeast cells should reflect differences in the frequency of replication initiation of these plasmids. Equivalent numbers of transformants per microgram of DNA (5,000 to 20,000) were obtained for all plasmids tested. This uniform transformation efficiency was presumably due to transient expression of the *URA3* gene in all cells that received plasmid DNA. Transformant colonies that would not grow when restreaked on selective media are indicated as NT (no transformants) in Fig. 7. Transformant colonies that did grow when restreaked on selective media were inoculated in liquid medium, and the plasmid loss rate was measured as described in Materials and Methods. The 138-bp *E* fragment (pJR928) provided ARS function when inserted into the *Sal*I site of the nonreplicating vector pJR615 in both *SIR* and *sir4* strains (*SIR4* loss rate, 0.26; *sir4* loss rate, 0.37), indicating that this form of the silencer was capable of promoting replication. The synthetic silencer, however, did not yield transformants that would restreak on selective media when inserted into the same site in the same orientation (pJR787). Thus, at least in this context, the synthetic silencer was unable to promote autonomous plasmid replication.

Because of the proposed link between silencer-mediated replication and transcriptional repression, it was of interest to determine whether the synthetic silencer had actually separated the ability to repress transcription from the ability to function in replication initiation, or whether the context effect imposed by different flanking sequences could influence the activities of the synthetic silencer in different ways. That is, were contexts in which the synthetic silencer was able to promote replication the same contexts in which it could repress transcription and vice versa? The flanking sequences surrounding the synthetic silencer in the chromosomes of strains JRY2149 and JRY2879 (Fig. 4), in which the synthetic silencer was competent to repress transcription, were different from the flanking sequences in plasmid pJR787, in which the synthetic silencer was ARS^{-}. To examine the possibility of context effects on the ARS activity of the synthetic silencer, plasmids were constructed in which the ARS function of the synthetic silencer could be evaluated in a context similar to that in which it could repress transcription. These plasmids, pJR947 and pJR950 (Fig. 7), contained the synthetic silencer flanked on either side by the same sequences that flanked the synthetic silencer in the chromosome where it was able to repress *HMR***a** transcription (JRY2149 and JRY2879). These *HMR* flanking sequences are indicated by the white boxes in Fig. 7. Interestingly, in this context, the synthetic silencer provided ARS function (pJR947 and pJR950 in Fig. 7), whereas a control plasmid containing the flanking sequences without the synthetic silencer (pJR954) lacked ARS function. These results indicated that sequences immediately flanking the synthetic silencer indeed could influence its ability to provide ARS function. To test this hypothesis further, a plasmid that contained the synthetic silencer in an ARS-incompetent context (indicated by the striped and black boxes in Fig. 7) flanked in turn by sequences that provided an ARS-competent context (indicated by the white boxes in Fig. 7) was constructed (pJR956). This plasmid was nearly incapable of autonomous replication, indicating that the proximity of the ARS-incompetent context to the synthetic silencer was dominant to the flanking ARS-competent context.

To investigate further the sequences responsible for the context effect on the ARS efficiency of the synthetic silencer, constructs were made in which the synthetic silencer is flanked only on the left side by pBR322 sequences (pJR955) and only on the right side by pBR322 sequences (pJR953). Each of these flanking sequences was in turn flanked by *HMR* sequences (Fig. 7). A continuum of plasmid loss rates was observed, with those plasmids containing the

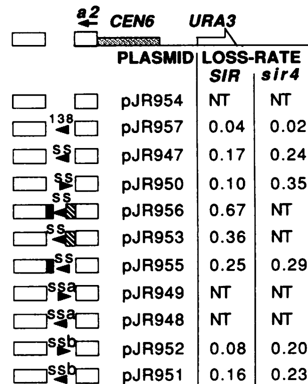

FIG. 7. Measurement of the ARS activity of various silencer alleles in the same context in which transcriptional repression was assayed in tne chromosome. The overall structure of the plasmids used is shown at the top. A 1.6-kb *CEN6* fragment (stippled box) ensured that segregation and copy number would be constant for each plasmid. The white boxes indicate the same *HMR*-derived flanking sequences depicted in Fig. 4. Note that the rightward *HMR* fragment does not include the promoter of the **a***2* gene so that transcriptional interference with the centromere was avoided. ss, synthetic silencer; 138, 138-bp *AluI-DraI* fragment of *HMR-E*; ssa, synthetic silencer with the *ssa* ARS consensus sequence mutation; ssb, synthetic silencer with the *ssb* ABF1 site mutation. The black and striped boxes indicate two different restriction fragments of pBR322 DNA. NT indicates that no transformants would restreak on selective media. The numbers listed under loss rate were determined as described in Materials and Methods.

most pBR322 sequence being the least stable and those with the least amount of pBR322 sequence being the most stable. pJR955, which had only the leftward 288 bp of pBR322 sequence, was slightly more stable than pJR953, which had only the rightward 466 bp of pBR322 sequence (Fig. 7). These results indicated that the context effect did not map to any particular element occurring in either the leftward or the rightward pBR322 sequences and thus were similar to results obtained for flanking sequence requirements of other ARS elements (5, 34).

Like the synthetic silencer, the 138-bp *E* silencer exhibited a context-dependent ARS activity. This silencer had a much lower loss rate in the *HMR* context (pJR957) than in the pBR322 context (pJR928). In either context, the 138-bp *E* silencer was a more efficient ARS than was the synthetic silencer. The loss rates for the 138-bp *E* silencer in the *HMR* context (pJR957) were comparable to those of plasmids containing *ARS1*, which has been shown to replicate once every S phase (16). Even in its best context (pJR950), the synthetic silencer had a higher loss rate and was presumably used as a replication origin in only a fraction of cell divisions.

The synthetic silencer was a context-dependent silencer. The results presented above indicated that a repression-competent context for the synthetic silencer was also replication competent. In addition, replication-incompetent contexts were found. If the replication function of the silencer is linked to transcriptional repression, then the replication-incompetent contexts would be repression incompetent. Therefore, the synthetic silencer in the ARS-incompetent context of pJR956 was substituted for the chromosomal copy of *HMR***a** and tested for silencer function. A high level of **a***1* mRNA was detected in RNA from both *SIR4* (JRY2387) and *sir4::HIS3* (JRY2389) strains bearing this allele of *HMR-E* (Fig. 5, lanes 1 and 2). Consistent with the high level of **a***1* mRNA detected, even the Sir^{+} strain mated with an efficiency 100-fold lower than that of a strain with the synthetic silencer flanked by *HMR* sequences (JRY2149; Fig. 4). Thus, a context in which the synthetic silencer was able to repress transcription was ARS competent, and a context in which the synthetic silencer was unable to provide ARS function was repression incompetent. A perfect correlation existed between ARS efficiency and silencer efficiency: the *HMR* alleles with intermediate levels of ARS activity (containing the synthetic silencer flanked only on the left [pJR955] or only on the right [pJR953] by pBR322) were gene replaced into the chromosome and led to intermediate levels of repression. The synthetic silencer flanked only on the left by pBR322 sequences (JRY2705) caused more repression than it did when flanked only on the right by pBR322 sequences (JRY2704; Fig. 5, lanes 3 and 5). The 138-bp *E* silencer flanked only on the left by pBR322 sequences caused complete repression of *HMR***a**. The mating efficiencies of these strains were all proportional to the degree of repression (Fig. 4 and 7).

The ARS consensus sequence but not the ABF1 binding site was required for the ARS activity of the synthetic silencer. Plasmids containing the synthetic silencer with the ARS consensus mutation (*ssa*), in either orientation with respect to *HMR* flanking sequences (pJR949 and pJR948; Fig. 7), yielded no transformants that formed colonies upon restreaking on selective media. This result indicated that the synthetic silencer's ARS activity depended completely on the ARS consensus sequence, even in the permissive *HMR* context. Plasmids containing the synthetic silencer with the ABF1 site mutation (*ssb*), in either orientation flanked by *HMR* sequences (pJR952 and pJR951; Fig. 7), had loss rates identical to those of the corresponding plasmids with intact ABF1 binding sites (pJR947 and pJR950). This result indicated that the ABF1 binding site played no role in the ARS activity of the synthetic silencer.

The synthetic silencer had *SIR*-dependent plasmid segregation properties. In addition to ARS function, the natural *HMR-E* silencer, as either a 490- or 138-bp fragment, provided a *SIR*-dependent segregation function to plasmids. To compare the segregation properties of the synthetic silencer with those of the 138-bp *E* silencer without demanding replication function, each fragment was inserted into the *URA3 ARS1*-containing plasmid YRp17 (described in Materials and Methods) and transformed into the isogenic wild-type and *sir4::HIS3* strains (DBY703 and JRY1263). The segregation function is best revealed by the plasmid loss rate, which is equal to 0.5 times the fraction of cell divisions in which one cell inherits all of the plasmids, providing a more direct measure than does the more commonly used mitotic stability measurement (discussed in reference 32). A perfect segregator would result in a loss rate of 0.0, and a complete lack of segregation (or replication) would result in a loss rate of 0.5. Results of these experiments are presented in Fig. 8. The relatively high loss rate of YRp17 was unaffected by the *SIR4* genotype of the cell (loss rates between 0.21 and 0.29 in *SIR4* and between 0.19 and 0.22 in *sir4::HIS3* strains). Similar results were observed for a plasmid containing the synthetic silencer (pJR781) in the *sir4::HIS3* strain (loss rate from 0.21 to 0.26). However, this plasmid had a much lower loss rate in the *SIR4* strain (0.07 to 0.08). Similar results were observed with plasmids containing the 138-bp *E* fragment (pJR783). The loss rates of this plasmid and of YRp17 were comparable when tested in the *sir4::HIS3* strain (0.15 to 0.25). In contrast, this plasmid had a dramatically reduced loss rate in the *SIR4* strain (0.04 to

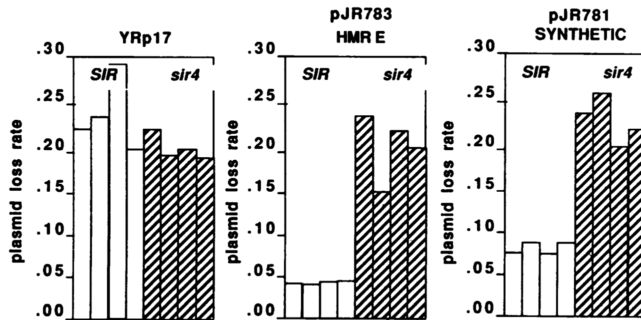

FIG. 8. Measurement of *SIR*-dependent plasmid segregation. Each bar indicates the plasmid loss rate determined from an independent transformant. Open bars indicate loss rates of plasmids transformed into a *SIR4* strain (DBY703), striped bars indicate loss rates of plasmids transformed into a *sir4*::*HIS3* strain (JRY1263). Plasmid pJR783 consisted of a 138-bp *Alu*I-*Dra*I *HMR-E* fragment inserted into YRp17; plasmid pJR781 consisted of a 142-bp synthetic silencer fragment inserted into YRp17.

0.05). These results demonstrated that the synthetic silencer was capable of providing a plasmid segregation function and that the segregation function was qualitatively indistinguishable from that provided by a wild-type silencer.

DISCUSSION

Previous work on the *HMR-E* silencer implicated three elements in the transcriptional repression function of the silencer: an ARS consensus sequence, an ABF1 binding site, and a RAP1 binding site (7, 24). Three questions concerning these elements were addressed by the experiments presented here: Are these three elements sufficient to constitute a functional silencer? Are all three of these elements involved in silencer function? Is ARS activity required for silencer function?

Experiments described above established that a synthetic silencer composed of heterologous binding sites for ABF1 and RAP1 and an 11-bp ARS consensus sequence was sufficient to substitute for the wild-type *HMR-E* silencer. This result clearly indicated that there are no additional elements hidden between previously identified sites that are required for function of the silencer. Furthermore, since the ABF1 and RAP1 sites in the synthetic silencer were chosen to be as different as possible from the RAP1 and ABF1 sites in the natural silencer, in all probability it is the RAP1 and ABF1 proteins that function at the silencer in vivo, rather than some rarer or more difficult to detect proteins that just happen to bind to the same range of binding-site sequences. Thus, these results place limits on the number of factors that must be considered in order to understand the molecular mechanism of silencing.

Genetic analysis revealed that mutation of the synthetic silencer in either the ABF1 binding site or in the ARS consensus sequence resulted in nearly complete derepression of *HMRa1* transcription. These results demonstrated that both the ABF1 binding site and the ARS consensus sequence were essential components of a functional silencer and that each had a unique role. However, mutations of the natural silencer in the ABF1 binding site or in the ARS consensus sequence had no derepression phenotype (7, 24). Thus, the functions of the ABF1 binding site and ARS consensus sequence were redundant in the wild-type *E* silencer but were not redundant in the synthetic silencer. Since mutations in the RAP1 binding site of the natural silencer cause partial derepression (6, 24), the function provided by the RAP1 site is not redundant and was not investigated in the synthetic silencer.

In addition to redundancy in silencer function, the natural silencer also exhibits redundancy in ARS function (6). Results from previous studies of ARS function provide potential insight into the nature of the redundant nature of the ARS function in the natural silencer. Multiple near matches to the ARS consensus sequence can function as an ARS without any perfect matches to the 11-bp ARS consensus sequence (53). The numerous near matches to the 11-bp ARS consensus found in the 490-bp natural *HMR-E* silencer may be sufficient for ARS function, thus explaining why an *HMR-E* restriction fragment lacking the 11-bp ARS consensus sequence was sufficient to confer ARS activity (7) and why two different mutations in the ARS consensus sequence of the *HMR-E* silencer fragment had no effect on the efficiency of this silencer as an ARS (25). In contrast, the synthetic silencer was completely dependent on the ARS consensus sequence for both ARS activity and transcriptional repression. Since the near matches to the ARS consensus were removed in the synthetic silencer constructions, it was likely that the lack of ARS redundancy in the synthetic silencer was due to a lack of site redundancy found in the natural *E* silencer. This interpretation was supported by the finding that the synthetic silencer was a much less efficient ARS than was the 138-bp *E* silencer. If this explanation is correct, then one might predict that it was the function of an ARS that was important for transcriptional repression by a silencer, rather than the ARS consensus sequence per se. Perhaps the *E* silencer must function as an origin of replication in the chromosome in order to repress transcription.

Further support for this model came from analysis of the *HMR* alleles with pBR322 sequences flanking the synthetic silencer. The allele with the highest plasmid loss rate, pJR956 (Fig. 7), was the most derepressed in the chromosome (JRY2387; Fig. 5). The alleles with intermediate loss rates, pJR953 and pJR955 (Fig. 7), showed intermediate repression in the chromosome (JRY2704 and JRY2705; Fig. 4 and 5). The allele with the lowest plasmid loss rate, pJR947 (Fig. 7), was the most repressed (JRY2149; Fig. 3 and 4). The flanking pBR322 sequences effectively reduced the efficiency of the silencer as an ARS on plasmids without altering the 11-bp consensus sequence and correspondingly reduced the efficiency of transcriptional repression.

So far, the results presented here suggested a working hypothesis in which DNA replication initiation at the *HMR-E* silencer was required for transcriptional repression of *HMR***a**. However, not all results were completely consistent with this hypothesis. Specifically, the synthetic silencer containing the ARS consensus sequence mutation had no ARS activity on plasmids yet still supported low-frequency mating when in the chromosome, indicative of a low level of residual repression of *HMR*. Since work by Miller and Nasmyth (31) suggests that replication plays a role in establishment of the repressed state, how can a replication defective silencer allow even a low level of mating? One consideration is that the ARS assay may be insensitive to very low levels of replication initiation. Thus, the low-frequency mating may reflect low frequency of replication initiation at the mutant silencer. Similarly, establishment of repression need occur only once in every 10 cell divisions in order to maintain a population of mating-competent cells (36). Thus, the small fraction of cells capable of mating in a strain in which the synthetic silencer harbors a mutation of

the ARS consensus sequence may be the result of a low frequency of replication initiation at the mutant silencer, or perhaps at *HMR-I*, resulting in establishment of repression, followed by the stable inheritance of the repressed state as suggested for *sir1* mutants (36).

Mutations in the ABF1 binding site of the synthetic silencer did not affect its ARS activity but did cause a strong derepression phenotype. Thus, it was unlikely that the ABF1 site mutation caused derepression by decreasing the frequency of replication initiation at the silencer. Therefore, ABF1 may play a role in the stability or inheritance of the repressed state. Alternatively, the ABF1 binding site may play a role in replication initiation in the chromosome but not on a plasmid, or it may play a replication-dependent role in establishment of repression.

One of the most curious results from this study was the extreme dependence of the synthetic silencer on *SIR1* for transcriptional repression. Results of quantitative mating experiments revealed that a *sir1* mutation resulted in a 1,000-fold reduction in the mating efficiency of cells with an *HMR* locus repressed by the synthetic silencer and only a 10-fold reduction in the mating efficiency of a strain with the wild-type silencer. In fact, the effect of the *sir1* mutation on repression by the synthetic silencer was even greater than the effect of a synthetic silencer containing a mutation in the ARS consensus sequence (*ssa*). *SIR1* plays a role in the establishment of repression (36), a process thought to be dependent on DNA replication (31). As judged by the plasmid replication data, the synthetic silencer provided replication function, albeit somewhat less efficiently than did the natural silencer. Furthermore, the degree of ARS function paralleled the degree of repression conferred by various synthetic silencer constructions. The synergistic phenotype caused by *sir1* mutations in synthetic silencer-containing strains is compatible with several interpretations involving DNA replication. Experiments are in progress to evaluate these hypotheses by measuring replication initiation events at *HMR* within the chromosome.

To understand the synthetic silencer's dependence on *HMR-I*, we imagine that an equilibrium exists between the repressed and derepressed states that is influenced by both the rate of establishment and the stability of the repressed state. By this hypothesis, the stability of the repressed state was compromised in strains lacking the *I* element. In I^- strains with a natural silencer, the frequency of replication initiation, and hence establishment, was sufficient to compensate for a slightly less stable repressed state. However, the synthetic silencer, with its reduced initiation frequency, could not compensate fully for the effect of the ΔI mutation on the stability of the repressed state, leading to more cells in the derepressed state.

The synthetic silencer was able to provide *SIR*-dependent plasmid segregation to a plasmid containing *ARS1*, an element known to initiate plasmid replication once every S phase. A major limitation in understanding the mechanism of *SIR*-dependent plasmid segregation is the lack of a mechanism to explain the mitotic instability (mother cell bias) for ARS plasmid segregation (32). One clue to the mechanism of *SIR*-dependent plasmid segregation can be inferred from this work. The synthetic silencer was able to confer *SIR*-dependent segregation in a context in which it was defective in both replication initiation and transcriptional repression. Therefore, the segregation function was genetically separable from replication initiation and transcriptional repression. Thus, *SIR*-dependent plasmid segregation may be the most sensitive assay for residual silencer function and may reflect the function of an intermediate in the assembly of the repressed chromatin structure at *HMR*.

The data presented here provide strong evidence that RAP1, ABF1, and replication initiation are, indeed, the activities that function at the *HMR-E* silencer. Nevertheless, it is still formally possible that other proteins with identical DNA binding specificities to ABF1 and ACBP function at the *HMR-E* silencer in vivo. Irrefutable resolution of this issue will require either analysis of the appropriate mutations in the genes encoding ABF1 and ACBP (as has been carried out for RAP1) or a *SIR*-dependent in vitro assay for silencer function. The simplicity of the synthetic silencer should facilitate an in vitro analysis of the mechanism of repression by *SIR* proteins.

ACKNOWLEDGMENTS

We thank A. Courey, M. Biggin, A. Buchman, D. Mahoney, and A. Brand for helpful discussions and communication of results prior to publication. We thank Dan Mytelka for constructing strain JRY2910. We also thank L. Pillus, P. Laurenson, and D. Rivier for critical reading of the manuscript.

Support for this work was provided by a National Science Foundation predoctoral fellowship to F.J.M., by grant GM31105 from the National Institutes of Health to J.R., and by National Institute of Environmental Health Sciences Center grant P30ESO1896-12.

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