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Replication and Segregation of Plasmids Containing *cis*-Acting Regulatory Sites of Silent Mating-Type Genes in *Saccharomyces cerevisiae* Are Controlled by the *SIR* Genes

WILLIAM J. KIMMERLY AND JASPER RINE*

Biochemistry Department, University of California, Berkeley, California 94720

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In *Saccharomyces cerevisiae*, two *cis*-acting regulatory sites called *E* and *I* flank the silent mating-type gene, *HMR*a, and mediate *SIR*-dependent transcriptional repression of the a*1*-a*2* promoters. It has been shown previously that the *E* and *I* sites have plasmid replicator (*ARS*) activity. We show in this report that the *ARS* activity of the *E* and *I* sites is governed by the *SIR* genotype of the cell. In wild-type cells, a plasmid carrying the *E* site from *HMR*a (*HMR E*) in the vector YIp5 exhibited very high mitotic stability at a copy number of approximately 25 per cell. However, in *sir2*, *sir3*, or *sir4* mutants, plasmids with *HMR E* had the low mitotic stability characteristic of plasmids containing *ARS1*, a *SIR*-independent replicator. Elevated mitotic stability of plasmids that carry *HMR E* is due to a segregation mechanism provided by *SIR* and *HMR E*. In *sir2* and *sir4* mutants, the plasmid copy number was significantly lowered, suggesting that these gene products also participate in the replication of plasmids carrying *HMR E*. The phenotype of point mutations introduced at an 11-base-pair *ARS* consensus sequence present at *HMR E* indicated that this sequence is functional but not absolutely required for autonomous replication of the plasmid and that it is not required for *SIR*-dependent mitotic stabilization. A plasmid carrying both a centromere and *HMR E* exhibited reduced mitotic stability in wild-type cells. This destabilization appeared to be due to antagonism between the segregation functions provided by the centromere and by *HMR E*.

The genes *HML* and *HMR* encode cryptic copies of the *MAT* genes. *HML*, located on the left arm of chromosome III, encodes an unexpressed copy of the *MAT*α genes (*HML*α). *HMR*, located distal to *MAT* on the right arm of chromosome III, contains an unexpressed copy of the *MAT*a genes (3, 23, 44, 59). These duplicated genes serve as donor templates in *HO*-mediated mating-type interconversion (reviewed in reference 24). The expression of the genes at *HML* and *HMR* is repressed by the products of *SIR1*, *SIR2* (*MAR1*), *SIR3* (*CMT*), and *SIR4* (21, 33, 35, 47, 48). Both *HML* and *HMR* are flanked by *cis*-acting sites, called *E* and *I*, that are also required for transcriptional repression. Deletion and insertion analyses indicate that the *E* site is contained within a 220-base-pair (bp) region and the *I* site within an 85-bp region at *HMR* (1, 19). Presumably, *E* and *I* are the sites through which the *SIR* proteins mediate transcriptional repression of *HML* and *HMR*. Indeed, DNA sequence analysis of the *E* and *I* elements has revealed several short conserved blocks of homology, some of which may be sites of *SIR* action (19).

In addition to their role in *SIR*-mediated transcriptional repression, the *E* and *I* elements allow plasmids to replicate autonomously in yeasts (1, 10, 19). The ability to promote autonomous replication has been observed for other yeast DNA fragments that are referred to as *ARS* (autonomous replication sequences). *ARS* elements may represent chromosomal origins of replication. The *E* and *I* sites each contain a perfect 11-bp match to an *ARS* consensus sequence (10). Estimates of the frequency of *ARS* elements in the yeast genome indicate that an *ARS* element is found on average every 40 kilobases (kb) (5, 14). Therefore, the coincidence of *ARS* elements with the *E* and *I* regions flanking both *HML* and *HMR* is unlikely to have occurred by chance and may represent a mechanistic link between the processes of DNA replication and transcriptional repression. In this regard, the onset of *SIR*-mediated repression at *HMR*a requires transit through the S phase of the cell cycle (42). It is not known whether the requirement for passage through the S phase is for DNA replication per se, for some other event that occurs in the S phase, or for some subsequent cell cycle event dependent on DNA replication.

To understand further the relationship between *SIR*-mediated repression and DNA replication, we characterized the *ARS* activity of the *E* and *I* sites which flank *HMR*. These studies were motivated by the observation that the mitotic stability of plasmids carrying *HMR*a is lowered in *sir1* mutants (J. Abraham, personal communication). We show that the *HMR E* site behaves as a conditional *ARS* and provides a segregation mechanism to plasmids in yeasts.

MATERIALS AND METHODS

Strains, media, and genetic methods. Yeast rich medium (YPD) and yeast minimal medium (YM) were prepared as described previously (4). Amino acid and base supplements were added at 30 μg/ml as needed. *Saccharomyces cerevisiae* strains are described in Table 1. Schematic representations of the insertional alleles of the *SIR* genes are shown in Fig. 1. Strains YWK178, YWK179, and YRS477 were constructed by the method of one-step gene replacement (49). The parent strain for these constructions was DBY703. Strain YWK18 is a *lys2* mutant isolated as a spontaneous α-aminoadipic acid-resistant derivative of DBY703 (15). Strain YWK55 (also isogenic with DBY703) was constructed as follows. Strain YWK18 was transformed with the plasmid pJR317, which carries the *sir3*::*LYS2* disruption allele in the vector YEp24 (see below for details of plasmid construction). YWK18 cells containing pJR317 were grown in YM medium lacking lysine. Cells were plated on solid YM

* Corresponding author.

TABLE 1. Strains used in this study

Strain	Genotype	Source
DBY703	*MATα trp1 his3 ura3-52* cir^0	D. Botstein
YWK18	*MATα trp1 his3 ura3-52 lys2-6* cir^0	This work
YWK178	*MATα trp1 his3 ura3-52 sir1::HIS3* cir^0	This work
YWK179	*MATα trp1 his3 ura3-52 sir2::HIS3* cir^0	This work
YWK55	*MATα trp1 his3 ura3-52 lys2-6 sir3::LYS2* cir^0	This work
YRS477	*MATα trp1 his3 ura3-52 sir4::HIS3* cir^0	R. Schnell
676 × 703	*MAT***a***/MATα his4-519/+ leu2-3,112/+ ura3-52/ura3-52 +/trp1 +/his3*	This work
227	*MAT***a** *lys1*	54
JRY184	*mat*a*1 HML***a** *HMR***a** *sir4-351 ade ura3 leu1 rme*	This work

medium containing 5-fluoro-orotic acid (8), histidine, tryptophan, and uracil and incubated at 30°C for 2 to 3 days. The resulting Lys$^+$ Ura$^-$ colonies were screened for their ability to mate with a tester lawn of strain 227. Nonmating isolates were candidates for gene replacement of *SIR3* with *sir3*::*LYS2*. Gene replacements were confirmed by genomic DNA blot hybridization analysis (51). Yeast DNA was prepared as previously described (27). Yeast spheroplasts were prepared with lyticase (a gift from the laboratory of R. Schekman) and were transformed by a slight modification of the method of Beggs (6). Diploids formed by mating *MAT***a** and *MATα* haploids were selected by complementation of parental auxotrophic markers. Plasmid DNA was prepared in *Escherichia coli* DH1 (22).

Plasmid constructions. All recombinant DNA manipulations were done by the method of Maniatis et al. (40). Restriction endonucleases, T4 DNA ligase, and Klenow fragment of *E. coli* DNA polymerase I were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). T4 polynucleotide kinase was purchased from P-L Biochemicals, Inc. (Milwaukee, Wis.). DNA restriction fragments for subcloning were isolated from Seaplaque agarose (FMC Corp., Philadelphia, Pa.) as suggested by the manufacturer.

The *SIR3*-containing plasmid pJR104 was isolated from a YEp24-based yeast genomic library by complementation of the *sir3-8* mutation and was shown to contain the authentic *SIR3* gene by integration at the *SIR3* locus (J. Rine, unpublished data). The restriction endonuclease map of the insert DNA correlates well with published maps of the *SIR3* gene (28, 52). This plasmid was cleaved with *Xho*I and ligated with a 5.6-kb *Sal*I fragment containing the *LYS2* gene. One orientation of *LYS2* with respect to *SIR3*, called pJR317, was chosen (Fig. 1).

The plasmid Δ60 (provided by J. Abraham) carries an *Xho*I linker adjacent to *HMR E* (1). This plasmid was cleaved with *Xho*I and *Xba*I to liberate a 490-bp restriction fragment. This fragment is operationally defined as the *E* site. The resulting 5′ protruding ends were filled in with Klenow fragment and deoxynucleotide triphosphates and ligated to *Hin*cII-cleaved pEMBL18 (constructed by H. Roiha by replacing the polylinker of pEMBL8 with that of pUC18 [16]). The orientation in which the former *Xba*I site is closest to the universal priming site was called pJR315 and chosen for further manipulations. This plasmid was cleaved with *Bam*HI and *Hin*dIII, and the insert carrying *HMR E* was isolated and ligated to *Bam*HI- and *Hin*dIII-cleaved YIp5 (58), creating the plasmid YRp315. The plasmids YRp315A and YRp315G were constructed in an analogous fashion with mutant derivatives of *HMR E* constructed in pJR315 (see below). The plasmid pUC7-*CEN6* (provided by P. Philippsen) was cleaved with *Eco*RI and *Hin*dIII, and the 1.6-kb restriction fragment carrying *CEN6* (46) was isolated. This fragment was ligated to YRp315 cleaved with both *Eco*RI and *Hin*dIII, creating the plasmid YCp6.315. The plasmids YRp320 and YRp321 were constructed by inserting a 1.45-kb *Eco*RI restriction fragment carrying *ARS1/TRP1* in both orientations (56) into the *Eco*RI site of YRp315.

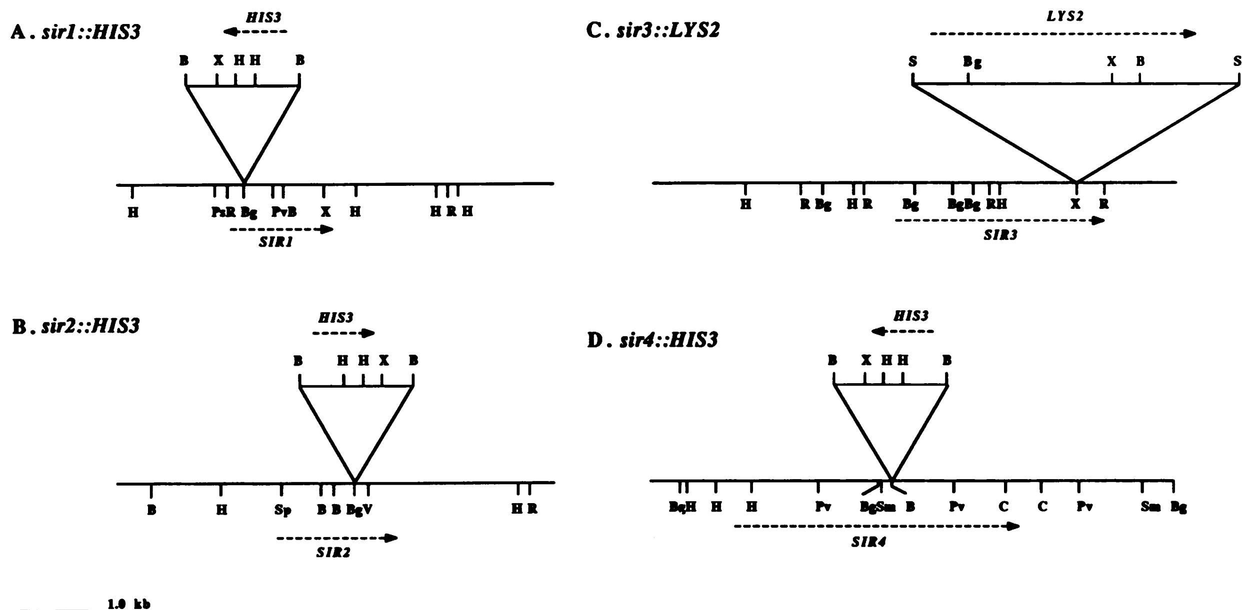

FIG. 1. Schematic representation of insertional null *SIR* alleles. The transplacement of mutant alleles was performed as described in Materials and Methods. The direction of transcription and approximate endpoints of mRNA transcripts are indicated by arrows (30, 57; R. Schnell, unpublished data). Restriction endonuclease cleavage sites: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; H, *Hin*dIII; Ps, *Pst*I; Pv, *Pvu*II; R, *Eco*RI; S, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; V, *Eco*RV; X, *Xho*I.

A 650-bp *Cla*I-*Xho*I fragment containing *HML E* was isolated from the 6.5-kb *Hin*dIII restriction fragment containing *HML*α (44). This fragment was ligated with YIp5 cleaved with *Cla*I and *Sal*I, creating the plasmid YRp415.

The plasmid Δ59 (obtained from J. Abraham; see reference 1) was cleaved with *Xho*I and *Hin*dIII. A 600-bp restriction fragment carrying *HMR I* was ligated with YIp5 cleaved with *Sal*I and *Hin*dIII, creating the plasmid YRp98.

The plasmid pRS62 (constructed by R. Schnell) carries a deletion of all *HMR***a** sequences between *E* and *I*. An *Xho*I restriction site separates the *E* site from the *I* site. This plasmid was constructed by ligating the 2.25-kb *Hin*dIII-*Xho*I fragment containing *HMR E* from the plasmid Δ224 (1) with the 600-bp *Hin*dIII-*Xho*I fragment containing *HMR I* from the plasmid Δ59. The resulting 2.85-kb fragment was ligated with *Hin*dIII-cleaved pSEY8 (18). pRS62 was cleaved with *Eco*RI and *Hin*dIII, and a 2.05-kb fragment carrying both *HMR E* and *HMR I* was isolated. This fragment was inserted into YIp5 cleaved with *Eco*RI and *Hin*dIII, creating the plasmid YRp99.

Oligonucleotide-directed mutagenesis. The 20-mer oligonucleotide 5'-CATTTTTTATATT(A,C,G)AGGTAT-3' was synthesized on an Applied Biosystems oligonucleotide synthesizer. This sequence, which is complementary to nucleotides 344 to 363 of *HMR E* (numbering after Abraham et al. [1]) contains the *ARS* consensus sequence (10) except at nucleotide 357 where mixed synthesis added A, G, or C in place of T. This degenerate position corresponds to the 10th base pair of the *ARS* consensus sequence. The full-length oligonucleotide was purified by thin-layer chromatography (2). Single-stranded pJR315 was prepared by superinfecting *E. coli* DIH101 (constructed by D. Ish-Horowicz; provided by S. Mount) carrying pJR315 with f1 bacteriophage as described previously (64) and used as the template for oligonucleotide-directed mutagenesis. Mutagenesis and screening by oligonucleotide hybridization were performed with slight modifications of published procedures (64). Potential mutations were characterized further by DNA sequencing (50). This procedure identified the plasmids pJR315A and pJR315G, which have T-to-A and T-to-G transversions, respectively, at nucleotide 357 of *HMR E*. These mutations will be referred to as *hmr***a** *e-357A* and *hmr***a** *e-357G*, respectively.

Plasmid mitotic stability and copy number determinations. Yeast cells transformed with the plasmid of interest were grown at 30°C in supplemented YM medium lacking uracil to an A_{600} of 2 to 3. Cells were diluted in sterile water, plated onto solid YPD medium, and allowed to form colonies at 30°C. Replicas were subsequently printed onto supplemented YM plates with uracil and onto plates lacking uracil. The mitotic stability of a plasmid is defined as the percentage of cells in a population that carry the plasmid as measured by the ratio of Ura^+ colonies to the total number of colonies. Four to six transformants were measured for each determination. Selectively grown cells were diluted 1000-fold into YPD broth and grown for 10 to 12 generations at 30°C, and the mitotic stability of the plasmid was determined as described above. When indicated, cells were grown nonselectively in the presence of methylbenzimidazole-2-yl-carbamate (MBC) at various concentrations in YPD at 30°C. MBC (Du Pont Co., Wilmington, Del.) was diluted from a 20-mg/ml stock solution in dimethyl sulfoxide and stored at 4°C. The viability of cells grown in the presence of 160 μg of MBC per ml was greater than 98%.

Plasmid copy number was determined by genomic DNA blot hybridization analysis (51). Total DNA from transformants was isolated as described previously (27) from 10-ml cultures grown at 30°C in YM broth containing 5% YPD broth, 0.5% Casamino Acids (Difco Laboratories, Detroit, Mich.), and 30 μg each of histidine and tryptophan per ml. These preparations were digested with *Hin*dIII, separated on a 1.0% agarose gel, and transferred to nitrocellulose filter paper. A 550-bp *Acc*I fragment from *URA3* was used for probe preparation. Probes of high specific activity (5×10^8 to 8×10^8 cpm/μg) were prepared with [α-^{32}P]dCTP (800 Ci/mmol) and the Multiprime DNA-labeling system (Amersham Corp., Arlington Heights, Ill.) used as recommended by the supplier. Plasmid copy number was estimated from densitometric scans of several autoradiographic exposures of the blots with Kodak XAR-5 film. The copy number was calculated as the ratio of hybridization of the probe to the plasmid relative to the chromosomal *ura3-52* restriction fragment. Alternatively, plasmid copy number was measured by excision of regions from the nitrocellulose filter that hybridized to the probe corresponding to either chromosomal or plasmid restriction fragments. The radioactivity that hybridized to the filters was quantitated by scintillation counting. After background radioactivity was subtracted, the plasmid copy number was estimated from the ratio of plasmid-to-chromosomal hybridization.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunological detection methods. Yeast protein extracts were prepared by glass bead disruption of spheroplasts at 4°C in 50 mM Tris hydrochloride (pH 7.5)–4% sodium dodecyl sulfate–10% glycerol–10 mM EDTA–1% β-mercaptoethanol–1 mM phenylmethylsulfonyl fluoride. Spheroplasts were suspended in 0.3 ml of buffer, transferred to a 0.5-ml polypropylene microcentrifuge tube containing 0.5 g of glass beads, and vortexed at high speed for 5 min at 4°C. After boiling for 5 min, extracts were centrifuged at 4°C for 10 min in a microcentrifuge at 12,000 × *g*. Equal amounts of protein from various samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (37). After electrophoresis, proteins were transferred to nitrocellulose filter paper (BA 85S597; Schleicher & Schuell, Inc., Keene, N.H.) by the procedure of Burnette (11). All subsequent manipulations were done in 50 mM Tris hydrochloride (pH 7.5)–150 mM NaCl–1% Nonidet P-40 (Sigma Chemical Co., St. Louis, Mo.)–0.02% NaN_3–1% nonfat dry milk (30). Rabbit polyclonal antiserum raised against yeast orotidine-5'-monophosphate (OMP) decarboxylase was provided by A. Buchman (Stanford University). ^{125}I-labeled *Staphylococcus aureus* protein A was a gift from the laboratory of R. Schekman.

RESULTS

Mitotic stability of plasmids carrying *HMR E*. The *E* and *I* sites from both *HML* and *HMR* act as *ARS* elements (1, 10, 19). To explore the properties of one of these sites in greater detail, we inserted a 490-bp *Xho*I-*Xba*I restriction fragment carrying *HMR E* into the plasmid YIp5 (58), creating the plasmid YRp315. This plasmid carries neither coding information from *HMR***a** nor the **a***1*-**a***2* promoter region. The wild-type strain DBY703 and all isogenic *sir* mutants tested yielded 5,000 to 10,000 Ura^+ transformants per μg of DNA when transformed with this plasmid. This high transformation frequency is characteristic of autonomously replicating plasmids, confirming that this *E*-site fragment acts as an *ARS*. Control experiments with YIp5 lacking the *E* site routinely gave 0 to 5 transformants per μg of DNA, a frequency characteristic of plasmid integration into the chro-

TABLE 2. Mitotic stability of plasmids in wild-type and *sir* mutant strains

Strain	*SIR* genotype	Plasmid	*ARS*	Mitotic stability	
				Selective[a]	Nonselective[a]
DBY703	*SIR*	YRp315	*HMR E*	89.4 ± 5.4	70.9 ± 4.3
YWK178	*sir1::HIS3*	YRp315	*HMR E*	55.7 ± 7.9	32.5 ± 8.8
YWK179	*sir2::HIS3*	YRp315	*HMR E*	12.5 ± 3.7	0.33 ± 0.31
YWK55	*sir3::LYS2*	YRp315	*HMR E*	15.8 ± 1.3	0.53 ± 0.20
YRS477	*sir4::HIS3*	YRp315	*HMR E*	5.4 ± 2.5	<0.06[b]
703 × 676	*SIR/SIR*	YRp315	*HMR E*	72.8 ± 2.0	57.0 ± 8.3
JRY184	*sir4-351*	YRp315	*HMR E*	12.8 ± 0.8	ND[c]
DBY703	*SIR*	YRp415	*HML E*	47.5 ± 6.0	17.1 ± 4.0
YWK55	*sir3::LYS2*	YRp415	*HML E*	6.6 ± 0.6	0.43 ± 0.33
YRS477	*sir4::HIS3*	YRp415	*HML E*	6.1 ± 1.3	0.32 ± 0.24
DBY703	*SIR*	YRp98	*HMR I*	18.0 ± 1.1	1.8 ± 0.2
YWK55	*sir3::LYS2*	YRp98	*HMR I*	1.7 ± 0.5	0.03[d]
YRS477	*sir4::HIS3*	YRp98	*HMR I*	2.2 ± 0.6	0.54 ± 0.36
DBY703	*SIR*	YRp17	*ARS1*	14.2 ± 1.7	1.4 ± 0.7
YWK178	*sir1::HIS3*	YRp17	*ARS1*	8.4 ± 3.8	0.55 ± 0.41
YWK179	*sir2::HIS3*	YRp17	*ARS1*	3.4 ± 0.9	0.08[d]
YWK55	*sir3::LYS2*	YRp17	*ARS1*	14.8 ± 7.7	0.43 ± 0.14
YRS477	*sir4::HIS3*	YRp17	*ARS1*	9.5 ± 0.7	0.20 ± 0.20
703 × 676	*SIR/SIR*	YRp17	*ARS1*	15.8 ± 2.1	2.4 ± 1.5

[a] In this and subsequent tables, mitotic stability is presented as the percentage of cells in the population that exhibit the plasmid-borne *URA3* marker. The standard error among four to six independent transformants is reported as a percentage.
[b] No cells carrying the plasmid were found of 1,781 screened.
[c] The mitotic stability after nonselective growth was not determined.
[d] The standard error was not determined.

mosome. However, transformation frequency is only a qualitative measure of *ARS* activity and does not allow accurate comparison between different *ARS* elements or among strains of different genotypes (13, 25, 36; this work). As a more sensitive index of *ARS* function, the mitotic stability of YRp315 was measured in each strain. Mitotic stability is defined as the percentage of cells that carry a plasmid in a population of transformed cells. The mitotic stability of YRp315 depended on the *SIR* genotype of the cell (Table 2). In the wild-type strain DBY703, YRp315 was found in approximately 90% of the cells in the population grown under selective conditions. When these cells were grown nonselectively, the plasmid was stably maintained in the population. In contrast, the same plasmid in an isogenic *sir2::HIS3* strain (YWK179), *sir3::LYS2* strain (YWK55), or *sir4::HIS3* strain (YRS477) exhibited a significantly lowered mitotic stability under all growth conditions. The plasmid was found in approximately 5 to 15% of cells in these populations when grown selectively and was lost rapidly during nonselective growth (Table 2). The plasmid YRp315 exhibited intermediate mitotic stability in an isogenic *sir1::HIS3* strain (YWK178) under all growth conditions. This intermediate value correlates well with the leaky transcriptional repression of the silent mating-type loci in *sir1* mutants (28).

One phenotypic difference between the wild-type parent strain and *sir* mutants is that the mutant exhibits the nonmating phenotype of an **a**/α diploid. As a control for general effects of mating type on plasmid stability, this analysis was repeated in an **a**/α diploid strain. YRp315 exhibited high mitotic stability during both selective and nonselective growth in the *SIR/SIR* diploid strain 703 × 676 (Table 2). Thus, simultaneous expression of both *MAT***a** and *MAT*α mating-type information is not a sufficient explanation for the destabilization of plasmids carrying *HMR E* in *sir* mutants. To determine whether the simultaneous expression of both *MAT***a** and *MAT*α mating-type information is necessary for the destabilization of plasmids carrying *HMR E* in *sir* mutants, we analyzed a strain containing only *MAT***a** mating-type information and the *sir4-351* mutation (JRY184). YRp315 exhibited the same low mitotic stability in this strain as in the other *sir* mutants (Table 2). Taken together, these results indicated that expression of both *MAT***a** and *MAT*α mating-type information was neither necessary nor sufficient to explain the mitotic instability of *HMR E*-containing plasmids in *sir* mutants.

The plasmid YRp17 is replicated by the *ARS1* element (55). The data in Table 2 show that the mitotic stability of YRp17 was not severely affected by the *SIR* genotype of the cell. In all cases, the plasmid was found in about 4 to 15% of cells under selective conditions. The low mitotic stability of this plasmid is due to a defect in its segregation between mother and daughter cells at mitosis, and not its replication (43). The slight, yet statistically significant effects of *sir2* and *sir4* mutations on the mitotic stability of YRp17 were unanticipated and remain unexplained. In summary, as a plasmid replicator, *HMR E* provides higher mitotic stability to plasmids than *ARS1* in wild-type cells. In *sir* mutants, the *ARS* activity of the *HMR E* site is similar to that of *ARS1*.

***SIR*-dependent mitotic stability of plasmids carrying *HML E* and *HMR I*.** To determine whether *SIR*-dependent plasmid stability is a general feature of regulatory regions involved in *SIR*-mediated repression, or whether only *HMR E* displays this property, we characterized the replicator properties of two other sites involved in *SIR*-dependent transcriptional repression. Like the *HMR* locus, *HML* is also flanked by *E* and *I* sites which mediate *SIR*-dependent transcriptional repression. These sites also contain functional *ARS* elements. A 650-bp *Cla*I-*Xho*I restriction fragment containing *HML E* was inserted into YIp5, creating the plasmid

TABLE 3. Mitotic stability of mutant *HMR E*-containing plasmids in wild-type and *sir* mutant strains

Strain	*SIR* genotype	Plasmid[a]	*ARS*	Mitotic stability	
				Selective	Nonselective
DBY703	*SIR*	YRp315	*HMR E*	89.4 ± 5.4	70.9 ± 4.3
YWK55	*sir3*::*LYS2*	YRp315	*HMR E*	15.8 ± 1.3	0.53 ± 0.20
YRS477	*sir4*::*HIS3*	YRp315	*HMR E*	5.4 ± 2.5	<0.06[b]
DBY703	*SIR*	YRp315A	*hmr***a** *e-357A*	84.1 ± 5.2	46.0 ± 3.2
YWK55	*sir3*::*LYS2*	YRp315A	*hmr***a** *e-357A*	10.8 ± 0.7	0.37 ± 0.31
YRS477	*sir4*::*HIS3*	YRp315A	*hmr***a** *e-357A*	1.5 ± 1.0	<0.03[c]
DBY703	*SIR*	YRp315G	*hmr***a** *e-357G*	71.3 ± 3.5	37.9 ± 3.8
YWK55	*sir3*::*LYS2*	YRp315G	*hmr***a** *e-357G*	2.8 ± 1.7	<0.02[d]
YRS477	*sir4*::*HIS3*	YRp315G	*hmr***a** *e-357G*	1.3 ± 0.6	0.02[e]

[a] The data for YRp315 are repeated from Table 2 and were obtained concurrently with additional data presented in this table.
[b] No cells carrying the plasmid were found of 1,781 screened.
[c] No cells carrying the plasmid were found of 3,464 screened.
[d] No cells carrying the plasmid were found of 5,414 screened.
[e] The standard error was not determined.

YRp415. The mitotic stability of YRp415 in wild-type and *sir* mutant strains was determined (Table 2). This plasmid was relatively stable and found in 48% of wild-type cells under selective growth conditions. In the isogenic *sir3* and *sir4* mutant strains, the mitotic stability was lowered by a factor of seven to eight. Since plasmids replicated by *HML E* also showed elevated mitotic stability that was *SIR* dependent, this property is not unique to *HMR E*.

HMR I is also required in *cis* for efficient *SIR*-mediated repression of *HMR***a**, although its role seems less critical (1, 9). *HMR I* also has *ARS* activity allowing plasmids to replicate autonomously in yeasts (1). To test whether *SIR* affects the *ARS* properties of *HMR I*, we inserted a 600-bp *Xho*I-*Eco*RI restriction fragment containing *HMR I* into YIp5, creating the plasmid YRp98. The mitotic stability of this plasmid in wild-type and *sir* mutant strains was determined (Table 2). In the wild-type strain DBY703, the plasmid was found in 18% of cells under selective conditions. Hence, *HMR I* was not as strong a plasmid replicator as either *HML E* or *HMR E*. Although *HMR I* was an intrinsically weaker *ARS* element, it was influenced by the *SIR* genotype of the cell since it exhibited an 8- to 11-fold-lower mitotic stability in either the *sir3*::*LYS2* or the *sir4*::*HIS3* mutant derivative. Hence, it appears that *SIR* influenced the replicator functions of both the *E* and *I* regions of *HMR***a**, although *HMR I* was a less efficient *ARS* element than *HMR E*. The efficiency of *E* and *I* as plasmid replicators parallels their relative contribution to *SIR*-dependent repression at *HMR***a** (1).

ARS consensus sequence is not essential for SIR-dependent plasmid stabilization by HMR E. Mutational and DNA sequence analyses of yeast *ARS* elements have identified several domains required for optimal function (10, 13, 31, 54, 57). The 11-bp *ARS* consensus sequence, 5′-$^{A}_{T}$TTTATRT TT$^{A}_{T}$-3′, found at all yeast *ARS* elements so far identified defines one domain (10). Both *ARS1* (13) and an *ARS* element tightly linked to the *HO* gene (31) are inactivated by single-base-pair changes that alter the 10th base pair of this consensus sequence. A perfect match to this consensus sequence is found at *HMR E* (nucleotides 348 to 358; numbering as in reference 1). To examine the importance of the *ARS* consensus sequence at *HMR E* for *SIR*-dependent plasmid stabilization, we altered this sequence by oligonucleotide-directed mutagenesis. The sequence 5′-TTTTATA TTTT-3′ found at *HMR E* was changed at the 10th base pair to either 5′-TTTTATATTAT-3′ or 5′-TTTTATATTGT-3′. The plasmids YRp315A and YRp315G contain these mutant *HMR E ARS* fragments, referred to as *hmr***a** *e-357A* and *hmr***a** *e-357G*, respectively. The mitotic stabilities of plasmids carrying the wild-type *HMR E* or either mutant *HMR E* are shown in Table 3. Neither mutation appeared to affect significantly the mitotic stability in the wild-type strain. Both mutant *E* sites still showed a requirement for *SIR* to achieve elevated mitotic stability. However, the *ARS* consensus sequence mutations behaved differently in the *sir3*::*LYS2* and the *sir4*::*HIS3* strains. In particular, the combination of the *hmr***a** *e-357A* allele with the *sir4*::*HIS3* mutation was striking. The point mutation alone resulted in no significant loss in plasmid stability in the wild-type strain. The *sir4*::*HIS3* allele resulted in a 17-fold decrease in stability of the wild-type plasmid (Table 2). However, the plasmid containing the *hmr***a** *e-357A* allele in the *sir4*::*HIS3* strain exhibited a 56-fold loss in mitotic stability (Table 3). A nearly equivalent synergistic effect was observed with the *hmr***a** *e-357G* allele in the *sir4* strain YRS477. Our interpretation of this observation is that *SIR4* acts at a site other than the *ARS* consensus sequence at *HMR E*. The synergistic effects of *sir4* and the *ARS* consensus sequence mutations established that the *ARS* consensus sequence was part of a functional *ARS* element. The absence of a strong phenotype of the *ARS* consensus sequence mutations in *SIR* cells implied that there was a second functional *ARS* in the *HMR E* fragment. Although the *ARS* consensus sequence chosen for mutagenesis was the only perfect 11-bp match at *HMR E*, computer-aided homology searches identified four 10-of-11-bp matches and thirty-nine 9-of-11-bp matches to the proposed *ARS* consensus sequence, raising the possibility that one or more of these sequences with a close match to the consensus sequence was also functional.

Plasmid stabilization by HMR E and SIR is cis dominant. Models that account for the stable mitotic inheritance of plasmid YRp315, which is replicated by *HMR E*, fall into two classes. First, *E* may be a site that *SIR* acts on to increase the mitotic stability of plasmids carrying this region. This model predicts that *E*-mediated plasmid stabilization would be *cis* dominant to other mitotically unstable *ARS* elements that do not respond to *SIR*. Alternatively, all other *ARS* elements may render plasmids intrinsically unstable owing to interactions between these sites and cellular factors, and *HMR E* would be defective in these interactions. This model predicts that elevated mitotic stabilization of

TABLE 4. Dominance test of *SIR*-dependent mitotic stabilization function of *HMR E*

Strain	*SIR* genotype	Plasmid	*ARS*	Mitotic stability	
				Selective	Nonselective
DBY703	*SIR*	YRp99	*HMR E/I*	89.9 ± 1.9	61.4 ± 7.6
YRS477	*sir4::HIS3*	YRp99	*HMR E/I*	3.5 ± 1.6	<0.02[a]
DBY703	*SIR*	YRp320	*HMR E/ARS1*	84.4 ± 2.4	54.0 ± 5.9
DBY703	*SIR*	YRp321	*HMR E/ARS1*	79.7 ± 3.3	61.3 ± 5.5
YWK55	*sir3::LYS2*	YRp320	*HMR E/ARS1*	5.7 ± 1.9	0.14[b]
YRS477	*sir4::HIS3*	YRp320	*HMR E/ARS1*	9.7 ± 1.4	0.21[b]

[a] No cells carrying the plasmid were found of 5,347 screened.
[b] The standard error was not determined.

plasmids containing *E* would be recessive to other *ARS* elements that do not respond to *SIR*. To distinguish between these two views, we constructed a series of plasmids carrying *HMR E* and another *ARS* element. The plasmids YRp320 and YRp321 carry both *HMR E* and *ARS1* in either orientation with respect to *HMR E*. The mitotic stabilities of these plasmids were determined in both wild-type and *sir* mutant strains (Table 4). The plasmids YRp320 and YRp321 exhibited high mitotic stability in the wild-type strain DBY703. A total of 80 to 84% of cells carried the plasmid during selective growth regardless of the orientation of *ARS1* relative to *HMR E*. The high mitotic stability of these plasmids was again dependent on the *SIR* genotype. This result indicated that *SIR*-dependent plasmid stabilization functions of *HMR E* were dominant to the inherent instability of plasmids containing the *SIR*-independent replicator, *ARS1*. YRp99 carries both *E* and *I ARS* elements from *HMR*a (all intervening coding sequences have been deleted). This plasmid was also extremely stable in wild-type cells (Table 4); 89% of cells carried the plasmid under selective conditions. This high mitotic stability was dramatically reduced in the *sir4::HIS3* strain. Therefore, the *SIR*-dependent plasmid stabilization mechanism provided by *E* was also dominant over the weakly *SIR*-dependent *ARS*, *HMR I*. Furthermore, the effect must be *cis* dominant as a chromosomal *E* site did not stabilize YRp17 (replicated by *ARS1*) in *trans* (Table 2).

Replication of YRp315 at high copy number. To characterize further the behavior of YRp315 in cells of different *SIR* genotypes, we determined the copy number of the plasmid by blot hybridization analysis. Total DNA from six independent transformants was isolated, cleaved with *Hin*dIII, separated by agarose gel electrophoresis, transferred to nitrocellulose filter paper, and hybridized with a radiolabeled probe made from a 550-bp *Acc*I fragment internal to the *URA3* gene. Two bands of hybridization were observed upon autoradiography (Fig. 2A, C to F). A 5.6-kb band corresponded to linearized plasmid DNA, and a 1.9-kb band corresponded to a genomic *ura3-52* restriction fragment. The average relative intensity of the two bands among the transformants analyzed is a measure of the total plasmid copy number. When corrected for the number of plasmid-bearing cells based on the mitotic stability data in Table 2, the relative plasmid copy number is obtained (Table 5). In the wild-type strain, the total copy number in the population was 25 plasmids per cell. When corrected for the mitotic stability of the plasmid, each cell that contained plasmid had a copy number of approximately 28 plasmids per Ura$^+$ cell.

The plasmid copy number was also determined in isogenic *sir* mutant strains containing YRp315. Figures 2C to F depict a similar analysis of six independent transformants of each *sir* mutant. Plasmid copy number results are summarized in Table 5. No *SIR* gene was absolutely required for plasmid replication. However, the most striking observation was that the plasmid copy number as measured in the entire population was significantly reduced in *sir2* and *sir4* mutants. This effect was most severe in *sir4* mutants. Nevertheless, the relative plasmid copy number (plasmids per Ura$^+$ cell) in *sir4* mutants remained comparable to the value observed in wild-type cells since the reduction in the total amount of plasmid DNA in the population was balanced by the decreased mitotic stability. The *sir3* mutant behaved differently. Only a slight drop in total plasmid copy number was observed in the population which, in combination with the low mitotic stability, resulted in a very high plasmid copy number in each plasmid-bearing cell in the *sir3* population (about 90 plasmids per Ura$^+$ cell). The *sir1* mutant had an intermediate effect on total plasmid copy number.

The copy number determination of YRp17 in the wild-type strain is shown in Fig. 2B. The total plasmid copy number was approximately 3 per cell, whereas the relative plasmid copy number was 22 plasmids per Ura$^+$ cell. Both of these properties are similar to YRp315 in a *sir2::HIS3* or *sir4::HIS3* background. This result strengthens the conclusion that neither *SIR2* nor *SIR4* is absolutely required for replication of plasmids containing *HMR E*. In the absence of the functions provided by *SIR2* and *SIR4*, *HMR E* behaved like a normal yeast chromosomal *ARS*.

Expression of plasmid-borne *URA3* gene is not repressed by *HMR E* and *SIR*. Since the high copy number of YRp315 in wild-type cells was unexpected, experiments were performed to determine whether the experimental procedure inadvertently selected for high-copy-number plasmid maintenance. Since *HMR E* can act as a "silencer" and repress the expression of other genes in its vicinity (9, 51), it was possible that *E* and *SIR* were acting to repress the expression of *URA3*, the selectable marker carried by YRp315. If the amount of expression per gene copy of *URA3* was repressed sufficiently, perhaps only cells that accumulated plasmid to a level of about 30 per cell would express enough *URA3* gene product to allow growth under selective conditions. Alternatively, if repression of *URA3* expression did not occur, high-copy-number plasmid maintenance would lead to overexpression of the *URA3* gene product. To distinguish between these possibilities, we measured the level of *URA3* expression from YRp315 in both wild-type and *sir* mutant cells. Antibody to yeast OMP decarboxylase, the product of the *URA3* gene, was used to compare levels of this protein in whole-cell extracts by immunological blotting procedures (Fig. 3). If *E* and *SIR* repressed *URA3* expression, then multicopy YRp315 should result in the accumulation of an amount of enzyme comparable to that obtained with cells transformed with YCp50, a single-copy plasmid bearing the

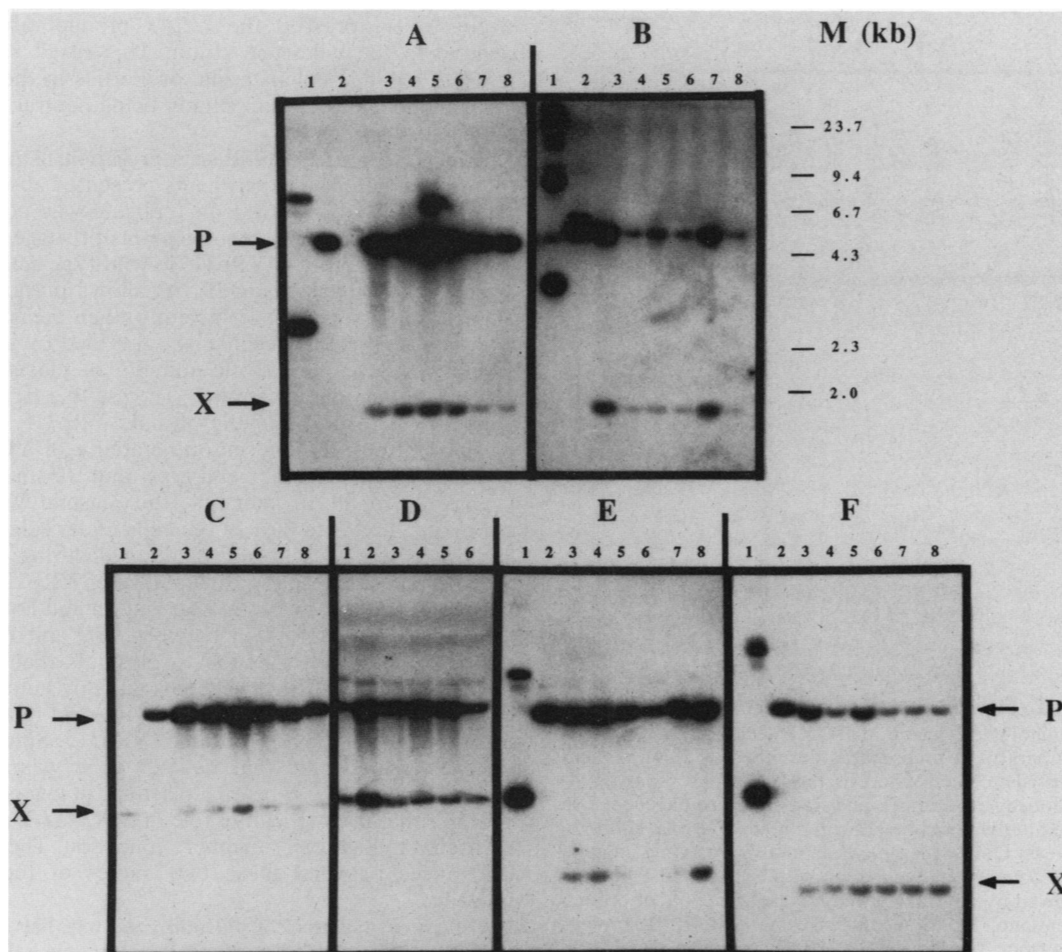

FIG. 2. Plasmid copy number determination by genomic DNA blot hybridization. Genomic DNA samples were cleaved with *Hin*dIII and subjected to electrophoresis through a 1.0% agarose gel, blotted to a nitrocellulose filter, and hybridized with a 550-bp ^{32}P-radiolabeled *Acc*I restriction fragment derived from the *URA3* gene. The copy number was estimated as described in Materials and Methods. The positions of linearized plasmid (P) and the chromosomal *ura3-52 Hin*dIII restriction fragments (X) are indicated in the figure. M, Molecular weight markers. (A) Lane 1, Uncut YRp315; lane 2, YRp315 cleaved with *Hin*dIII; lanes 3 to 8, genomic DNA from independent transformants of DBY703 containing YRp315. (B) Lane 1, Uncut YRp17; lane 2, YRp17 cleaved with *Hin*dIII; lanes 3 to 8, genomic DNA from independent transformants of DBY703 (*SIR*) containing YRp17. (C) Lane 1, Genomic DNA from untransformed DBY703 (*SIR*); lane 2, YRp315 cleaved with *Hin*dIII; lanes 3 to 8, genomic DNA from independent transformants of YWK178 (*sir1*) containing YRp315. (D) Lanes 1 to 6, Genomic DNA from independent transformants of YWK179 (*sir2*) containing YRp315. (E) Lanes 1 and 2, As in panel A; lanes 3 to 8, genomic DNA from six independent transformants of YWK55 (*sir3*) containing YRp315. (F) Lanes 1 and 2, As in panel A; lanes 3 to 8, genomic DNA from six independent transformants of YRS477 (*sir4*) containing YRp315.

TABLE 5. Plasmid copy number in wild-type and *sir* mutant strains

Strain	*SIR* genotype	Plasmid	Copy no.	
			Total[a]	Relative[b]
DBY703	*SIR*	YRp315	25	28
DBY703	*SIR*	YRp17	3	22
YWK178	*sir1::HIS3*	YRp315	11	20
YWK179	*sir2::HIS3*	YRp315	4	32
YWK55	*sir3::LYS2*	YRp315	15	94
YRS477	*sir4::HIS3*	YRp315	2	37

[a] Total plasmid copy number was determined as described in Materials and Methods and reported as plasmids per cell averaged over the entire population.

[b] Relative plasmid copy number was obtained by dividing the total plasmid copy number by the mitotic stability of the plasmid in the particular strain (Table 2) and reported as plasmids per Ura$^+$ cell.

URA3 gene which would not be subject to *SIR* repression. Lanes 2 and 4 in Fig. 3 measure the relative expression of *URA3* in wild-type cells carrying YCp50 and YRp315, respectively. Significantly more cross-reacting material was present in extracts of wild-type cells carrying YRp315 than in those carrying the single-copy plasmid, YCp50. Therefore, *E* and *SIR* did not significantly repress the expression of *URA3* from YRp315, implying that high-copy-number maintenance of YRp315 in *SIR* cells was not due solely to the achievement of a threshold level of the *URA3* gene product. The level of accumulation of OMP decarboxylase in wild-type cells transformed with YCp6.315 (Fig. 3, lane 6) was nearly equivalent to that seen by the same strain transformed with YCp50 (lane 2). Therefore, *SIR*-mediated repression of *URA3* was not detectable at the single-copy plasmid level.

Unexpectedly, greater accumulation of OMP decarboxylase was observed in isogenic *sir3::LYS2* and *sir4::HIS3*

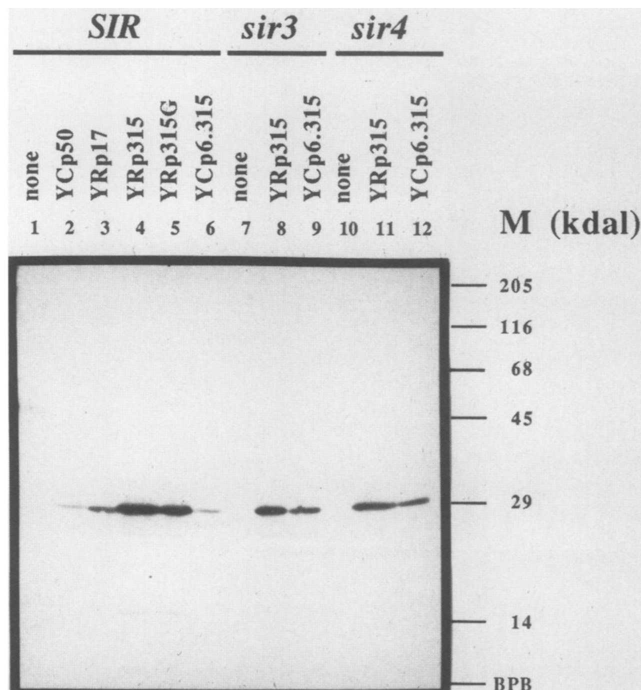

FIG. 3. Immunological detection of OMP decarboxylase. Whole-cell protein extracts were prepared as described in Materials and Methods, subjected to electrophoresis through a 12.5% polyacrylamide-sodium dodecyl sulfate gel, and transferred to nitrocellulose filter paper. The filter was incubated with rabbit polyclonal antibody raised against yeast OMP decarboxylase, and the resulting immune complexes were detected by incubation with ^{125}I-labeled *S. aureus* protein A, followed by autoradiography. Equal amounts of protein were added per lane. M, Molecular size markers; BPB, bromophenol blue dye front. Lane 1, DBY703 (*SIR*) with no plasmid; lane 2, DBY703 containing YCp50; lane 3, DBY703 containing YRp17; lane 4, DBY703 containing YRp315; lane 5, DBY703 containing YRp315G; lane 6, DBY703 containing YCp6.315; lane 7, YWK55 (*sir3*) with no plasmid; lane 8, YWK55 containing YRp315; lane 9, YWK55 containing YCp6.315; lane 10, YRS477 (*sir4*) with no plasmid; lane 11, YRS477 containing YRp315; lane 12, YRS477 containing YCp6.315.

strains transformed with the centromere-containing plasmid, YCp6.315 (Fig. 3, compare lane 6 with lanes 9 and 12). Although the plasmid copy number was one per cell in these three isogenic strains (see below, and Fig. 4), both *sir* mutant strains overexpressed the *URA3* product about fivefold relative to the wild-type strain. This result suggests that *HMR E* exhibits enhancerlike properties in the absence of *SIR* function, rather than simply being neutral with respect to gene expression.

Evidence for *SIR*-dependent segregation of plasmids containing *HMR E*. The experiments presented above indicated that the ability of *HMR E* to replicate was not absolutely dependent on any *SIR* gene. This result suggested that the high mitotic stability of YRp315 in wild-type cells was due to a segregation mechanism. If *SIR* allows plasmids carrying *HMR E* to be segregated efficiently, then the addition of an alternate segregation mechanism provided by a centromere should increase the mitotic stability of plasmids carrying *HMR E* in *sir* mutant strains. To test this hypothesis, we inserted *CEN6* (46) into the plasmid YRp315, creating the plasmid YCp6.315. The mitotic stability of YCp6.315 was determined in isogenic wild-type and *sir* mutant strains (Table 6). In all *sir* mutants, the plasmid was found in approximately 63 to 80% of the cells under selective growth conditions and was stably maintained during nonselective growth. Thus, the mitotic instability of YRp315 in *sir* mutants was due to a defect in segregation and not replication. Therefore, the interaction between *SIR* and *HMR E* provides a segregation function to plasmids that contain this regulatory region. However, plasmid copy number analyses (above) suggested roles for *SIR2* and *SIR4* (and to a lesser extent, *SIR1*) in the replication of YRp315. Since the results in Table 6 indicated that no *SIR* gene was required for replication of *HMR E*-bearing plasmids at low copy number, we infer that the replicative role of *SIR1*, *SIR2*, and *SIR4* is restricted to high-copy-number replication. The mechanistic differences between these two modes of replication are unclear.

Centromere control of plasmid copy number is epistatic to *SIR*-dependent high-copy-number maintenance. In addition to providing a segregation mechanism to plasmids, a centromere also provides copy number control at one or two copies per cell (60). An experiment was performed to determine whether the *CEN* or *SIR* control of plasmid copy number was epistatic. The experiment shown in Fig. 4 is a plasmid copy number analysis of wild-type and *sir* mutant strains transformed with YCp6.315. In all cases the plasmid was present at approximately one copy per cell regardless of the *SIR* genotype (compare wild-type transformants in Fig. 4, lanes 3 to 8, with *sir3* transformants in lanes 9 to 14 and *sir4* transformants in lanes 15 to 20). This result indicated that the centromere control of copy number was epistatic to high-

TABLE 6. Mitotic stability of centromere-containing plasmids in wild-type and *sir* mutant strains

Strain	*SIR* genotype	Plasmid	*ARS/CEN*	Mitotic stability	
				Selective	Nonselective
DBY703	*SIR*	YCp50	*ARS1/CEN4*	82.3 ± 1.5	64.0 ± 1.3
YWK178	*sir1::HIS3*	YCp50	*ARS1/CEN4*	88.3 ± 5.5	80.2 ± 6.1
YWK179	*sir2::HIS3*	YCp50	*ARS1/CEN4*	85.9 ± 4.3	71.3 ± 12.7
YWK55	*sir3::LYS2*	YCp50	*ARS1/CEN4*	83.2 ± 4.9	54.3 ± 6.7
YRS477	*sir4::HIS3*	YCp50	*ARS1/CEN4*	81.3 ± 4.5	51.4 ± 3.2
DBY703	*SIR*	YCp6.315	*HMR E/CEN6*[a]	71.0 ± 7.2	14.5 ± 8.3
YWK178	*sir1::HIS3*	YCp6.315	*HMR E/CEN6*	62.9 ± 8.4	62.7 ± 5.0
YWK179	*sir2::HIS3*	YCp6.315	*HMR E/CEN6*	62.3 ± 4.5	63.4 ± 7.2
YWK55	*sir3::LYS2*	YCp6.315	*HMR E/CEN6*	78.4 ± 3.3	63.9 ± 1.6
YRS477	*sir4::HIS3*	YCp6.315	*HMR E/CEN6*	81.2 ± 2.1	63.3 ± 1.4

[a] A plasmid containing the *CEN6* restriction fragment inserted into YIp5 exhibited a mitotic stability of <1% under selective growth conditions owing to the absence of an *ARS* element. After 10 to 12 generations of nonselective growth, the mitotic stability was <0.01% (data not shown).

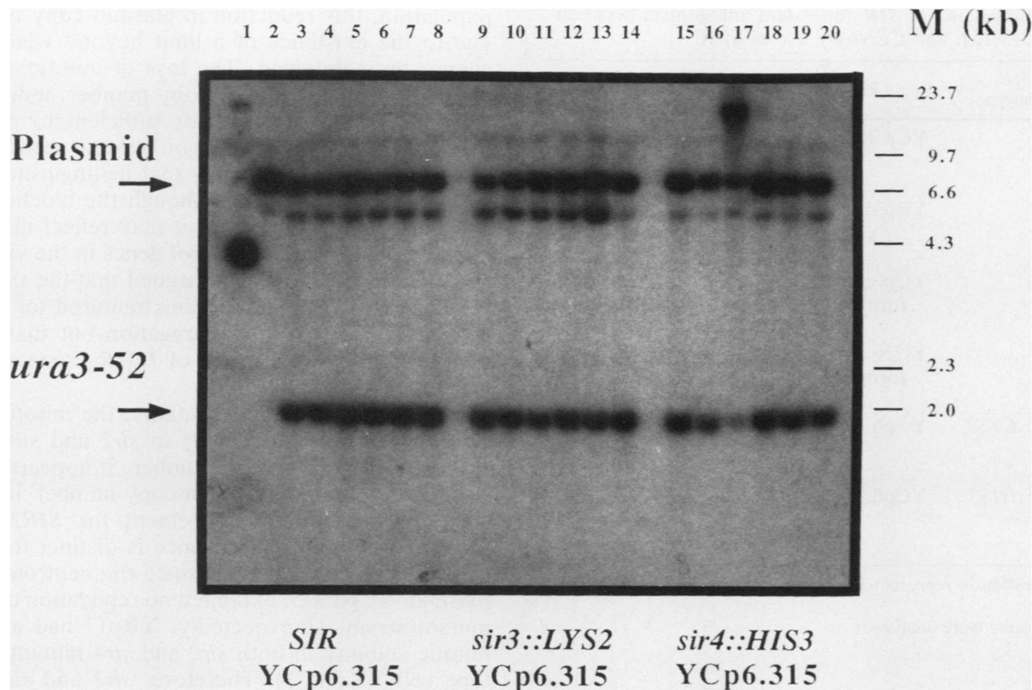

FIG. 4. Copy number determination of centromere-containing plasmids by blot hybridization. Details are as described in the legend to Fig. 2. Lane 1, Uncut YCp6.315; lane 2, YCp6.315 cleaved with *Hin*dIII; lanes 3 to 8, genomic DNA from independent transformants of DBY703 (*SIR*) containing YCp6.315 cleaved with *Hin*dIII; lanes 9 to 14, genomic DNA from independent transformants of YWK55 (*sir3*) containing YCp6.315 cleaved with *Hin*dIII; lanes 15 to 20, genomic DNA from independent transformants of YRS477 (*sir4*) containing YCp6.315 cleaved with *Hin*dIII. The band with slightly faster mobility than linearized plasmid is of unknown origin. Presumably it is a plasmid isomer, as this band was observed when purified YCp6.315 was cleaved with *Hin*dIII (lane 2). Lane M, Molecular weight markers.

copy-number plasmid maintenance provided by *SIR* and *HMR E*. This experiment also provided further direct evidence that the replication of plasmids carrying *HMR E* at the single-copy level was not perturbed in *sir3* or *sir4* strains. A similar single-copy-level maintenance was seen for the plasmid YCp50 in wild-type and *sir* mutant strains (data not shown).

Antagonism between *CEN*- and *SIR*-mediated plasmid segregation. In principle, if a plasmid were able to respond to two different segregation mechanisms, then the mitotic stability of that plasmid may be greater than that of plasmids able to respond to only one segregation mechanism. However, for *HMR E* and *CEN6*, the opposite result was obtained. In the wild-type strain, the plasmid YCp6.315 was present in 71% of cells under selective growth conditions, and when grown under nonselective conditions for 10 to 12 generations, it was present in less than 15% of the cells (Table 6). The rapid loss of the plasmid during nonselective growth was not observed in isogenic *sir* mutant strains. The decrease in mitotic stability caused by the combination of *CEN6* and *HMR E* was paradoxical since each element alone when present on a plasmid provided high mitotic stability. This observation suggested an antagonism between the mechanisms by which *CEN6* and *HMR E* provide segregation and high mitotic stability to plasmids.

As a test of this hypothesis, if either segregation function were to be inactivated, the mitotic stability of the plasmid YCp6.315 should be increased. One prediction of this hypothesis had already been realized: YCp6.315 showed high mitotic stability during nonselective growth in all *sir* mutant strains in comparison with the wild-type strain. In these strains, the *E-SIR* mechanism of plasmid segregation had been inactivated by mutation. By analogy, if the centromere-mediated mechanism of plasmid segregation could be selectively inactivated or perturbed, the mitotic stability of YCp6.315 should increase in wild-type cells. To this end, the drug MBC was used to disrupt the interaction between the spindle and *CEN6* carried on YCp6.315 (62). In this experiment, cells containing YCp6.315 were grown under nonselective conditions in the absence of MBC, or in the presence of 40 or 160 μg of MBC per ml for 10 to 12 generations. Samples were diluted, plated onto solid YPD agar, and allowed to form single colonies. The mitotic stability was determined by replica plating single colonies onto supplemented YM agar plates with and without uracil. For two independent transformants of the wild-type strain, the mitotic stability of YCp6.315 increased steadily as the concentration of MBC was increased (Table 7). This result indicated that as the centromere function was disrupted, proper segregation of the plasmid was restored during nonselective growth by the interaction between *SIR* and *HMR E*. As expected, a decrease in mitotic stability was observed with YCp6.315 transformants of both isogenic *sir* mutant strains, since the only segregation mechanism available to this plasmid, that provided by *CEN6*, was disrupted by MBC. This result also indicated that antagonism during mitotic transmission of this plasmid requires *SIR3* and *SIR4*. The simplest explanation for these observations is that two independent segregation mechanisms imparted to a single plasmid molecule resulted in lowered mitotic stability.

In control experiments, this analysis was repeated with the wild-type strain containing either YCp50 or YRp315. In the case of YCp50, as the concentration of MBC was increased, the mitotic stability of the plasmid decreased.

TABLE 7. Suppression of *SIR*-dependent antagonism between *HMR E* and *CEN6* by use of MBC

Strain	*SIR* genotype	Plasmid	MBC concn[a]	Mitotic stability
DBY703	*SIR*	YCp50	None	64.0 ± 1.3
			40	55.5 ± 2.9
			160	42.5 ± 2.9
DBY703	*SIR*	YRp315	None	64.5 ± 2.9
			40	65.0 ± 4.5
			160	61.9 ± 1.1
DBY703	*SIR*	YCp6.315 (transformant 1)[b]	None	16.3 ± 1.6
			40	24.1 ± 1.6
			160	29.2 ± 1.3
DBY703	*SIR*	YCp6.315 (transformant 2)[b]	None	3.3 ± 1.0
			40	6.9 ± 1.8
			160	14.4 ± 2.2
YWK55	*sir3::LYS2*	YCp6.315	None	63.9 ± 1.6
			40	60.5 ± 0.7
			160	50.4 ± 1.1
YRS477	*sir4::HIS3*	YCp6.315	None	62.3 ± 1.4
			40	61.7 ± 1.7
			160	54.8 ± 1.0

[a] MBC concentration is represented as micrograms per milliliter in YPD broth.
[b] Two transformants were analyzed.

Under identical conditions, the mitotic stability of YRp315 was not significantly affected, providing evidence that the *E-SIR* mechanism of segregation does not involve the mitotic spindle.

DISCUSSION

HMR E* provides *SIR*-dependent replication and segregation functions to plasmids.** Experiments presented in this paper indicate that the regulatory region *HMR E*, required for *SIR*-mediated transcriptional repression of the silent mating-type gene *HMRa**, affects the mitotic stability of plasmids that contain this region. Specifically, the *ARS* activity of *HMR E* is influenced by the *SIR* genotype of the cell. In wild-type cells, a plasmid carrying *HMR E* was maintained at high copy number (28 per cell) in approximately 90% of the cells in the population. The high level of mitotic stability of this plasmid was correlated with two properties not shared with other chromosomal *ARS* elements: a segregation or partitioning mechanism and a high-copy-number maintenance. Both of these properties depended on the products of the *SIR* genes. Although the *HMR E*-bearing plasmids are replicated at 28 copies per cell, the chromosomal *HMR* and *HML* loci are not amplified. Clearly, some property of the plasmid is critically different from the chromosomal locus. An explanation for this difference may require an understanding of the events that occur in the first few divisions after transformation.

Mutations in any *SIR* gene resulted in loss of mitotic stability of plasmids containing *HMR E*. The *sir3* mutation disrupted the plasmid segregation mechanism, but had very little effect on the copy number of YRp315 in the population. However, since the plasmid was asymmetrically distributed in the population, the fraction of the population that had at least one plasmid must have had, on average, 94 plasmids per cell. The very high plasmid copy number in a subset of *sir3* cells can be explained by the absence of the *SIR*-dependent segregation mechanism, with only a slight effect on replication. Although there appeared to be a twofold reduction in plasmid copy number among all cells in the *sir3* population, this reduction in plasmid copy number may be due to the existence of a limit beyond which the plasmid cannot be maintained. The loss of mitotic stability in *sir3* mutants, despite the high copy number, indicates that high plasmid copy number is not sufficient to provide mitotic stability. The different effect on plasmid replication and copy number is the first property that distinguishes *sir3* mutants from other *sir* mutants. Although the biochemical basis of this difference is unknown, it may reflect different roles of these proteins in repression of genes in the vicinity of *HMR E*. Alternatively, it may be argued that the *sir3::LYS2* allele (Fig. 1) disrupted the functions required for transcriptional repression and plasmid segregation but that the functions required for the replication of *HMR E*-containing plasmids were not affected.

In contrast to the *sir3* mutants, the mitotic instability of *HMR E*-containing plasmids in *sir2* and *sir4* mutants was associated with low copy number. It appears that *SIR2* and *SIR4* are required for high-copy-number maintenance of such plasmids. The requirement for *SIR2* and *SIR4* in high-copy-number maintenance is distinct from any role in general replication per se, since the centromere-containing plasmid, YCp6.315, exhibited no replication defect in any *sir* mutant strain. Unexpectedly, YRp17 had a slightly lower mitotic stability in both *sir2* and *sir4* mutants than in wild-type cells (Table 2). Therefore, *sir2* and *sir4* may have a slight, yet statistically significant effect on the segregation of plasmids containing *ARS1*. The biological relevance of the effect on *ARS1* is unclear.

The plasmid segregation function provided by *HMR E* is clearly different from the centromere-mediated mechanism in many respects. First, plasmids replicated and segregated by *HMR E* were not subject to the copy number control characteristic of yeast centromeres. They were maintained at a level of approximately 28 per cell, whereas centromere-containing plasmids are maintained at 1 to 2 per cell (60) (Fig. 4). Second, the mitotic stability of plasmids segregated by a centromere was sensitive to the microtubule inhibitor MBC. Plasmids containing only *HMR E* were insensitive to MBC. Hence, *HMR E* probably does not interact with the spindle to provide segregation. Finally, no DNA sequence homology between *E* and identified centromeric elements was found (7, 26). The addition of *CEN6* to YRp315 reduced the copy number of the plasmid to approximately 1 to 2 per cell in the wild-type strain. Therefore, the copy number control provided by the centromere is epistatic to the mechanism of the *SIR*-dependent high-copy-number plasmid maintenance. The epistasis of the centromere in control of plasmid copy number has also been observed for the 2μm plasmid (60). This property of yeast centromeres is remarkable considering that the mechanism of 2μm plasmid copy number amplification (20) is most certainly distinct from the mechanism responsible for copy number elevation of *HMR E*-containing plasmids.

***HMR E* and *SIR*-mediated segregation antagonizes centromere-mediated segregation.** A surprising result from these experiments was that plasmids containing both *HMR E* and *CEN6* are less mitotically stable than are plasmids containing either *HMR E* or a centromere. The loss of mitotic stability by the plasmid YCp6.315 implies the existence of antagonism between two mechanisms of plasmid segregation, since selective perturbation of either the *HMR E-SIR* interaction or the centromere-spindle interaction resulted in increased plasmid stability. Some possible explanations of this antagonism are unlikely. In principle, the *SIR*-dependent antagonism of centromere function could be

viewed as another example of the ability of *SIR* to repress site-specific protein-nucleic acid interactions in the vicinity of an *E* site (34, 51). In this case, *SIR* could interfere with the assembly of a kinetochore complex at the centromere. However, the plasmid copy number determinations of YCp6.315 in wild-type cells indicated that *SIR* had no adverse effect on copy number control provided by the centromere. Furthermore, experiments with *CEN6* flanked by both an *E* site and an *I* site gave no indication of *SIR*-dependent interference with meiotic centromere function (R. A. Schnell and J. Rine, unpublished data). Therefore, it is unlikely that *SIR* directly interferes with *CEN6* function in YCp6.315. Another explanation of the antagonism between *E* and *CEN6* is that *SIR* mediates enhanced recombination between sister chromatids resulting in dicentric plasmids which would be mitotically unstable and lead to broken or rearranged plasmids (41). The plasmid copy-number determinations would have detected structurally altered plasmids in wild-type cells, but none were found. Therefore, recombination does not explain the antagonism between *HMR E* and *CEN6* segregation functions.

The antagonism between the *CEN6*-mediated segregation mechanism and the *HMR E-SIR*-mediated mechanism provides further insight into the mitotic instability of YRp315 in the *sir4* mutant strain. Since the copy number of YRp315 was reduced in *sir4* mutants, it was difficult to conclude whether the instability was due exclusively to reduced copy number or to a defect in the *HMR E-SIR*-dependent segregation mechanism. If the segregation mechanism was still operational in *sir4* mutants, YCp6.315 should exhibit lowered mitotic stability during nonselective growth owing to the antagonism between two segregation mechanisms. However, since the *sir4* mutation relieved the antagonism, *SIR4* must contribute to the segregation mechanism in addition to its role in high-copy-number maintenance. Therefore, it is likely that mitotic instability of the plasmid in *sir4* mutants is primarily due to the segregation defect.

A model that reconciles *SIR*-dependent plasmid segregation and transcriptional repression functions of *HMR E*. Our working hypothesis for the *HMR E-SIR* interaction is attachment of plasmids containing the *E* site to structural components of the nucleus that partition to both cells during mitosis. *SIR* proteins may either be components of that structure or mediate attachment of plasmids to it. The antagonism provided by the centromere would be a consequence of the reduction in plasmid copy number. If fewer plasmids were available to be attached to this nuclear structure, some nuclei would inherit a portion of the structure lacking an attached plasmid. Conversely, some of the plasmids would attach to this nuclear structure and not to the spindle. Hence, the centromere function would also be compromised. In *sir* mutants the lack of attachment to sites other than to the spindle would alleviate antagonism. A weakness of this model is that the increase in mitotic stability of YCp6.315 upon MBC treatment would be difficult to understand unless the control of copy number by the centromere is MBC sensitive.

The endogenous yeast plasmid 2μm circle also possesses a plasmid-specific segregation mechanism. This plasmid carries an 85-bp region required in *cis* for replication (10) that contains an *ARS* consensus sequence. It has been shown that plasmid-encoded *trans*-acting factors, *REP1* and *REP2*, increase the stability of plasmids carrying another *cis*-acting region, *REP3*, without increasing plasmid copy number (12, 29, 32). Biochemical evidence suggests that the *REP1* protein is associated with the nuclear matrix and may also have affinity for DNA (63). It has been proposed that the equipartitioning of 2μm derivatives is accomplished by the interaction of the plasmid via *REP3* with matrix-associated *REP1* (61, 63). This plasmid-partitioning mechanism is similar in many respects to that involving *HMR E* and *SIR* described in this paper. Both require a *cis*-acting locus (*REP3* or *E*) and *trans*-acting factors (*REP1*, *REP2* or *SIR3*, *SIR4*). By analogy, the *HMR E-SIR*-mediated mechanism of segregation may also involve an interaction between *E* and the nuclear matrix via *SIR* protein(s). The attachment of *HMR E* to a nuclear structure would have implications toward an understanding of *SIR*-mediated repression of the silent mating-type loci. The stable association of these loci with a nuclear structure may be directly required for initiation or maintenance of the repressed state. Alternatively, attachment may be required to confine the repressive effect of *SIR* to a limited region.

The coincidence of regulatory regions involved in both transcription and replication is a recurring theme. An enhancer region required for early viral transcription of polyoma virus is also required in *cis* for viral replication (17). Bovine papillomavirus carries two *cis*-acting elements that promote stable episomal maintenance of viral genomes in transformed cells (38). One of these regions also acts as a transcriptional enhancer and is required in *cis* for replication (39). In *Drosophila melanogaster*, two clusters of chorion genes are amplified by localized, developmentally regulated DNA replication (53). For one of these clusters, amplification requires a region also involved in transcriptional control. Similarly, the *REP3* region of 2μm circle has promoter activity (29). Although the transcriptional regulation of *HMR***a** involves repression, whereas the above examples involve transcriptional activation, perhaps both positive and negative transcriptional regulation and the regulation of DNA replication share common factors.

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