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Roles of two DNA-binding factors in replication, segregation and transcriptional repression mediated by a yeast silencer

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The *HMR E* silencer is required for *SIR*-dependent transcriptional repression of the silent mating-type locus, *HMR*. The silencer also behaves as an origin of replication (*ARS* element) and allows plasmids to replicate autonomously in yeast. The replication and segregation properties of these plasmids are also dependent on the four *SIR* genes. We have previously characterized two DNA-binding factors in yeast extracts that recognize specific sequences at the *HMR E* silencer. These proteins, called ABFI (*ARS*-Binding Factor) and GRFI (General Regulatory Factor), are not encoded by any of the *SIR* genes. To investigate the biological roles of these factors, single-base-pair mutations were constructed in both binding sites at the *HMR E* silencer that were no longer recognized by the corresponding proteins *in vitro*. Our results indicate that the GRFI-binding site is required for the efficient segregation of plasmids replicated by the *HMR E* silencer. *SIR*-dependent transcriptional repression requires either an intact ABFI-binding site or GRFI-binding site, although the GRFI-binding site appears to be more important. A double-mutant silencer that binds neither ABFI nor GRFI does not mediate transcriptional repression of *HMR*. The replacement of *HMR E* with a chromosomal origin of replication (*ARS1*) allows partial *SIR*-dependent transcriptional repression of *HMR*, indicating a role for replication in silencer function. Together, these results suggest that the *SIR* proteins influence the properties of the *HMR E* silencer through interactions with other DNA-binding proteins.

Key words: transcription/repression/silencer/replication/yeast

Introduction

The yeast *Saccharomyces cerevisiae* can exist in any of three different cell types: the **a** and the α haploid mating types, which can mate with each other to form the **a**/α diploid, which cannot mate. The mating type of each cell is determined by a co-dominant allele of a single locus, *MAT* (Strathern *et al.*, 1981; reviewed by Herskowitz and Oshima, 1981). The *MATa* and *MAT*α alleles each encode regulatory proteins that control the expression of unlinked cell-type-specific genes. Although the allele present at the *MAT* locus is expressed and determines the cell type, there are additional copies of the *MATa* and *MAT*α sequences elsewhere in the yeast genome that are not expressed. The *HML*α locus contains a copy of *MAT*α, and the *HMRa* locus contains a copy of *MATa*. The *HML* and *HMR* loci serve as donor templates for *HO*-mediated mating-type interconversion.

Transcriptional silencers mediate repression of the *HML* and *HMR* loci. These silencers, known as the E site and the I site, lie to the left and right respectively of both *HML* and *HMR*, but are absent at *MAT* (Abraham *et al.*, 1984; Feldman *et al.*, 1984). Further analysis of one silencer, *HMR E*, indicates that this site mediates repression in a distance- and orientation-independent manner, and hence the term 'silencer' (Brand *et al.*, 1985). In addition to the E and I sites, transcriptional repression of *HML* and *HMR* also requires the action of four genes called *SIR1*, *SIR2*, *SIR3* and *SIR4* (Klar *et al.*, 1979; Ivy *et al.*, 1986; Rine and Herskowitz, 1987). Transcriptional repression presumably results from the action of the *SIR* proteins at the E and I sites.

Transcriptional repression by the *SIR* proteins is not limited to the mating-type promoters located at *HML* and *HMR*. The gene *SUP3a*, which encodes an amber-suppressing tRNA and is transcribed by RNA polymerase III, is also repressed by *SIR* when inserted at *HMR*, indicating that *SIR*-mediated transcriptional repression is a regional effect that is neither gene-specific nor polymerase-specific (Schnell and Rine, 1986). Furthermore, the promoters for the *LEU2* and *TRP1* genes are also repressed when placed at *HMR*, providing further evidence for the lack of gene specificity of *SIR*-mediated repression (Brand *et al.*, 1985). *SIR* also prevents the cleavage of *HML* and *HMR* DNA by the HO endonuclease, indicating that *SIR* can affect DNA−protein interactions that are not involved in transcription (Klar *et al.*, 1984). It has thus been proposed that *SIR* mediates the functional inactivation of DNA in the vicinity of the E and I sites (Schnell and Rine, 1986).

In addition to their role in *SIR*-mediated regional repression, the E and I sites also allow plasmids to replicate autonomously in yeast (Broach *et al.*, 1982; Abraham *et al.*, 1984; Kimmerly and Rine, 1987). DNA fragments that possess this activity are generally referred to as *ARS* (Autonomously Replicating Sequence) elements and may represent chromosomal origins of replication. Both the E and I sites are unusual *ARS* elements. Plasmids replicated by these mating-type silencers show higher mitotic stability than plasmids replicated by the *ARS1* chromosomal origin of replication. Mitotic stabilization of these plasmids is due to the provision of a plasmid segregation mechanism that is dependent on the *SIR* genes (Kimmerly and Rine, 1987). The coincidence of *ARS* elements with the E and I sites suggests a mechanistic link between the processes of DNA replication and transcriptional repression. In support of this notion, it has been observed that the onset of *SIR*-mediated transcriptional repression at *HMR* requires passage through the S phase of the cell cycle, suggesting a requirement for DNA replication (Miller and Nasmyth, 1984).

In order to understand the mechanism by which *SIR* influences the replication, segregation and transcriptional repression properties of the E and I sites, it is necessary to

identify the precise sequences and *trans*-acting factors that mediate these properties. Using deletion and linker-insertion mutagenesis, Brand *et al.* (1987) have dissected the *HMR E* silencer into three functional domains which are contained within a 138-bp region of the silencer. Repression by *SIR* requires the integrity of any two of the three domains. If any two domains are mutated, repression of *HMR* by *SIR* is lost. Two abundant proteins have also been identified that recognize specific regions of the *HMR E* silencer, neither of which is the product of any *SIR* gene (Shore and Nasmyth, 1987; Shore *et al.*, 1987; Buchman *et al.*, 1988). The binding sites for these proteins roughly correspond to two of the domains identified by Brand *et al.* (1987). One of these proteins, called GRFI (General Regulatory Factor; Buchman *et al.*, 1988) or RAP1 (Repressor−Activator Protein; Shore and Nasmyth, 1987), recognizes variants of the consensus sequence 5′-(A/G)(A/C)ACCCANN(C/A)A(T/C)(T/C)-3′. A match to this consensus sequence is found at both the *HML E* and *HMR E* silencers (Shore *et al.*, 1987; Buchman *et al.*, 1988) and hence may be required for the *SIR*-dependent properties of *HMR E*. A second protein that binds the *HMR E* silencer, called ABFI (*ARS*-Binding Factor; Buchman *et al.*, 1988) or SBF-B (Silencer-Binding Factor; Shore *et al.*, 1987), recognizes variants of the consensus sequence 5′-TATCATTNNNNACGA-3′. ABFI also binds to the *HMR I* and *HML I* regions, and is associated with other *ARS* elements (Shore *et al.*, 1987; Buchman *et al.*, 1988). However, other binding sites for ABFI have been identified that are not associated with a known *ARS* element, suggesting that the role of ABFI may not be limited to DNA replication.

In this report, we address the roles of GRFI and ABFI in the *HMR E*-dependent properties of *HMR E*. The analysis of single base-pair mutations in the binding sites for each of these factors indicates that an intact GRFI-binding site, but not an ABFI-binding site, is required for *SIR*-dependent mitotic stabilization of plasmids that contain *HMR E*. Also, transcriptional repression mediated by *SIR* and *HMR E* is more dependent on GRFI than ABFI. Our results imply that *SIR* influences the properties of *HMR E* through interactions with the bound GRFI factor.

Results

Two DNA-binding proteins recognize the HMR E silencer

Two *trans*-acting factors, called ABFI and GRFI, have been identified in yeast extracts that are able to bind specific sequences of the *HMR E* silencer (Shore *et al.*, 1987; Buchman *et al.*, 1988). The binding sites for both proteins have been precisely mapped by DNase I footprinting (Shore *et al.*, 1987; Buchman *et al.*, 1988; Figure 1). When a ^{32}P-labeled probe containing the *HMR E* silencer was incubated with a wild-type (*SIR*) extract, three complexes were resolved by gel mobility-shift electrophoresis (Figure 2A, lane 2). Complex 1 consisted of ABFI bound to the probe, complex 2 consisted of GRFI bound to the probe, and complex 3 was a ternary complex which consisted of both proteins bound to the probe. The identities of these complexes were established by competition experiments using an excess of a double-stranded oligonucleotide that encoded either binding site. The competitor oligonucleotide A contains the ABFI-binding site at *HMR E*, and the oligonucleotide G

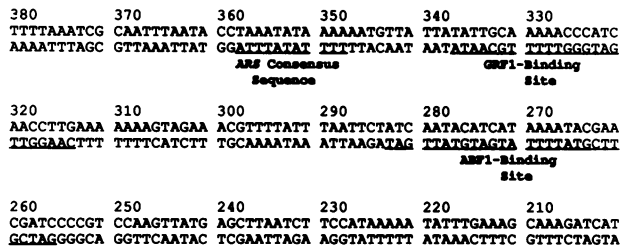


Fig. 1. Nucleotide sequence of *HMR E*. The numbering system of Abraham *et al.* (1984) is followed. The nucleotide sequences represented extend from nucleotide 201 to nucleotide 380. The sequences represented are internal to the 490-bp *HMR E* restriction fragment used in the determination of plasmid mitotic stability and in the reconstruction of *HMRa* alleles for transcriptional studies. The *ARS* consensus sequence (348−358), GRFI-binding site (314−337) and ABFI-binding site (256−283) are indicated as underlined sequences.

contains the GRFI-binding site at *HMR E* (Buchman *et al.*, 1988; Table I). When an excess of the A oligonucleotide was included in the DNA-binding reaction, only a single protein−DNA complex was observed with a wild-type extract (Figure 2A, lane 3). Both the ABFI complex and the ternary complex were eliminated. Similarly, when the competitor oligonucleotide G was included in the binding reaction, both the GRFI complex and the ternary complex were eliminated and only the ABFI complex was detected (Figure 2A, lane 4). When this analysis was repeated with whole-cell extracts of strains that carried null mutations in any *SIR* gene, qualitatively and quantitatively similar patterns of complexes were observed (Figure 2A, lanes 5−16). Therefore, neither ABFI nor GRFI were encoded by any *SIR* gene. Furthermore, mutations in any *SIR* gene did not detectably alter the mobility of either complex, indicating that *SIR* was neither an accessory component of either binding activity, nor caused a post-translational modification of either ABFI or GRFI that was detectable as a change in mobility of either complex. These results confirm and extend previous observations on the identities of *HMR E*-binding proteins.

To investigate the roles of ABFI and GRFI in the functions of *HMR E*, site-specific mutagenesis was used to alter each binding site so that recognition by the corresponding factor would be abolished. As a first step towards characterizing the properties of each mutation, oligonucleotides containing mutant-binding sites were tested for their ability to compete with a labeled *HMR E* restriction fragment containing the wild-type binding site. ABFI binding was not competed by the double-stranded oligonucleotide mtIA, which contained a single-base-pair insertion in the central variable core of the ABFI consensus sequence (Buchman *et al.*, 1988; Table I). One strand of the mtIA oligonucleotide was used as a mutagenic primer to create the corresponding mutation at *HMR E*, named *hmr e-IA*. When a ^{32}P-labeled probe containing this mutant silencer was used in a DNA mobility-shift assay, a single complex was observed (Figure 2B, lane 6). The remaining protein−DNA complex corresponded to GRFI bound to the probe as it was competed by the G oligonucleotide, but not by the A oligonucleotide (Figure 2B, lanes 7 and 8). Similarly, mutations in the GRFI-binding site were constructed. The double-stranded oligonucleotide mtIIA (Buchman *et al.*, 1988) carried a C/G-to-A/T trans-

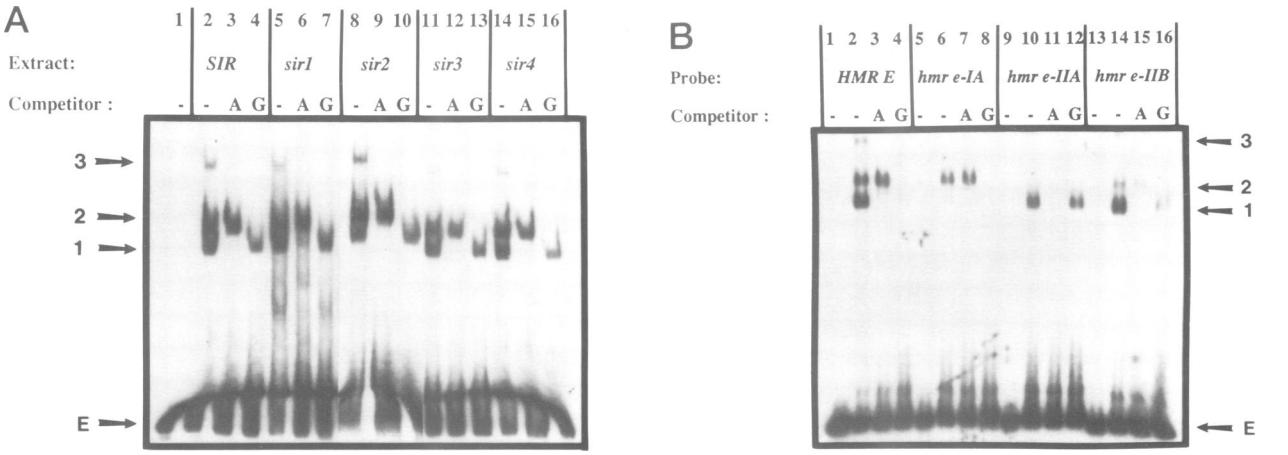


Fig. 2. (**A**) Detection of *HMR E*-binding proteins by DNA mobility-shift electrophoresis. DNA-binding reactions included 3–5 μg of protein from whole-cell extracts of strains of the indicated *SIR* genotypes and were performed as described in Materials and methods. Where indicated, 100 ng of competitor oligonucleotides (Table I) were included in the DNA-binding reactions. A, ABFI-binding site; G, GRFI-binding site. **Lane 1**, no extract; **lanes 2–4**, *SIR* extract (DBY703); **lanes 5–7**, *sir1::HIS3* extract (YWK178); **lanes 8–10**, *sir2::HIS3* extract (YWK179); **lanes 11–13**, *sir3::LYS2* extract (YWK55); **lanes 14–16**, *sir4::HIS3* extract (YRS477). Protein–DNA complexes are indicated by the arrows to the left of the figure; **Complex 1**, ABFI complex; **Complex 2**, GRFI complex; **Complex 3**, ternary complex; **E**, unbound *HMR E* probe. (**B**) Characterization of DNA–protein complexes formed with wild-type and mutant *HMR E* probes by DNA mobility-shift electrophoresis. Oligonucleotide competitors and DNA–protein complexes are indicated as described in (A). **Lanes 1, 5, 9** and **13** contained no extract. **Lanes 2–4, 6–8, 10–12** and **14–16** contained <5 μg of protein supplied by a whole-cell extract of a wild-type strain (DBY703).

Table I. Oligonucleotides used in this study

Oligonucleotide	Length	Sequence[a]
A	28	G A T C C A A T A C A T C A T A A A A T A C G A A C G A G T T A T G T A G T A T T T T A T G C T T G C T T C G A
mtIA	29	G A T C C A A T A C A T C A T A A A A A T A C G A A C G A G T T A T G T A G T A T T T T T A T G C T T G C T T C G A
G	31	G A T C T T A T A T T G C A A A A A C C C A T C A A C C T T G A A T A T A A C G T T T T T G G G T A G T T G G A A C T T A A
mtIIA	31	G A T C T T A T A T T G C A A A A A C A C A T C A A C C T T G A T A T A A C G T T T T T G T G T A G T T G G A A C T T A A
mtIIB	31	G A T C T T A T A T T G C A C A A A C C C A T C A A C C T T G A A T A T A A C G T G T T T G G G T A G T T G G A A C T T A A

[a]Both strands of the oligonucleotides are shown with the top strand listed 5′ to 3′ from left to right.

version within the core of the GRFI site consensus sequence (Table I). This oligonucleotide did not compete for GRFI binding to a labeled *HMR E* probe (data not shown; Buchman *et al.*, 1988). The corresponding mutant silencer was constructed and named *hmr e-IIA*. When ^{32}P-labeled probe containing this mutant silencer was used in a gel mobility-shift experiment, only a single complex was observed (Figure 2B, lane 10). This remaining complex corresponded to ABFI bound to the *hmr e-IIA* probe, as it was competed by the A, but not by the G oligonucleotide (Figure 2B, lanes 11 and 12). A second mutation consisting of an A/T-to-C/G transversion adjacent to the consensus sequence of the GRFI-binding site, called *hmr e-IIB*, was also constructed (Table I). When *hmr e-IIB* was used as a probe in a DNA mobility-shift experiment, both the ABFI and GRFI protein–DNA complexes were observed; however, GRFI recognized this mutant site with lower affinity than the wild-type site (Figure 2B, compare lanes 2 and 14). A double-mutant silencer containing both the *hmr e-IA* and *hmr e-IIA* mutations was also constructed and named *hmr e-IA,IIA*. The double-mutant silencer bound neither ABFI nor GRFI (data not shown).

Roles of protein-binding sites at HMR E in plasmid replication and segregation

The wild-type *HMR E* silencer provides for both autonomous replication and efficient segregation of plasmids in yeast, resulting in their high mitotic stability. These properties of silencer-related *ARS* elements depend on the products of the four *SIR* genes and are quantitatively distinct from the properties of other *ARS* elements such as *ARS1* (Kimmerly and Rine, 1987). To determine whether binding of either ABFI or GRFI is required for the *SIR*-dependent mitotic stabilization of such plasmids, the mutant silencers were subcloned into the non-replicating yeast vector YIp5. The resulting plasmids were transformed into wild-type and *sir* mutant yeast strains, and the mitotic stabilities of the plasmids were determined (Kimmerly and Rine, 1987). The mitotic stability of a plasmid is defined as the percentage of cells

Table II. Mitotic stabilities of plasmids carrying wild-type and mutant silencers

Strain	Genotype	Plasmid	*ARS*	Selective (%)[a]	Non-selective (%)[b]
DBY703	*SIR*	YRp315	*HMR E*	89.4 ± 5.4	70.9 ± 4.3
		YRp315-IA	*hmr e-IA*	85.3 ± 3.2	53.8 ± 3.2
		YRp315-IIA	*hmr e-IIA*	11.2 ± 2.9	1.4 ± 0.8
		YRp315-IIB	*hmr e-IIB*	60.7 ± 15.4	33.9 ± 14.0
		YRp353	*hmr e-IA,IIA*	8.7 ± 2.0	ND[c]
YWK55	*sir3::LYS2*	YRp315	*HMR E*	15.8 ± 1.3	0.52 ± 0.20
		YRp315-IA	*hmr e-IA*	6.8 ± 3.1	0.09 ± 0.21
		YRp315-IIA	*hmr e-IIA*	4.9 ± 1.2	0.19 ± 0.28
		YRp315-IIB	*hmr e-IIB*	3.7 ± 1.9	0.02[d]
		YRp353	*hmr e-IA,IIA*	8.0 ± 2.9	ND[c]
YRS477	*sir4::HIS3*	YRp315	*HMR E*	5.4 ± 2.5	<0.06[e]
		YRp315-IA	*hmr e-IA*	8.1 ± 2.4	0.25 ± 0.23
		YRp315-IIA	*hmr e-IIA*	3.9 ± 1.9	0.19 ± 0.31
		YRp315-IIB	*hmr e-IIB*	7.0 ± 2.4	0.26 ± 0.21
		YRp353	*hmr e-IA,IIA*	8.9 ± 2.2	ND[c]

[a]In this and subsequent tables, selective mitotic stability is presented as the percentage of cells in a population that exhibit the plasmid-borne *URA3* marker in YM broth without uracil. Values are presented as the mean percentage of 4–6 transformants. The standard error among these transformants is presented as a percentage.
[b]Cells grown in selective broth were diluted 1:1000 in YPD broth and grown for an additional 10–12 generations. Non-selective mitotic stability is presented as the percentage of cells in the population that exhibit the plasmid-borne *URA3* marker after this growth period in YPD broth.
[c]Mitotic stability after non-selective growth was not determined.
[d]The standard error was not determined.
[e]No cells with the plasmid were found out of 1781 screened.

that carry a plasmid, in a population of transformed cells, during mitotic growth. A plasmid containing *HMR E*, YRp315, is present in ~90% of wild-type (*SIR*) cells under selective growth conditions. The same plasmid has significantly reduced mitotic stability in isogenic *sir4* strains (Kimmerly and Rine, 1987). The plasmid YRp315-IA, which carried *hmr e-IA*, was present in 85% of wild-type cells under selective growth conditions (Table II), indicating that the ABFI-binding site was not required for the high mitotic stability of plasmids that carry *HMR E*. The mitotic stability of this plasmid was still dependent on *SIR*, since only 4–8% of cells carried the plasmid when the analysis was repeated using isogenic *sir3* and *sir4* mutants (YWK55 and YRS477, Table II). The plasmid YRp315-IIA, which carried the *hmr e-IIA* allele, was present in only 11.2% of wild-type cells under selective growth conditions (Table II), indicating that the GRFI-binding site was required for high mitotic stability of *HMR E*-containing plasmids. This lowered mitotic stability was moderately affected by the *sir4::HIS3* mutation (Table II). The additional decrease may reflect residual binding of GRFI to this mutant site *in vivo*, which was not detected *in vitro* (Figure 2B). Alternatively, *SIR* may influence the properties of *HMR E* independent of the binding of GRFI (see Discussion).

The plasmid YRp315-IIB, which carried the *hmr e-IIB* silencer, exhibited an intermediate mitotic stability in the wild-type strain, and this level of plasmid stability was dependent on the *SIR* genotype of the cell (Table II). This observation correlated well with the slightly reduced binding affinity of GRFI observed *in vitro* (Figure 2B). The plasmid YRp353, which contained the double-mutant silencer (*hmr e-IA,IIA*) exhibited a low mitotic stability comparable to that exhibited by the GRFI-site mutant plasmid, YRp315-IIA (Table II). The absence of additive or synergistic effects with the double-mutant silencer indicated that no additional roles of ABFI in *SIR*-dependent plasmid stabilization were revealed in the absence of GRFI binding. This result is in contrast to the roles of these factors in transcriptional repression (see below).

The roles of these DNA-binding proteins in the replication of plasmids carrying *HMR E* were investigated by two methods. First, the replication properties of the mutant silencers were evaluated by measuring the mitotic stability of plasmids that carry both *HMR E* and a centromere (*CEN6*). In this assay, changes in mitotic stability are a direct measurement of a plasmid's ability to replicate because the centromere provides an efficient plasmid-segregation mechanism that is independent of *SIR*. The mitotic stabilities of centromere-containing plasmids that carried mutant silencers are reported in Table III. Under selective growth conditions, centromere-containing plasmids that contained either the ABFI-site mutant (YCp6.316-IA) or the GRFI-site mutant (YCp6.315-IIA) exhibited a mitotic stability of 70–75%, comparable to a control plasmid that carried the wild-type silencer (YCp6.315). Therefore, at the single-copy level there appeared to be no requirement for either binding site for efficient replication. However, the centromeric plasmid that contained the double-mutant silencer (YCp6.353) exhibited a mitotic stability of 62%, somewhat lower than plasmids that contained the wild-type or either single-mutant silencers. This result indicated that a functional binding site for either ABFI or GRFI was required for optimum replication of *HMR E*-containing plasmids at the single-copy level in wild-type cells. The centromere-containing plasmid YCp6.315-IIB carrying the *hmr e-IIB* silencer behaved unexpectedly. This mutant was still recognized by GRFI *in vitro*, albeit with lower affinity than the wild-type (Figure 2B). The mitotic stability of this plasmid was 86% in the wild-type strain, significantly higher than plasmids that carried either the wild-type or the other single-mutant

Table III. Mitotic stabilities of centromere-containing plasmids carrying wild-type or mutant silencers

Strain	Genotype	Plasmid	*ARS*	Selective (%)	Non-selective (%)
DBY703	*SIR*	YCp6.315	*HMR E*	71.2 ± 7.2	14.5 ± 8.3
		YCp6.315-IA	*hmr e-IA*	72.8 ± 2.8	29.5 ± 4.6
		YCp6.315-IIA	*hmr e-IIA*	73.4 ± 2.9	26.9 ± 4.2
		YCp6.315-IIB	*hmr e-IIB*	85.2 ± 4.8	49.8 ± 4.5
		YCp6.353	*hmr e-IA,IIA*	62.0 ± 3.4	54.7 ± 3.8
YWK55	*sir3::LYS2*	YCp6.315	*HMR E*	78.4 ± 3.3	63.9 ± 1.6
		YCp6.315-IA	*hmr e-IA*	77.3 ± 5.3	61.2 ± 5.3
		YCp6.315-IIA	*hmr e-IIA*	84.2 ± 4.1	64.6 ± 9.2
		YCp6.315-IIB	*hmr e-IIB*	77.3 ± 5.0	52.4 ± 1.1
		YCp6.353	*hmr e-IA,IIA*	76.9 ± 3.4	60.4 ± 7.2
YRS477	*sir4::HIS3*	YCp6.315	*HMR E*	81.2 ± 2.1	63.3 ± 1.4
		YCp6.315-IA	*hmr e-IA*	71.8 ± 6.3	49.8 ± 3.5
		YCp6.315-IIA	*hmr e-IIA*	82.1 ± 6.1	61.6 ± 6.9
		YCp6.315-IIB	*hmr e-IIB*	80.3 ± 3.7	52.9 ± 5.6
		YCp6.353	*hmr e-IA,IIA*	78.6 ± 3.6	68.5 ± 2.6

silencers (Table III). The results obtained with this plasmid remain unexplained. Perhaps the *hmr e-IIB* allele exhibits neomorphic properties in its ability to replicate and segregate plasmids.

Plasmids that contain *HMR E* can replicate at high copy number (in the absence of a centromere) or at low copy number (in the presence of a centromere). These two processes are affected differently by the *SIR* genes (Kimmerly and Rine, 1987). To determine the roles of ABFI and GRFI in high-copy number plasmid maintenance, the plasmid copy number was measured for plasmids that carried either wild-type or mutant silencers in the wild-type strain, DBY703. YRp315, which carried *HMR E*, was present at an average of 20 plasmids/cells in the population, in agreement with previous determinations. A plasmid that carried a mutation in either the ABFI-binding site or the GRFI-binding site was present at a slightly lower copy number (7 and 10/cell, respectively) indicating that *HMR E* required the binding of both proteins for maximum high-copy-number maintenance. The plasmid that carried the double-mutant silencer, YRp353, was present at an average of six plasmids/cell. Since the double-mutant silencer exhibited a replication defect that was not appreciably greater than either single-mutant silencer, neither binding site was absolutely required for high-copy-number maintenance.

Roles of the ABFI- and GRFI-binding sites in antagonism between HMR E- and CEN6-mediated segregation

Plasmids that carry both *HMR E* and *CEN6* are less stable during non-selective mitotic growth than plasmids that carry only one of these elements. That is, a plasmid that carries both *HMR E* and a centromere is lost at a higher rate than a plasmid that carries only *HMR E*. This phenomenon is due to antagonism between the mechanisms by which a centromere and a silencer provide segregation to plasmids (Kimmerly and Rine, 1987). Antagonism can be relieved by disrupting either segregation function. For instance, introduction of a *sir4::HIS3* mutation inactivates the *HMR E*-mediated segregation thereby raising the observed mitotic stability during non-selective growth. Similarly, if the anti-

Table IV. Antagonism between *HMR E*- and *CEN6*-mediated plasmid segregation requires either ABFI or GRFI

Strain	Genotype	Plasmid	Non-selective mitotic stability (%) in the presence of benomyl (μg/ml)[a]				
			0[b]	20	40	80	160
DBY703	*SIR*	YCp6.315	28.6	34.4	40.6	34.3	38.8
		YCp6.315-IA	26.8	27.1	26.0	30.3	38.9
		YCp6.315-IIA	22.2	28.3	28.3	44.8	33.8
		YCp6.353	55.5	51.3	47.1	49.9	48.6
YRS477	*sir4::HIS3*	YCp6.315	61.9	56.9	54.8	55.6	56.3
		YCp6.353	79.5	75.6	77.7	62.6	58.0

[a]Cells were diluted 1:1000 from a stationary-phase YM-grown culture into YPD broth with benomyl at the indicated concentrations. After growth for 10–12 generations, plasmid mitotic stability was determined as in Kimmerly and Rine (1987). Benomyl was diluted from a 20 mg/ml stock in DMSO.
[b]Cells were grown in YPD broth with 0.8% DMSO, equivalent to the DMSO concentration obtained in the presence of 160 μg/ml benomyl.

microtubule drug 1-butylcarbamoyl-2-benzimidazolecarbamate methyl ester (benomyl) is included in the medium at sub-lethal levels during non-selective growth, the centromere– spindle interaction is perturbed and centromere-mediated plasmid segregation is disrupted and the mitotic stability of the plasmid increases (Kimmerly and Rine, 1987). The binding-site mutants of *HMR E* were tested to determine whether they played a role in *SIR*-dependent antagonism between *HMR E* and the centromere. During non-selective growth the plasmids YCp6.315-IA and YCp6.315-IIA were significantly less stable in the wild-type strain (DBY703) than in either isogenic *sir3::LYS2* (YWK55) or *sir4::HIS3* (YRS477) derivatives, suggesting that antagonism between the centromere and the single-mutant silencers still existed (Tables III and IV). To extend this observation, the same strains were grown non-selectively in YPD medium with several concentrations of benomyl, and the mitotic stabilities were determined after 10–12 generations (Table IV). The centromere-containing plasmid that carried *HMR E* (YCp6.315) showed increased mitotic

stability in the *SIR* strain as the concentration of benomyl in the medium was increased. Similarly, centromere-containing plasmids that carried either single-mutant silencer, YCp6.315-IA or YCp6.315-IIA, also showed increased mitotic stability in the wild-type strain (DBY703) as the benomyl concentration was increased during non-selective

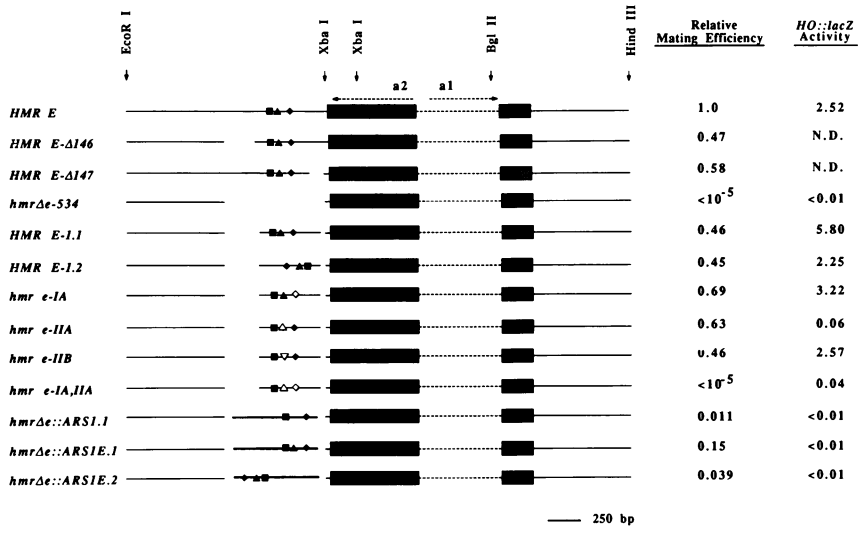
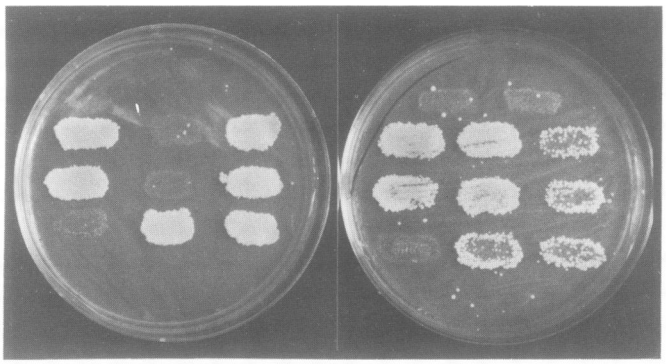


Fig. 3. Structures of reconstructed *HMRa* alleles. All alleles were replaced to the normal chromosomal location as described in Materials and methods. The relative locations of the *ARS* consensus sequence (■), GRFI-binding site (▲) and ABFI-binding site (◆) are indicated in each construction. Filled symbols denote wild-type sites and open symbols denote mutant sites. The large and small shaded boxes indicate the X and Z1 regions, which are homologous among all mating-type-related genes (Astell *et al.*, 1981; Nasmyth and Tatchell, 1980). The dotted arrows indicate the approximate limits of the a1 and a2 RNA transcripts. The bold lines replacing *HMR E* in the representations of the last three alleles indicate *ARS1*-derived sequences. All strains containing reconstructed *HMRa* alleles are isogenic with DBY703. The corresponding strain numbers are listed in Table V. The left data column lists the quantitative mating efficiencies of strains containing reconstructed *HMRa* alleles. Diploids formed by mating with an excess of JRY676 (*MATa*) cells were quantitated and normalized to the control strain DBY703. The right data column lists β-galactosidase activity expressed from an *HO::lacZ* fusion gene. Strains containing different *HMRa* alleles were transformed with the plasmid p*HO*-c12-*lacZ* (Russell *et al.*, 1986). The mean activity of four independent transformants measured in duplicate is reported. The standard errors among these determinations were 10−20%. β-Galactosidase assays were performed in the strain JRY1707 which contains a replacement of *HMR E* with *LYS2* (*hmrΔe::LYS2*) rather than a deletion as shown (JRY1731). β-Galactosidase activity is reported in Miller units (Miller, 1972). ND, not determined.



			hmrΔe::ARS1.1 *sir4::HIS3*	*hmrΔe::ARS1E.1* *sir4::HIS3*	
HMR E	*hmrΔe::LYS2*	*HMR E-Δ146*	*hmr e-IA*	*hmr e-IIB*	*hmrΔe::ARS1.1*
MATα	*hmrΔe-534*	*HMR E-1.1*	*hmr e-IIA*	*sir1::HIS3*	*hmrΔe::ARS1E.1*
MATa	*HMR E-Δ146*	*HMR E-1.2*	*hmr e-IA,IIA*	*sir1::HIS3* *hmr e-IIA*	*hmrΔe::ARS1E.2*

Fig. 4. Qualitative patch-mating test. Strains containing reconstructed alleles of *HMRa* were grown as patches on YPD−2% agar plates, then replica-plated to YM−2% glucose−2% agar plates seeded with ~10^7 mating-type tester cells (*MATa*; strain 227). The plates were incubated at 30°C for 2−3 days to allow formation and growth of diploids. The relevant *HMR E* and *SIR* genotypes of the strains indicated at the bottom of the figure are arranged in an array which corresponds to the pattern of the strains on the petri plates. The corresponding strain numbers are listed in Table V.

growth. The centromere-containing plasmid YCp6.353, which carried the double-mutant silencer, exhibited higher mitotic stability than YCp6.315 or either single-site mutants during non-selective growth and decreased mitotic stability in the presence of benomyl. These results established that the *HMR E*-mediated segregation mechanism was fully abolished by the double-mutant silencer, and that at least one of the two plasmid-binding sites was required for antagonism.

In the *sir4::HIS3* strain (YRS477), centromere-containing plasmids that carried either the wild-type or the double-mutant silencer both showed decreased mitotic stability during non-selective growth in the presence of benomyl. This result indicates that *CEN6* was the only functional segregator available to this plasmid. In the case of the plasmid that contained the double-mutant silencer, YCp6.353, a greater effect of benomyl on the mitotic stability in the *sir4::HIS3* strain than in the wild-type strain was observed. This enhanced effect may result from either residual antagonism in the wild-type strain due to slight leakiness of these site mutations *in vivo*, or to an antagonism by *SIR* at *HMR E* that is independent of both binding sites.

Roles of ABFI- and GRFI-binding sites in SIR-dependent transcriptional repression

To understand further the connections between the different *SIR*-dependent functions of *HMR E*, the effects of protein-binding site mutations at *HMR E* on transcriptional repression of *HMR* were examined. A series of *HMRa* alleles were reconstructed *in vitro* and transplaced to their normal chromosomal location (see Materials and methods for details). The structures of these reconstructed alleles are shown in Figure 3. The expression of a1 mRNA was evaluated and quantitated by three methods. No single method provided a comprehensive view of silencer function. First, the expression of a1 mRNA was measured directly by S1 nuclease protection. In addition, other assays were developed. Assays which measure the mating ability of strains containing mutant silencers were best for detecting slight residual silencer function in strains that had lost most silencer function, whereas the measurement of expression of a haploid-specific gene, *HO*, was best for detecting slight losses of silencer function.

Silencer function was evaluated by qualitative and quantitative measurement of the mating ability of strains containing mutant silencers. All strains that contained reconstructed alleles of *HMRa* also contained the *MATα* allele. If sufficient a1 mRNA were expressed from *HMR*, the cells would exhibit a non-mating phenotype. Hence, expression of a1 mRNA could be indirectly evaluated by a patch-mating test with cells of the opposite mating type. The results of this analysis are shown in Figure 4. Strains that contained wild-type *HMR E* (DBY703) or reconstructed versions of *HMRa* with the E site in either orientation (JRY1723 and JRY1724) mated strongly with the *MATa* lawn (strain 227). No mating was observed with a *MATα* lawn (data not shown). However, strains that contained either a deletion of *HMR E* (JRY1731) or a replacement of E by the *LYS2* (JRY1707) gene did not mate with either lawn. Strains that contained mutations in either the ABFI-binding site (JRY1752) or the GRFI-binding site (JRY1727) also mated with the *MATa* lawn. However, a strain that contained the double-mutant E site (JRY1877) did not mate at all with the *MATa* lawn. This result indicated that the binding sites for either ABFI or GRFI alone were sufficient to allow transcriptional repression of *HMR* by *SIR*, but when both binding sites were defective, transcriptional repression was abolished. This result suggested that ABFI and GRFI may have partially redundant roles in *SIR*-mediated transcriptional repression. Brand *et al.* (1987) have reached a similar conclusion from an independent analysis of *HMR E*.

In addition to patch-mating tests, a quantitative mating experiment was performed with all strains that contained reconstructed alleles of *HMRa*. These results are presented in Figure 3 along with the structures of the corresponding alleles. All strains that contained a wild-type version of *HMR E* (*HMR E, HMR-E-Δ146, HMR E-Δ147, HMR E1.1* and *HMR E1.2*) mated with the *MATa* strain JRY676 with an efficiency near that of the wild-type control strain (DBY703). Also, strains that contained the single-mutant silencers, *hmr e-IA, hmr e-IIA* or *hmr e-IIB*, mated with the *MATa* strain with efficiencies similar to the wild-type control strain. A strain that contained the double-mutant silencer, *hmr e-IA, IIA* (JRY1877), did not mate detectably with the *MATa* strain in this analysis. The quantitative mating data (Figure 3) correlated well with the qualitative mating results presented in Figure 4. The value of this assay for the detection of low-level silencer function was most evident in the following series of experiments aimed at determining the minimal requirements for silencer function.

The mating-type silencers are composed of different combinations of *ARS* elements, GRFI-binding sites and ABFI-binding sites (Brand *et al.*, 1987; Shore and Nasmyth, 1987; Shore *et al.*, 1987; Buchman *et al.*, 1988). All three elements appear to be involved in silencer function (Miller and Nasmyth, 1984; Brand *et al.*, 1987). However, binding sites for both of these factors have been identified that are not associated with any of the silencers (Shore and Nasmyth, 1987; Shore *et al.*, 1987; Buchman *et al.*, 1988). Also, numerous other *ARS* elements have been identified, that are not associated with the silencers, throughout the yeast genome. To determine whether *SIR*-dependent transcriptional repression is a general property of combinations of *ARS* elements, ABFI-binding sites and GRFI-binding sites, or whether silencer function requires these elements to be present in a special context, strains were constructed that carried derivatives of another yeast *ARS* element, *ARS1*. *ARS1* is located adjacent to the *TRP1* gene on chromosome IV, and hence is unlinked to any of the mating-type silencers, all of which are located on chromosome III. In addition, *ARS1* has been shown to be an origin of replication *in vivo* (Brewer and Fangman, 1987). *ARS1* also carries a binding site for the ABFI factor (Shore *et al.*, 1987; Buchman *et al.*, 1988). The strain JRY1728, which carried *ARS1* in place of *HMR E* (*hmrΔe::ARS1.1*), allowed weak mating in a patch-mating test (Figure 4), as evidenced by the appearance of several papillae. Quantitative mating determinations indicated that *ARS1* was nearly 100-fold less effective in repression of *HMR* than the wild-type E site (Figure 3). However, this level of mating was at least three orders of magnitude greater than that provided by the double-site-mutant silencer, *hmr e-IA,IIA*. This result indicated a role for the function of a replication origin, and perhaps the ABFI-binding site, in the mechanism of *SIR*-dependent transcriptional repression. A derivative of *ARS1*, called *ARS1E*, was constructed which carried an oligonucleotide-derived GRFI-binding site in addition to the *ARS* consensus

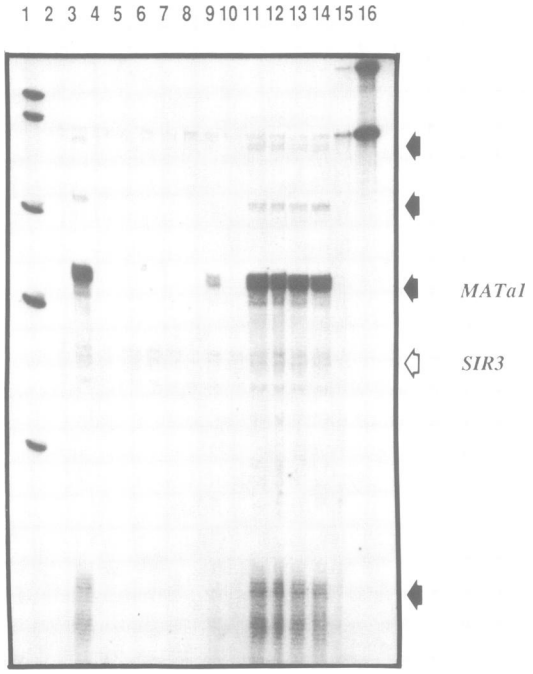


Fig. 5. Detection of *MATa1* and *SIR3* by S1 nuclease protection. Twenty micrograms of total RNA from various strains were hybridized to uniformly labeled, single-stranded probes homologous to the *MATa1* and *SIR3* genes, and then digested with S1 nuclease. The S1 nuclease protection products were separated by electrophoresis on a 5% polyacrylamide−50% urea sequencing gel. The *SIR3*-specific probe was included as an internal control for verifying equivalent amounts of RNA in different lanes. The *SIR3*-specific protection products are indicated by the open arrowhead. The *MATa1*-specific protection products are indicated by the solid arrowheads. The *MATa1* gene contains two introns (Miller, 1984), therefore several different sizes of protected fragments are detected with a uniformly labeled probe. **Lane 1**, size markers (end-labeled pBR327 *Hin*fI fragments; 517 bp, 450 bp, 298 bp, 220 bp and 154 bp); **lane 2**, *HMR E* (DBY703); **lane 3**, *hmr Δe-534* (HRY1731); **lane 4**, *HMR E-Δ146* (JRY1732); **lane 5**, *HMR E-Δ147* (JRY1733); **lane 6**, *HMR E-1.1* (JRY1723); **lane 7**, *HMR E-1.2* (JRY1724); **lane 8**, *hmr e-IA* (JRY1752); **lane 9**, *hmr e-IIA* (JRY1727); **lane 10**, *hmr e-IIB* (JRY1876); **lane 11**, *hmr e-IA,IIA* (JRY1877); **lane 12**, *hmrΔe::ARS1.1* (JRY1728); **lane 13**, *hmrΔe::ARS1E.1* (JRY1729); **lane 14**, *hmrΔe::ARS1E.2* (JRY1730); **lane 15**, *E.coli* tRNA; **lane 16**, probes alone.

sequence and the ABFI-binding site provided by *ARS1* (see Figure 3 and Materials and methods for details). Strains that carried *ARS1E* in either orientation relative to *HMR* (JRY1729 and JRY1730), also allowed weak mating with the *MATa* strains (Figures 3 and 4). Quantitative matings with these strains indicated that *ARS1E* was 4- to 15-fold more effective as a silencer than *ARS1*, implicating GRFI binding in the transcriptional repression of *HMR*. The repression provided by either *ARS1* and *ARS1E* was still *SIR* dependent since the introduction of a *sir4::HIS3* mutation into strains that carried *hmrΔe::ARS1.1* (JRY1735) or *hmrΔe::ARS1E.1* (JRY1736) completely abolished mating in a patch test (Figure 4).

A method to quantitate expression of a1 mRNA and designed to detect slight defects in silencer functions measured β-galactosidase activity expressed from an *HO::lacZ* fusion gene in strains that contained mutant silencers. The yeast *HO* gene, which encodes a site-specific endonuclease involved in mating-type switching, is regulated by the cell type. *HO* is expressed in *MATa* and *MATα* haploids, but is repressed in a/α diploids by the concerted action of the a1 and α2 products (Jensen *et al.*, 1983). Therefore, in *MATα* strains, the expression of a1 mRNA from *HMR* can be quantitated by a decrease in β-galactosidase activity expressed from the *HO::lacZ* fusion gene. A centromere plasmid that carried an *HO::lacZ* fusion (p*HO*-c12-*lacZ*; Russell *et al.*, 1986) was transformed into strains that contained mutated silencers and β-galactosidase activities of these transformants were measured (Figure 3). Strains that contained wild-type *HMR E* in either orientation expressed 2−6 units of activity. This level of β-galactosidase activity correlated well with *HO::lacZ* expression observed previously in haploid strains (Russell *et al.*, 1986). The strain that carried *hmr e-IA* (JRY1752) expressed similar levels of β-galactosidase activity from the *HO::lacZ* fusion gene, indicating again that the ABFI-binding site was not required for repression of a1 transcription by *SIR*. However, a strain that carried *hmr e-IIA* (JRY 1727) exhibited nearly 100-fold less β-galactosidase activity than the wild-type control, providing further evidence that the GRFI-binding site was required for efficient transcriptional repression. Although this strain exhibited a mating efficiency similar to strains that contained a wild-type silencer (Figures 3 and 4), the low levels of a1 mRNA accumulated in this mutant were sufficient to repress *HO::lacZ* expression. Apparently this level of a1 mRNA was insufficient to perturb α mating (see below). A strain that contained the *hmr e-IIB* silencer (JRY1876) exhibited wild-type levels of activity from the *HO::lacZ* fusion gene, suggesting that weaker recognition by GRFI of its site at *HMR E* was sufficient to allow full transcriptional repression of *HMR*. Strains that contained *ARS1* or *ARS1E* in place of *HMR E* exhibited undetectable levels of β-galactosidase activity. Similarly a deletion of *HMR E* (JRY1707) also resulted in undetectable levels of β-galactosidase. A strain that contained the double-mutant silencer (*hmr e-IA,IIA*) exhibited *HO::lacZ* expression that was barely detectable in this assay.

As a direct method of quantitation, the a1 mRNA levels were measured by S1 nuclease-protection analysis in strains that contained mutant silencers (Figure 5). The wild-type control strain (DBY703) exhibited no accumulation of a1-specific protection products (Figure 5, lane 2), whereas a strain that carried a deletion of *HMR E* expressed significant levels of a1 mRNA. Strains that contained reconstructed versions of *HMR E* in either orientation, or deletions that flanked *HMR E*, showed complete transcriptional repression since no accumulation of a1 mRNA was observed (Figure 5, lanes 4−7). Also, no a1 mRNA accumulated in the strain that contained the *hmr e-IA* silencer (Figure 5, lane 8). However, in the strain that contained the *hmr e-IIA* silencer (JRY1727), intermediate levels of a1 mRNA were observed (Figure 5, lane 9). This result provides further evidence for a significant role of the GRFI-binding site in *SIR*-dependent transcriptional repression of *HMRa*. This intermediate level of a1 transcription was apparently sufficient to repress transcription of the *HO::lacZ* fusion, but insufficient to inhibit α mating. The strain that contained the double-mutant silencer exhibited fully derepressed levels of a1 mRNA (Figure 5, lane 11). Therefore, in the absence of bound GRFI, ABFI presumably can interact with *SIR* to allow partial repression of *HMR*. By the S1 protection analysis, the repression of *HMR* observed with the mating assays in strains that contained derivatives of *ARS1* in place of *HMR E* was not evident at the level of a1 mRNA (Figure 5, lanes

Table V. Strains used in this study

Strain	Genotype	Source
DBY703	*MATα trp1 his3 ura3-52 cir°*	D.Botstein
YRS477	*MATα trp1 his3 ura3-52 sir4::HIS3 cir°*	Kimmerly and Rine (1987)
YWK18	*MATα trp1 his3 ura3-52 lys2-6 cir°*	
YWK55	*MATα trp1 his3 ura3-52 lys2-6 sir3::LYS2 cir°*	
YWK178	*MATα trp1 his3 ura3-52 sir1::HIS3 cir°*	
YWK179	*MATα trp1 his3 ura3-52 sir2::HIS3 cir°*	
JRY1707	*MATα trp1 his3 ura3-52 lys2-6 hmrΔe::LYS2 cir°*	This work
JRY1723	*MATα trp1 his3 ura3-52 lys2-6 HMR E-1.1 cir°*	
JRY1724	*MATα trp1 his3 ura3-52 lys2-6 HMR E-1.2 cir°*	
JRY1727[a]	*MATα trp1 his3 ura3-52 lys2-6 hmr e-IIA cir°*	
JRY1728	*MATα trp1 his3 ura3-52 lys2-6 hmrΔe::ARS1.1 cir°*	
JRY1729	*MATα trp1 his3 ura3-52 lys2-6 hmrΔe::ARS1E.1 cir°*	
JRY1730	*MATα trp1 his3 ura3-52 lys2-6 hmrΔe::ARS1E.2 cir°*	
JRY1731	*MATα trp1 his3 ura3-52 lys2-6 hmrΔe-534 cir°*	
JRY1732	*MATα trp1 his3 ura3-52 lys2-6 HMR E-Δ146 cir°*	
JRY1733	*MATα trp1 his3 ura3-52 lys2-6 HMR E-Δ147 cir°*	
JRY1734[a]	*MATα trp1 his3 ura3-52 lys2-6 hmr e-IIA sir1::HIS3 cir°*	
JRY1735	*MATα trp1 his3 ura3-52 lys2-6 hmrΔe::ARS1.1 sir4::HIS3 cir°*	
JRY1736	*MATα trp1 his3 ura3-52 lys2-6 hmrΔe::ARS1E.1 sir4::HIS3 cir°*	
JRY1752	*MATα trp1 his3 ura3-52 lys2-6 hmr e-IA cir°*	
JRY1876	*MATα trp1 his3 ura3-52 lys2-6 hmr e-IIB cir°*	
JRY1877[a]	*MATα trp1 his3 ura3-52 lys2-6 hmr e-IA,IIA cir°*	
JRY676	*MATa his4-519 leu2-3,112 ura3-52*	
70	*MATα thr3*	I.Herskowitz
227	*MATa lys1*	

[a]These strains have been shown by several genetic criteria to be *MATα/MATα* diploids. All markers are homozygous. Presumably these diploids resulted from the fusion of spheroplasts during transformation.

12–14). Therefore, the molecular assay for residual silencer function was less sensitive than the patch-mating bioassay.

Discussion

ABFI- and GRFI-binding sites mediate the SIR-dependent properties of HMR E

The regulatory site *HMR E* is required for transcriptional repression of *HMR* by the four *SIR* genes. Two protein factors called ABFI and GRFI recognize specific sequences at the *HMR E* silencer. However, neither of these factors is encoded by any *SIR* gene. The work presented here establishes the role of the ABFI- and GRFI-binding sites in the different properties of the *HMR E* silencer: transcriptional repression, plasmid segregation and centromere antagonism. The major finding is that both sites have a role, but the relative importance of the two sites varies for each property. For these studies, single-base-pair mutations in the binding sites for each of these proteins were constructed. Neither mutant site was bound by the corresponding factor *in vitro*. The phenotypes of these mutants indicates that the binding site for GRFI is required for *SIR*-dependent mitotic stabilization of plasmids that carry *HMR E*. Specifically, the mitotic stability of a plasmid containing a mutant silencer that does not bind GRFI (YRp315-IIA) is 8-fold lower than a plasmid containing the wild-type silencer in a Sir^+ strain. Furthermore, the introduction of a *sir3* or *sir4* mutation does not appreciably reduce the mitotic stability of YRp315-IIA. In contrast, the binding site of ABFI is not required for high mitotic stability. Taken together, these effects of GRFI site mutations imply that the *in vivo* binding of GRFI is required for *SIR*-dependent mitotic stabilization of plasmids that carry *HMR E*.

The requirements for the ABFI- and GRFI-binding sites are not identical for the transcriptional repression function of *HMR E*. A mutation in the ABFI-binding site alone has no effect on repression of *HMRa* transcription by *SIR*. In contrast, a mutation in the GRFI-binding site disrupts repression and leads to the accumulation of intermediate levels of a1 mRNA. However, since the mutant silencer *hmr e-IIA*, which binds ABFI but not GRFI, is partially functional, the binding of GRFI is not the only contributor to the silencer property of *HMR E*. Although α strains carrying this mutation (*hmr e-IIA*) still display an α-mating phenotype, enough a1 mRNA is expressed such that transcription of an *HO::lacZ* fusion gene is repressed by the concerted action of a1 and α2 (Jensen *et al.*, 1983). A strain containing a mutant silencer that binds neither ABFI nor GRFI derepresses a1 transcription to a level similar to a deletion of *HMR E*. Therefore, an intact GRFI-binding site appears to be a crucial requirement for *SIR*-dependent transcriptional repression, but the ABFI site is also required for full silencer function. This effect is unlike the apparent roles for ABFI- and GRFI-binding sites in plasmid stabilization, as the loss of plasmid stability exhibited by the double-mutant silencer is no more severe than the GRFI-site mutant silencer, *hmr e-IIA*. Therefore, transcriptional repression and plasmid stabilization are not equivalent measures of silencer function.

Brand *et al.* (1987) have defined three domains of the *HMR E* silencer: A (*ARS* element), B (ABFI-binding site or SBF-B-binding site) and E (GRFI-binding site or RAP1-binding site). Using deletion and linker-insertion analyses, they observed that transcriptional repression by *SIR* requires any two of these three domains. Although we have not analyzed the effect of mutations of the *HMR E ARS* element on transcriptional repression, the results for the binding site

mutations are qualitatively similar in both studies. However, there is a difference in the apparent requirements for plasmid stabilization by *SIR* and *HMR E*. We observed that a point mutation in the ABFI-binding site had no effect on the mitotic stability of silencer-containing plasmids, whereas Brand *et al.* (1987) observed that a deletion of the ABFI-binding site (element B) lowered the mitotic stability of silencer-containing plasmids. Brand *et al.* (1987) used a smaller restriction fragment for assessment of the plasmid stabilization effect (138 bp versus 490 bp). Perhaps the larger fragment used in our analysis contained additional elements that were able to replace the plasmid stabilization function of ABFI. However, the phenotype of the double-mutant silencer suggests that if another element exists that is able to fulfil the role of the ABFI-binding site in plasmid stabilization, it is unable to fulfil its role in transcriptional repression. Furthermore, no protein–DNA complexes other than those described in this paper have been observed using *HMR E* probes. At present there is no clear explanation for this difference.

Either ABFI- or GRFI-binding sites can mediate SIR-dependent segregation antagonism

Our previous study found evidence for a *SIR*-dependent segregation mechanism for *HMR E*-containing plasmids that is independent of the mitotic spindle. This segregation mechanism may involve *HMR E*-mediated plasmid attachment to a structural component of the nucleus (i.e. the nuclear matrix or scaffold). As a consequence of this alternate segregation mechanism, plasmids that carry both *HMR E* and a centromere are less stable in a Sir^+ strain than are plasmids that carry only one of these elements. This lowered stability provides evidence for antagonism between the centromere-based segregation mechanism and the *SIR*-dependent mechanism (Kimmerly and Rine, 1987). Centromere plasmids containing either single-mutant silencer still exhibit antagonism of centromere function by several criteria. First, both plasmids (YCp6.315-IA and YCp6.315-IIA) are unstable during non-selective mitotic growth in a Sir^+ strain, but are stable in a *sir3* or *sir4* mutant strain. Second, the mitotic stability of both plasmids is increased by the presence of the anti-microtubule drug, benomyl, during non-selective growth. Furthermore, the centromere plasmid containing the double-mutant silencer, YCp6.353, does not exhibit segregation antagonism; it is stable during non-selective mitotic growth in a Sir^+ strain, and this stability is decreased in the presence of benomyl. Therefore, the requirements of the ABFI- and GRFI-binding sites for centromere antagonism are distinguishable from their requirements for both transcriptional repression and for plasmid stabilization.

These results allow a refined model for plasmid stabilization and centromere antagonism. If attachment to a nuclear structure is sufficient to cause segregation antagonism, then since both single-mutant silencers are able to antagonize centromere function, the *SIR*-dependent attachment may be mediated by *either* ABFI or GRFI. According to this view, attachment alone is insufficient to explain the elevated mitotic stability of plasmids that contain only *HMR E*, since the GRFI-site mutant has low mitotic stability. *SIR*-dependent plasmid stabilization would require both an attachment to the nuclear structure and another event that requires GRFI-binding site. For instance, bound GRFI may be required for efficient decatenation of plasmids after replication so that the progeny molecules can be segregated independently.

Heterologous ARS elements provide partial silencer function

The establishment of *SIR*-dependent transcriptional repression of *HMR* requires passage through the S phase of the cell cycle (Miller and Nasmyth, 1984). However, it is not known whether this observation reflects a requirement for DNA replication, or for some other cell-cycle-regulated event that is coincident with or dependent on DNA replication. The *ARS1* element has been shown to be an authentic origin of replication (Brewer and Fangman, 1987). Therefore, the demonstration that *ARS1* can act as a weak, *SIR*-dependent silencer strengthens the suggestion of a mechanistic connection between DNA replication and transcriptional repression. A requirement for initiation of DNA replication at the *HMR E ARS* element may be the reason that S phase transit is required for transcriptional repression of *HMR* by *SIR*. A requirement for replication initiation could accommodate the bidirectional nature of repression mediated by *HMR E* and *SIR*. The *ARS1E* element is also a weak silencer, although it is somewhat better than *ARS1* alone. Although *ARS1E* contains all the defined elements of a silencer, it has only partial silencer function. Therefore, either additional sequences are required for full silencer function, or the exact spacing and relative orientation of the *ARS* consensus sequence, the ABFI-binding site and the GRFI-binding site is critical. The apparent flexibility in the spacing of binding sites and the *ARS* consensus sequence among different silencers favors the notion that additional sequences are required for full silencer function. Another issue raised by the observation that *ARS1* provides partial silencer function is whether initiation of DNA replication *per se* causes a general repression of transcription of nearby genes. In this regard, the analysis of *ARS1* mutations (Celniker *et al.*, 1984; Strich *et al.*, 1986) on the transcription of the neighboring *TRP1* gene would be useful.

Silencer-binding proteins are involved in other functions

Characterization of the DNA-binding properties of ABFI and GRFI suggests that these factors may have diverse functions in the cell (Buchman *et al.*, 1988). ABFI-binding sites are found near silencer-related *ARS* elements (*HMR E, HMR I* and *HML I*) and non-silencer-related *ARS* elements (*ARS1* and *ARS2*). The existence of ABFI-binding sites in the vicinity of many known *ARS* elements suggests a role in DNA replication, although there is no direct demonstration of a connection between ABFI and replication. Tandem binding sites for ABFI are also found between the *HIS3* and *DED1* genes, several kilobases from an *ARS* element. The role of ABFI at these sites is unknown, since the sites may be deleted with little effect on *DED1* or *HIS3* transcription (Struhl, 1985a,b). However, synthetic ABFI-binding sites can function as UAS elements in specially constructed plasmids (Brand *et al.*, 1987; Buchman *et al.*, 1988). It has also been suggested that ABFI may function in transcription termination (Buchman *et al.*, 1988; Snyder *et al.*, 1988). Functional versatility for ABFI, would not be unique since other DNA-binding proteins have been shown to participate in different processes. For example, mammalian cells contain a protein implicated in the replication of adenovirus, nuclear factor 1 (Nagata *et al.*, 1982; Rawlins *et al.*, 1984), which is identical to a CAAT-binding transcription factor, CTF (Jones *et al.*, 1987).

Similarly, the locations of GRFI-binding sites suggest roles in transcriptional repression (*HMR E* and *HML E*), transcriptional activation (*MATα2* UAS, RPG-box UAS) and telomere function (Brand *et al.*, 1987; Shore *et al.*, 1987; Buchman *et al.*, 1988). The results presented in this paper suggest that *SIR* mediates transcriptional repression of *HMR* by interacting with GRFI bound to the E site. However, not all GRFI sites are affected by *SIR*. For instance, the UAS element of the ribosomal protein gene *RP39A* (Rotenberg and Woolford, 1986) matches the consensus sequence for GRFI-binding sites (Buchman *et al.*, 1988). Nevertheless, no effect of a *sir4* mutation was observed on the transcription of an *rp39A::lacZ* fusion gene (unpublished results). Therefore, other factors in addition to GRFI are required to create a *SIR*-dependent silencer. The relative positions of the GRFI-binding sites and other promoter determinants may dictate whether GRFI will act as a transcriptional activator or repressor. For instance, the bovine papillomavirus (BPV1) regulatory protein encoded by the E2 open reading frame can either activate or repress transcription mediated by the human papillomavirus (HPV18) enhancer depending on the relative positions of the HPV18 enhancer and other promoter elements (Thierry and Yaniv, 1987). Also, sequences that mediate transcriptional induction of a human interferon gene (IFNα) by virus can suppress transcriptional induction mediated by the SV40 enhancer when the IFNα promoter elements are placed between the enhancer and the TATA box (Kuhl *et al.*, 1987). Another possibility is that the function of GRFI at different binding sites depends on sequences that are not well conserved among sites, or perhaps sequences that lie adjacent to the binding sites. There is considerable flexibility in sequences that flank the core GRFI-binding site consensus sequence (Buchman *et al.*, 1988). These sequences, although apparently not important for binding, may be involved in specifying transcriptional activation, repression or other functions. In support of this notion, Martin *et al.* (1986) have identified mutations in the *araI* site (a binding site for the regulator encoded by *araC*) which affect *araC*-mediated repression, but not induction in the presence of arabinose, of the *araBAD* operon. These investigators propose that the conformation of the AraC protein, and therefore its function, is different when bound to the mutant site.

Does SIR act directly or indirectly at the silencer?

The properties of the ABFI and GRFI DNA-binding proteins and the phenotypes of mutations in their binding sites suggest a model in which *SIR* acts indirectly on the silencer through bound ABFI and GRFI. This model is based on the assumption that the same proteins that bind *HMR E in vitro*, also bind *in vivo*. Although a strong case can be made for the roles of the GRFI and ABFI proteins in the *in vivo* properties of the *HMR E* silencer, the data presented here illustrate only that the binding sites for these factors are required. Although the intermediate *in vivo* effects of the *hmr e-IIB* allele correlate well with the intermediate level of GRFI binding *in vitro*, there is still a possibility that these proteins, defined by their activity *in vitro*, are irrelevant to silencer function *in vivo*. Conceivably, other proteins may bind these sites *in vivo* and occlude the binding of ABFI and GRFI. Since ABFI and GRFI are fairly abundant in extracts, their binding properties may obscure the binding of less abundant or weaker binding factors. Although the genetic data would require that the binding of both the identified and the hypothetical factors are affected by the same site mutations, there is precedent for two proteins recognizing the same sequence in both prokaryotes (λ cI and cro proteins; Ptashne *et al.*, 1980) and eukaryotes (yeast HAP1 and RC2 proteins; Pfeifer *et al.*, 1987; also mammalian transcription factors Sp1 and AP-2; Gidoni *et al.*, 1984; Mitchell *et al.*, 1987). Definition of the *in vivo* roles of GRFI and ABFI will require suppressor mutations in the genes for these factors that restore silencer function in strains containing site mutations in the silencer. Alternatively, an *in vitro* assay for silencer function would allow the roles of these factors to be evaluated.

Even if ABFI and GRFI have the *in vivo* roles implied by the phenotypes associated with mutations in their binding sites, the analysis presented here is likely to be an oversimplification of the functional organization of silencers in general. This point is best illustrated by comparing the structure of the *HML E* silencer with the *HMR E* silencer. The residual repression provided by the *hmr e-IIA* allele, and the much stronger phenotype caused by the double-mutant allele, *hmr e-IA,IIA*, strongly implies that ABFI plays an important role in silencer function. However, the *HML E* silencer contains an *ARS* consensus sequence and a GRFI-binding site, but lacks an ABFI-binding site, and yet is still a fully functional silencer. How can ABFI be important for *HMR E* silencer function, yet be missing entirely from *HML E*? Part of the answer appears to be that additional accessory factors influence GRFI function at one site more than at the other site. For example, *ard1* mutations (Whiteway and Szostak, 1985) result in strong derepression of *HML* but only slight derepression of *HMR* (Whiteway *et al.*, 1987). Recent experiments indicate that *ARD1* regulates the silent mating-type loci through the GRFI-binding site (unpublished observations). Therefore, it appears that *ARD1* may encode a protein that facilitates GRFI function at *HML E* and thus compensates for the lack of an ABFI-binding site. In this regard, *ARD1* and *SIR* perform analogous functions in that both influence the properties of GRFI when bound to the silencers. However, the mechanisms are likely to be quite different. Although neither the *HMR I* nor the *HML I* silencers contain a GRFI-binding site (Buchman *et al.*, 1988), both contribute to transcriptional repression (Abraham *et al.*, 1984; Feldman *et al.*, 1984). Both patch-mating tests (Figure 4) and quantitative mating experiments (data not shown) indicated a synergistic interaction between the mutant silencer *hmr e-IIA* and the *sir1::HIS3* mutation. Therefore *SIR1* at least can act independently of GRFI. Presumably in the case of the I sites, the ABFI-binding site is required for the *SIR*-mediated repression properties. In this regard *ARD1* and *SIR* are different in that *ARD1* does not influence the properties of silencers that contain only an ABFI-binding site (unpublished results). Perhaps GRFI is influenced by yet other regulators when bound to non-silencer-related binding sites such as the UAS element of *RP39A*. There is evidence that a single *trans*-acting factor may have different roles in the regulation of functionally diverse genes in yeast and in other organisms (Arndt *et al.*, 1987; Imagawa *et al.*, 1987).

Materials and methods

Strains, media and genetic methods

Yeast rich media (YPD) and yeast minimal media (YM) containing 2% glucose were prepared as described by Barnes *et al.* (1984). Amino acid and base supplements were added at 30 μg/ml as needed. Yeast sphero-

plasts were prepared with lyticase (provided by R.Schekman's laboratory) and transformed by a slight modification of the procedure of Beggs (1978).

Saccharomyces cerevisiae strains used in this study are described in Table V. The strain JRY1707 was constructed by one-step gene replacement (Rothstein, 1983) as follows. YWK18 was transformed with *Bgl*II-cleaved pΔ*E::LYS2* (described below) and Lys$^+$ transformants were selected. Candidates for replacement of *HMR E* by *hmr*Δ*e::LYS2* were confirmed by genomic DNA blot-hybridization analysis. Yeast strains carrying mutant alleles of *HMR E* were constructed by gene conversion from plasmids in the following way. JRY1707 was transformed with one of a series of plasmids that contained an altered *HMRa* fragment (Figure 3) in the *URA3*-containing vector pSEY8 (Emr *et al.*, 1986). Transformants were selected in the absence of uracil and grown to stationary phase in supplemented YM broth containing 2% glucose, histidine, tryptophan and lysine. Approximately 10^7 cells were plated on solid minimal media containing 0.2% α-amino-adipic acid, 24 μg/ml lysine, and other supplements as required in order to select against the function of *LYS2* (Chattoo *et al.*, 1979). The plates were incubated at 30°C for 2–3 days. The resulting Lys$^-$ colonies contained either a replacement of *hmr*Δ*e::LYS2* by the plasmid-encoded allele through gene conversion, or *lys2* mutations. The replacement alleles were identified by genomic DNA blot-hybridization analysis.

Plasmid constructions

All recombinant DNA manipulations were carried out as described by Maniatis *et al.* (1982). Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase and the Klenow fragment of DNA polymerase I were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN) and used as suggested by the supplier. T4 gene-32 protein was from P-L Biochemicals (Milwaukee, WI). DNA restriction fragments used for subcloning and probes were isolated from agarose gels using Geneclean (BIO 101, La Jolla, CA).

The plasmids Δ60 and Δ77-224 are *Xho*I linker insertion–deletion derivatives of *HMRa* (Abraham *et al.*, 1984). The left-most *Eco*RI–*Xho*I fragment of Δ60 and the right-most *Xho*I–*Hin*dIII fragment of Δ77-224 were ligated to *Eco*RI- and *Hin*dIII-cleaved pSEY8 (Emr *et al.*, 1986) creating a plasmid p8ΔE, which carries an 800-bp deletion of the *HMR E* silencer marked by a unique *Xho*I restriction site. p8ΔE was cleaved with *Xho*I and ligated to a 5.6-kb *Sal*I fragment carrying the *LYS2* gene. One orientation of *LYS2* with respect to *HMR* was chosen and called pΔ*E::LYS2*. To reconstruct *HMRa* fragments carrying various mutant silencers, p8ΔE was cleaved with *Xho*I and the 5′-protruding ends were filled in using the Klenow fragment of DNA polymerase I and deoxynucleotide triphosphates. This vector was ligated to various blunt-ended restriction fragments that carried mutated *HMR E* sites. The *HMR E* sequences inserted into the *Xho*I restriction site of p8ΔE corresponded to a 490-bp region between the *Xho*I site of Δ60 and an *Xba*I site flanking *HMRa* (Abraham *et al.*, 1984; Kimmerly and Rine, 1987). In a parallel series of constructions, a 600-bp *Eco*RI–*Hin*dIII fragment carrying *ARS1* was inserted into the *Xho*I site of p8ΔE. One orientation was chosen for analysis and the corresponding allele named *hmr*Δ*e::ARS1.1*. An analogous fragment denoted *ARS1E* was constructed by removing 39 bp between the *Bgl*II and *Pst*I sites at *ARS1* and inserting a 39-base oligonucleotide-derived GRFI-binding site from *HMR E*. The *ARS1E* fragment was inserted into the *Xho*I site of p8ΔE. Both orientaions of *ARS1E* with respect of *HMRa* were chosen and the alleles denoted *hmr*Δ*e::ARS1E.1* and *hmr*Δ*e::ARS1E.2*. Schematic representations of these alleles are shown in Figure 3. A centromere-containing plasmid that carried *ARS1E* as the only replicator exhibited a partial replication defect due to the removal of sequences corresponding to Domain B of *ARS1* (Celniker *et al.*, 1984; Strich *et al.*, 1987), although the mitotic stability of this plasmid did respond to the *SIR* genotype of the cell (data not shown).

Site-specific mutagenesis

Mutagenic oligonucleotides were synthesized using an Applied Biosystems Oligonucleotide Synthesizer. Full-length oligonucleotides were purified from partial-synthesis products by polyacrylamide–urea gel electrophoresis. The 30-mer 5′-CTTTTTTTCAAGGTTGATGTGTTTTTGCAA-3′ contains a C/G-to-A/T transversion at nucleotide 325 of *HMR E* (numbering after Abraham *et al.*, 1984). The 31-mer 5′-TTCAAGGTTGATGTGGGTTTG-TGCAATATAA-3′ contains an A/T-to-C/G transversion at nucleotide 330 of *HMR E*. Both mutations created by these oligonucleotides are within the binding site for GRFI at *HMR E* (Buchman *et al.*, 1988) and were named *hmr e-IIA* and *hmr e-IIB* respectively. The 29-mer 5′-AGCTTCGTTCGT-ATTTTTATGATGTATTG-3′ contains an insertion of an additional A/T base pair in the central variable core of the ABFI-binding site at *HMR E* (nucleotides 267–270; numbering after Abraham *et al.*, 1984). The mutation created by this oligonucleotide was named *hmr e-IA*. The plasmid pJR315 (Kimmerly and Rine, 1987) was used as the template for construction of all single-site mutants. Single-stranded template DNA was prepared by the deoxyuridine-incorporation protocol of Kunkel (1985) using super-infection by wild-type f1 phage. Approximately 20 ng of phosphorylated primer were annealed with 200 ng of deoxyuridine-labeled template at 65°C for 15 min in 10 mM Tris–HCl, 50 mM NaCl, 20 mM $MgCl_2$, 1 mM DTT, pH 7.5. Second-strand synthesis reactions included, in addition to the above components, 0.4 mM deoxynucleotide triphosphates, 0.5 mM ATP, 1 μg T4 gene-32 protein, 2 units Klenow fragment, and 0.5 units T4 DNA ligase, and were performed at room temperature for 3 h in a final volume of 10 μl. Single-stranded templates were prepared by published procedures (Zoller and Smith, 1983). The desired mutations were identified by DNA sequencing (Sanger *et al.*, 1977) using [α-^{35}S]dCTP (Amersham, Arlington, IL). The double-mutant silencer was constructed using pJR315-IA (which contained the *hmr e-IA* allele) as template and the 30-base oligonucleotide that encoded the appropriate GRFI-site mutation as the mutagenic primer. All four mutant silencers were inserted into YIp5 to assess the replication and segregation phenotypes of the mutants. Mitotic stability and plasmid copy number were determined as previously described (Kimmerly and Rine, 1987). Where indicated, 1-butylcarbamoyl-2-benzimidazolecarbamate methyl ester (benomyl; Du Pont, Wilmington, DE) was included in the medium during non-selective growth. Benomyl was diluted from a 20 mg/ml stock solution in DMSO (Aldrich Chemicals, Milwaukee, WI).

DNA mobility-shift electrophoresis

Whole-cell protein extracts used in mobility-shift assays were prepared from 40 ml YPD-grown cells (A_{600} = 2–3). Cells were collected by centrifugation and washed once with sterile water. Cells were suspended in 0.3 ml Buffer A containing 0.3 M $(NH_4)_2SO_4$, 1 mM PMSF, 0.6 μM leupeptin and 2 μM pepstatin (Buffer A contained 25 mM Hepes, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, 10% glycerol, pH 7.5) and transferred to a 0.5-ml microcentrifuge tube containing 0.5 g of acid-washed glass beads. The samples were vortexed at high speed at 4°C for three intervals of 5 min. After each interval of vortexing, the tubes were incubated on ice for 5 min. The extracts were finally centrifuged at 4°C for 10 min in a microcentrifuge at 12 000 *g*. Whole-cell extracts prepared in this manner were stable for several months at −70°C. Multiple freeze–thaw cycles caused no apparent loss of either ABFI or GRFI binding activities.

For probe preparation, a plasmid carrying the wild-type or mutant *HMR E* site was cleaved with *Bam*HI and *Hin*dIII. A 490-bp fragment was isolated and end-labeled with [α-^{32}P]dCTP (800 Ci/mmol; Amersham).

DNA-binding reactions (20 μl) were performed at room temperature in Buffer A containing 10 000 c.p.m. probe (~4 fmol), 0.5 μg poly d(A-T) (Sigma, St Louis, MO) and 3–5 μg protein supplied by the extract. Where indicated in the figure legends, double-stranded oligonucleotide competitors that encoded either the ABFI- or the GRFI-binding sites were also included at 100 ng/reaction. The DNA-binding reactions were loaded directly onto 4% polyacrylamide gels cast in 0.5 × TBE (45 mM Tris-borate, 1 mM EDTA, pH 8.3) and subjected to electrophoresis at 200 V for 3–4 h at room temperature. The gels were pre-run at 200 V for 1 h before loading the samples. The gels were dried under vacuum at 80°C and exposed to Kodak XAR-5 film overnight at −70°C with an intensifying screen.

Assay of β-galactosidase activity

Strains carrying various reconstructed alleles of *HMR E* were transformed with the plasmid p*HO*-c12-*lacZ* (provided by R.Jensen, University of California, San Diego). This plasmid carries a fusion of the *Escherichia coli lacZ* gene to the 12th codon of the yeast *HO* gene in the vector YCp50 (Russell *et al.*, 1986). Transformants were grown overnight in appropriately supplemented YM broth at 30°C. These saturated cultures were diluted 1:20 into fresh medium and were grown for an additional 6 h at 30°C. The cells were permeabilized and assayed as described (Hagen and Sprague, 1984). In all cases, the plasmid was present in >90% of cells in the population.

Quantitative mating determination

Strains to be assayed were grown to stationary phase in YPD broth at 30°C, then diluted 1:1000 in the same medium and grown for an additional 12 h. Approximately 10^7 cells of the strain to be tested were mixed with an excess of strain JRY676 (*MATa*) in a final volume of 1 ml of YPD broth. The mating mixtures were incubated at room temperature for 6 h without agitation. The mixtures were then vortexed vigorously and appropriate dilutions were plated on solid YM medium containing uracil. Matings were detected by prototroph selection due to complementation in the diploid of auxotrophic markers present in each haploid parent.

Measurement of a1 mRNA by S1 nuclease protection

Total yeast RNA was prepared by the method of Nasmyth (1983). Single-stranded DNA templates used for preparation of labeled probes specific for the a1 and *SIR3* mRNAs were provided by A.Brand (Harvard University, Cambridge, MA). Probe preparation and S1 nuclease-protection analysis

were performed as previously described (Brand *et al.*, 1985). Hybridizations with 20 μg of total RNA included 20 000 c.p.m. of each probe. Annealing was performed at 68°C for 16 h.

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