REP3-Mediated Silencing in *Saccharomyces cerevisiae*

Laurie Ann Papacs,[1] Yu Sun,[2] Erica L. Anderson, Jianjun Sun[3] and Scott G. Holmes[4]

Department of Molecular Biology and Biochemistry, Wesleyan University, Middletown, Connecticut 06459

Manuscript received July 30, 2002
Accepted for publication October 3, 2003

ABSTRACT

In yeast the Sir proteins and Rap1p are key regulators of transcriptional silencing at telomeres and the silent mating-type loci. Rap1 and Sir4 also possess anchoring activity; the rotation of plasmids bound by Sir4 or Rap1 is constrained *in vivo*, and Rap1 or Sir4 binding can also correct the segregation bias of plasmids lacking centromeres. To investigate the mechanistic link between DNA anchoring and regulation of transcription, we examined the ability of a third defined anchor in yeast, the 2μ circle *REP3* segregation element, to mediate transcriptional silencing. We find that placement of the *REP3* sequence adjacent to the *HML* locus in a strain deleted for natural silencer sequences confers transcriptional repression on *HML*. This repression requires the Sir proteins and is decreased in strains lacking the *REP3*-binding factors Rep1 and Rep2. The yeast cohesin complex associates with *REP3*; we show that *REP3* silencing is also decreased in strains bearing a mutated allele of the *MCD1/SCC1* cohesin gene. Conventional silencing is increased in some strains lacking the 2μ circle and decreased in strains overexpressing the Rep1 and Rep2 proteins, suggesting that the Rep proteins antagonize conventional silencing.

YEAST employs a transcriptional silencing mechanism to control the expression of genes specifying cell type (for reviews see Huang 2002; Rusche *et al.* 2003). A mechanistically related but weaker silencing effect is observed when genes are placed adjacent to yeast telomeres. Silencing in yeast requires several *trans*-acting factors that interact directly with *cis*-acting "silencer" sequences, including Rap1p, Abf1p, and Orc. One function of these factors is to recruit a complex of Sir proteins; the Sir complex includes Sir2, a histone deacetylase (Imai *et al.* 2000; Landry *et al.* 2000; Smith *et al.* 2000), and the Sir3 and Sir4 proteins, which can bind the N terminus of histones H3 and H4 (Hecht *et al.* 1995; Carmen *et al.* 2002). These activities of the Sir proteins likely establish an altered chromatin structure that restricts transcription.

Silencing factors have additional properties that have not been directly linked to their role in repressing transcription. First, the Rap1 and Sir4 proteins have "anchoring" capability *in vivo*, defined as the ability to constrain the rotation of plasmid molecules to which these proteins are bound (Ansari and Gartenberg 1997; Mirabella and Gartenberg 1997). Second, plasmids containing yeast replication origins (ARSs) typically require centromeres to be segregated efficiently during mitosis; however, these plasmids can be stabilized by the inclusion of silencer sequences (Kimmerly *et al.* 1988), by telomere repeats, which bind the Rap1 protein (Longtine *et al.* 1992), or by tethering the Sir4 protein using a heterologous DNA-binding domain (Ansari and Gartenberg 1997).

Finally, several studies have shown that Rap1p, Sir3p, and Sir4p are localized to discrete foci found at the nuclear periphery (Palladino *et al.* 1993; Cockell *et al.* 1995). These foci can also include Sir2 (Gotta *et al.* 1997) and are coincident with the location of telomere sequences (Gotta *et al.* 1996). A weakly silenced *HMR* locus becomes more strongly repressed when artificially tethered to the nuclear envelope (Andrulis *et al.* 1998), suggesting that localization directly influences transcriptional repression. However, the cause-and-effect relationship of localization to the nuclear periphery and Sir protein-dependent silencing is not yet clear (Tham *et al.* 2001; Hediger *et al.* 2002b).

To examine the contribution of anchoring to silencing, we tested the ability of the *REP3* anchor to mediate transcriptional repression. *REP3* is a *cis*-acting element found on the 2μ circle, an endogenous yeast plasmid. Stable mitotic segregation of the 2μ circle relies on *REP3* and two plasmid-encoded proteins, Rep1 and Rep2. Like Sir4 and Rap1, *REP3* is also known to have plasmid anchoring ability, as defined above (Gartenberg and Wang 1993). Here we demonstrate that the *REP3* sequence also has silencing activity; our characterization of *REP3*-mediated silencing suggests that anchoring and gene repression are mechanistically related functions.

[1]*Present address:* University of Connecticut Health Center, Farmington, CT 06030.

[2]*Present address:* CuraGen, New Haven, CT 06511.

[3]*Present address:* Department of Microbiology and Molecular Genetics, Medical College of Wisconsin, Milwaukee, WI 53226.

[4]*Corresponding author:* Department of Molecular Biology and Biochemistry, Hall-Atwater Laboratories, Wesleyan University, Middletown, CT 06459-0175. E-mail: sholmes@wesleyan.edu

TABLE 1

Description of yeast strains

Strain	Genotype	Source[a]
DMY1	*MAT***a** *ura3 ade2 lys1 his5 leu2 can1*	Mahoney and Broach (1989)
DMY26	DMY1; *HML*α *E*Δ*79-113 I*Δ*242*	Mahoney and Broach (1989)
YSH392	DMY1; *HML*α *E*Δ*79-113::REP3 I*Δ*242*	
YSH393	YSH392, [cir^0]	
YSH422	YSH392; Δ*sir2::URA3*	
YSH417	YSH392; Δ*sir3::LEU2*	
YSH418	YSH392; Δ*sir4::URA3*	
DMY19	DMY1; *HML*α *E*Δ*79-113::SUP4 I*Δ*242*	Mahoney and Broach (1989)
YSH406	DMY19; [cir^0]	
YSH409	*hmr*Δ*A::ADE2 ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1-1 ura3-1*[cir^0]	
YSH426	YSH409; *leu2-3,112::LEU2-GAL10-REP1*	
YSH481	YSH409; *trp1::TRP1-GAL10-REP2*	
YSH425	YSH409; *leu2-3,112::LEU2-GAL10-REP1 trp1::TRP1-GAL10-REP2*	
YSH316	*HMLa MAT*α *HMRa ade2 leu2 lys2 trp1-289 ura3-52*	Holmes *et al.* (1997)
YSH323	YSH316; Δ*sir2::URA3*	Holmes *et al.* (1997)
YSH496	YSH316; Δ*sir3::URA3*	
YSH312	YSH316; Δ*sir4::URA3*	Holmes *et al.* (1997)
BY4741	*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*	Brachmann *et al.* (1998)
YSH509	YSH392; Δ*mlp1::URA3*	
YSH510	YSH392; Δ*mlp2::URA3*	
YSH511	YSH392; Δ*mlp1::URA3* Δ*mlp2:KAN*	
YSH561	YSH392; Δ*mig1::URA3*	
YSH560	YSH392; Δ*hst3::URA3*	
YSH558	YSH392; Δ*yku70::URA3*	
YSH559	YSH392; Δ*yku80::URA3*	
YSH572	YSH392; Δ*esc1::URA3*	
YSH554	YSH392; Δ*scc1::scc1-73-URA3*	
YSH555	YSH392; Δ*scc1::scc1-73^{REV}-URA3*	
YSH482	BY4741; Δ*mlp1::KAN*	
YSH512	BY4741; Δ*mlp2::URA3*	
YSH508	BY4741; Δ*mlp1::KAN* Δ*mlp2::URA3*	
YSH565	BY4741; Δ*yku70::URA3*	
YSH566	BY4741; Δ*yku80::URA3*	
YSH564	BY4741; Δ*esc1::URA3*	

[a] Unless noted, strains were constructed during the course of this work.

MATERIALS AND METHODS

A 712-bp DNA fragment containing the genetically defined 2μ circle *REP3* sequence (Kikuchi 1983; Jayaram *et al.* 1985) was amplified from plasmid YEp24 by polymerase chain reaction (PCR) using primers SP69 (GATAGGATCCGCACTTC TACAATGGCTG) and SP70 (GATAGGATCCATAGAGCGC ACAAAGGAG). This fragment includes a core element consisting of five repeats of a 62-bp sequence (Kikuchi 1983; Jayaram *et al.* 1985). This fragment was subcloned into plasmid pCR2.1 (Invitrogen, San Diego) to form pLXB1. The *REP3* sequences were then removed from pLXB1 as a *Bam*HI fragment and subcloned into the *Bgl*II site of pDM11 (Mahoney and Broach 1989) to form pLXB2. Plasmid pAW2, used to delete the *SIR2* gene, is based on pRS416 (Sikorski and Hieter 1989) and contains ~300 bp upstream and downstream of the *SIR2* open reading frame, separated by the *URA3* gene. Plasmid stability was determined by fluctuation analysis; loss rates shown are the means of three or more independent experiments.

All yeast strains used to assess *REP3* silencing are derived from DMY1 (Mahoney and Broach 1989; Table 1). YSH392, in which the *HML-E* silencer is replaced by the *REP3* sequence, was constructed via a two-step gene replacement procedure using DMY19 as the starting strain (Mahoney and Broach 1989). In strain DMY19, the *HML-E* silencer is replaced by the *SUP4* gene. In step 1, pLXB2 was cut with *Xho*I and transformed into DMY19, producing uracil prototrophs by integration at the *HML* locus. In step 2, ura$^-$ recombinants were selected for by plating this strain on 5-fluoroorotic acid media; ura$^-$ isolates that lost *SUP4*-suppressing activity were then tested for the presence of the *REP3* sequence at *HML-E* by Southern blot. Strain YSH409 is an isolate of strain YLS404 (Chi and Shore 1996) that has lost the 2μ circle. Strains lacking the 2μ circle (cir^0 strains) were identified by selecting for isolates that survived overexpression of the 2μ *FLP1* gene (Volkert and Broach 1986). These isolates were tested for the presence of 2μ by Southern blot or a PCR assay using 2μ-specific primers. Deletions or disruptions of the *SIR2*, *SIR3*, and *SIR4* genes were made using pAW2, pAR78 (Braunstein *et al.* 1993), and pCTC77 (Chien *et al.* 1993), respectively. Strains containing galactose-inducible *REP1* and *REP2* genes were made by transforming YSH409 with *Kpn*I-digested pKA610 DNA (containing *GAL10-REP1*) and/or *Eco*RI-digested pKA620 plasmid DNA (containing *GAL10-REP2*; Som *et al.* 1988). The *YKU70*, *YKU80*, *HST3*, *MIG1*, *MLP1*, *MLP2*, and *ESC1* open

Strain	Genotype	Mating efficiency
DMY1	*E-HML-I*	0.42
DMY26	*ΔE -HML-ΔI*	2.0×10^{-4}
YSH392	*ΔE::REP3-HML-ΔI*	1.5×10^{-2}

FIGURE 1.—*REP3*-mediated silencing. A partial genotype is shown, as well as the results of qualitative and quantitative mating assays. Mating assays were performed as described (SHEI and BROACH 1995; DULA and HOLMES 2000).

reading frames were removed from strain YSH392 by PCR-mediated deletion (WACH *et al.* 1994). The wild-type *MCD1/SCC1* gene was replaced with the *scc1-73* allele (MICHAELIS *et al.* 1997) by one-step gene replacement. A DNA fragment containing both *scc1-73* and the *URA3MX* gene with ends homologous to sequences 5′ and 3′ of *SCC1* was made by hybrid PCR (HORTON *et al.* 1989) and transformed into YSH392. Isolates that were uracil prototrophs and temperature sensitive for growth were screened by PCR for incorporation of the *URA3MX* gene adjacent to the *SCC1* locus.

RESULTS

***REP3*-mediated silencing:** The Rap1 and Sir4 silencing factors can function as anchors in yeast, mediating stable segregation of episomes and restraining the rotation of plasmids bound to these factors (ANSARI and GARTENBERG 1997; MIRABELLA and GARTENBERG 1997). To examine if silencing is a general property conferred by noncentromeric anchors, we determined the ability of a third anchor, the 2μ circle *REP3* sequence, to mediate silencing. The 2μ circle is a naturally occurring, high-copy plasmid present in almost all strains of *Saccharomyces cerevisiae* (see BROACH and VOLKERT 1991 for review). Efficient segregation of the 2μ circle likely occurs by anchoring the plasmid to a nuclear structure, which is then equally partitioned during cell division (WU *et al.* 1987; SCOTT-DREW and MURRAY 1998; VELMURUGAN *et al.* 2000; MEHTA *et al.* 2002; SCOTT-DREW *et al.* 2002). This partitioning requires two plasmid-encoded *trans*-acting factors, Rep1p and Rep2p, which bind a *cis*-acting sequence, *REP3* (VELMURUGAN *et al.* 1998). Plasmids containing *REP3* are topologically constrained *in vivo* in a Rep1- and Rep2-dependent way (GARTENBERG and WANG 1993), an observation consistent with the proposal that Rep1p and Rep2p fix *REP3* to a nuclear structure. If anchoring helps mediate silencing, we reasoned then that the Rep system might substitute for some or all functions of the silencing factors. To test this possibility, we replaced the silencer sequences at *HML* with the *REP3* sequence, creating strain YSH392. This strain is missing all functional *HML-I* silencer sequences, as well as the Rap1- and Orc-binding sites at *HML-E* (MAHONEY and BROACH 1989). A defect in silencing causes a reduction in mating efficiency; therefore, we compared the mating ability of strain YSH392 to an otherwise identical strain lacking the *REP3* sequences. The results of these experiments are shown in Figure 1. Our results indicate that the *REP3* sequence significantly improves the mating ability of this strain.

***REP3* silencing requires Rep1p and Rep2p:** *REP3* silencing could be due to an interaction with Rep1p and Rep2p, as predicted by the premise of our approach, or could be due to an independent mechanism. To determine if *REP3* silencing requires 2μ-encoded gene products, we eliminated the 2μ circle in our test strain and repeated the mating assays. As shown in Figure 2, we found that silencing is markedly reduced in this strain, indicating that *REP3* silencing does depend on the 2μ circle. However, we note that mating efficiency in the *REP3* cir^0 strain still exceeds that in the control strain lacking *REP3* adjacent to *HML*. This could reflect a function of the *REP3* sequence that is independent of the Rep1 and Rep2 proteins or could be a more indirect effect of losing the 2μ circle (see below).

***REP3* silencing requires the *SIR* gene products:** Our results are consistent with the hypothesis that anchoring DNA sequences has a repressive effect on gene expression. There are two general models for this effect. First, anchoring *per se* could be repressive, perhaps by sequestering sequences from the transcriptional machinery. Alternatively, anchoring could aid the association of transcriptional repressors, such as the Sir proteins. To discriminate between these models, we determined whether *REP3* silencing depends on the presence of the Sir proteins. We made deletions or disruptions of the *SIR2*, *SIR3*, or *SIR4* genes in a strain exhibiting *REP3* silencing and measured the effects on mating ability. As shown in Figure 3, elimination of any one of the *SIR* genes eliminates *REP3* silencing. We also found that the silencing retained in YSH393, the cir^0 isolate of YSH392, also depends on Sir proteins (not shown). Therefore, our results suggest that *REP3* either recruits Sir proteins or relocalizes *HML* to a location of high Sir protein concentration.

An alternative explanation for these results is that the Sir proteins are required for *REP3* anchoring function. In this model the role of Sir proteins is indirect, and

Strain	Genotype	Mating efficiency
YSH392	*ΔE::REP3-HML-ΔI* [cir$^+$]	1.5×10^{-2}
YSH393	*ΔE::REP3-HML-ΔI* [cir^0]	1.9×10^{-3}

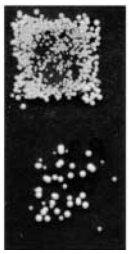

FIGURE 2.—*REP3* silencing requires the 2μ circle. The mating ability of [cir$^+$] and [cir^0] strains is shown.

anchoring remains the dominant event in *REP3* silencing. To examine the influence of Sir proteins on *REP3* function, we determined the stability of plasmid YEp51 in a set of congenic Sir$^+$ and Sir$^-$ strains. YEp51 is a 2μ-derived vector that depends on the *REP3* sequence for efficient segregation; plasmids using *REP3* for segregation function are unstable in strains lacking the 2μ circle (KIKUCHI 1983; JAYARAM *et al.* 1985). We predicted that if *REP3* anchoring requires Sir proteins, then this plasmid should also be unstable in a strain lacking Sir protein function. However, we found that the loss rate of YEp51 in wild-type cells was essentially identical to the loss rate in strains lacking the *SIR* genes (Table 2). Therefore, we conclude that Sir proteins are not required for *REP3*'s anchoring ability.

Rep1 and Rep2 antagonize conventional silencing: As controls for our experiments demonstrating a requirement for the 2μ circle to mediate *REP3* silencing, we also isolated cir^0 versions of a number of strains lacking chromosomal *REP3* sequences. Unexpectedly, the loss of the 2μ circle increased the mating ability of some of these strains. This effect was most pronounced when comparing strains DMY19 and YSH406, which differ only by the absence of the 2μ circle in YSH406. In these strains, the Orc- and Rap1-binding sites of the *HML-E* silencer are replaced by the *SUP4* gene (MAHONEY and BROACH 1989; see Figure 4). One possible explanation for this increase is that the Rep proteins antagonize conventional silencing; the loss of Rep1 and Rep2 proteins in the cir^0 strain would then lead to improved silencing. This model predicts that elevating the expression of the Rep1 and Rep2 proteins might decrease conventional silencing. To test this prediction, we constructed cir^0 strains that had integrated copies of the *REP1* and *REP2* genes, each fused to the inducible *GAL10* promoter. These strains also contain the yeast *ADE2* gene integrated at the *HMR* locus, placing its transcription under control of the *HMR* silencer sequences. Yeast strains that do not express the Ade2 protein accumulate a red pigment when grown on media containing limiting adenine. Therefore, a change in silencing in these strains produces detectable changes in colony color. We compared a control strain with strains overexpressing *REP1*, *REP2*, or both *REP1* and *REP2* (Figure 5). We observed that increasing the levels of either Rep1 or Rep2 proteins had no effect on expression of the *ADE2* reporter, while overexpression of both *REP1* and *REP2* appears to disrupt silencing, allowing increased expression of *ADE2*. A straightforward model for this antagonism is that the Rep proteins bind and titrate a limiting factor for silencing, such as the Sir proteins. To further investigate the interaction of the Rep and Sir proteins, we examined whether overexpression of *SIR3* or *SIR4* affects the stability of plasmids that rely on the *REP3* sequence (and Rep proteins) for segregation function (Table 2). In this experiment we observed no effects of elevating Sir proteins on *REP3* plasmid stability and therefore failed to find evidence for a direct Rep-Sir interaction. However, interactions with a Sir protein complex could depend on normal ratios of individual Sir proteins and not be detected in these experiments.

Strain	Genotype
YSH392	*ΔE::REP3-HML-ΔI SIR$^+$*
YSH422	*ΔE ::REP3-HML-ΔI Δsir2*
YSH417	*ΔE ::REP3-HML-ΔI Δsir3*
YSH418	*ΔE ::REP3-HML-ΔI Δsir4*

FIGURE 3.—*REP3* silencing is Sir dependent. The mating ability of a set of strains deleted for different *SIR* genes is shown.

TABLE 2

***REP3* plasmid stability assays**

Strain	Genotype	Loss rate (% per generation)
YSH316	Wild type	2.47 ± 0.69
YSH323	Δ*sir2*	1.83 ± 0.94
YSH496	Δ*sir3*	1.56 ± 0.73
YSH312	Δ*sir4*	3.09 ± 0.44
YSH480	*GAL-SIR3*	3.33 ± 1.07
YSH479	*GAL-SIR4*	2.96 ± 0.29
YSH474	Wild type	0.59 ± 1.08
YSH482	Δ*mlp1*	0.87 ± 0.88
YSH512	Δ*mlp2*	0.91 ± 0.35
YSH508	Δ*mlp1* Δ*mlp2*	2.16 ± 1.11
YSH565	Δ*yku70*	0.63 ± 0.44
YSH566	Δ*yku80*	1.20 ± 0.33
YSH564	Δ*esc1*	1.77 ± 0.63

The loss rates of YEp51 or YEp53 were measured in the indicated strains; a partial genotype is shown. YEp51 and YEp53 depend on *REP3* for segregation function. Plasmid stability assays with YSH480 and YSH479 were carried out in raffinose/galactose media, causing overexpression of *SIR3* or *SIR4*, respectively.

***Trans*-factors influencing *REP3* silencing:** During the course of our experiments an independent study was published describing a form of transcriptional repression mediated by the 2μ circle replication origin sequences (Grunweller and Ehrenhofer-Murray 2002). This silencing depended on the Sir proteins, as well as on the Mig1 and Hst3 proteins; the 2μ origin sequence mediating silencing contains likely binding sites for the Mig1 protein (Grunweller and Ehrenhofer-Murray 2002). To examine the similarity of this silencing to the one we describe here, we determined the influence of Mig1p and Hst3p on *REP3* silencing (Figure 6). We find that deletion of the *HST3* gene has no measurable effect on *REP3* silencing, while deletion of *MIG1* increases *REP3* silencing. There are no close matches to the Mig1p-binding site in the *REP3* sequence used in our experiments, so the effect of deleting the *MIG1* gene is likely to be indirect. We conclude that *REP3*-mediated silencing is distinct from that caused by the 2μ origin.

Prior studies have defined distinct candidates for proteins that serve as tethering sites involved in silencing and/or plasmid anchoring. The Mlp1 and Mlp2 proteins are associated with the nuclear pore complex and have been reported to influence both telomere localization and telomere silencing (Galy *et al.* 2000; Feuerbach *et al.* 2002), although this effect has not been observed in all cases examined (Andrulis *et al.* 2002; Hediger *et al.* 2002a). The Ku heterodimer, coded for by the *YKU70* and *YKU80* genes, interacts with telomeres and influences both telomere localization and silencing (Gravel *et al.* 1998; Martin *et al.* 1999; Hediger *et al.* 2002b). Finally, Esc1 is a protein located at the nuclear periphery that binds Sir4 and is required for Sir4-mediated plasmid segregation (Andrulis *et al.* 2002). We next tested the influence of these proteins on *REP3* silencing and *REP3* plasmid stability (Figure 6 and Table 2). *REP3* silencing is not affected in a strain missing both *MLP1* and *MLP2*, nor is *REP3* silencing decreased in strains lacking *YKU70* or *YKU80*. Instead, deletion of *YKU80* improves *REP3* silencing. This could be due to release of Sir proteins from the telomere, increasing their concentration at other locations (Martin *et al.* 1999), although prior studies generally observed identical phenotypes when deleting *YKU70* or *YKU80*. Finally, strains lacking the *ESC1* gene show no changes in *REP3* silencing. None of these genes had significant effects on *REP3* plasmid stability (Table 2).

The *REP3* sequence has been shown to associate with members of the cohesin protein complex (Mehta *et al.* 2002). The specific role of cohesins in *REP3*'s segregation function is not yet clear, but cohesins may mediate an association of the 2μ circle with chromosomes (Mehta *et al.* 2002). The genes coding for cohesins are essential; therefore, to examine their influence on *REP3* silencing we replaced the wild-type copy of the *MCD1/SCC1* cohesin gene with the *scc1-73* temperature-sensitive allele (Michaelis *et al.* 1997) in a strain exhibiting *REP3* silencing. We tested the effects of this mutation at 30°, a temperature at which the strain exhibits wild-type growth properties. Figure 7 shows that this mutation markedly reduces *REP3* silencing. This is unlikely to be an indirect effect of reducing the copy number of 2μ circle in our strain; all gene deletions described in this work had no effects on 2μ copy number, with the exception of the *MIG1* knockout, in which 2μ copy

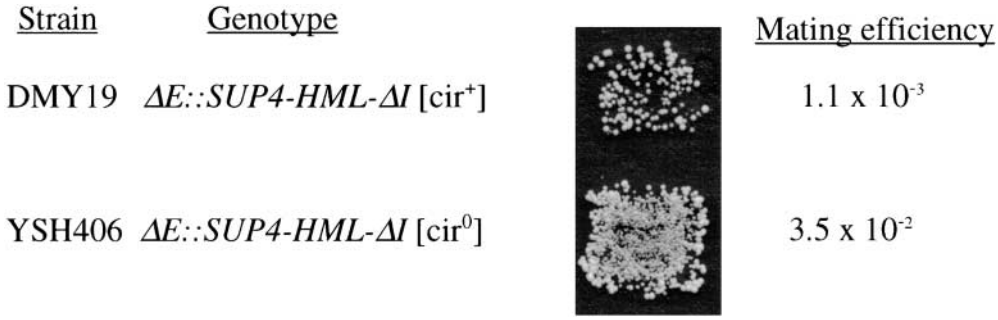

Figure 4.—Increased mating of a [cir^0] strain. The mating ability of congenic [cir$^+$] and [cir^0] strains is compared.

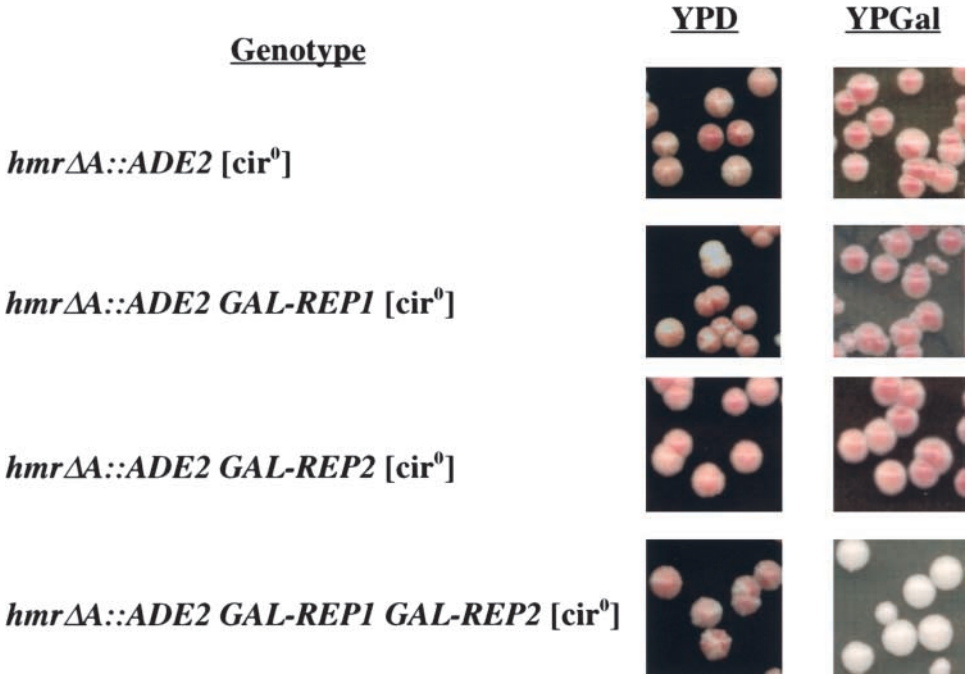

FIGURE 5.—Overexpression of the Rep1 and Rep2 proteins decreases silencing. The results of a colony color assay for silencing at *HMR* are shown. Transcription of the *ADE2* gene is placed under control of the *HMR* silencer sequences. A decrease in the expression of *ADE2* leads to an increase in red color. A partial genotype of each strain is listed. *hmrΔA* strains lack the *HMR-E* ACS element (the ORC-binding site). Overexpression of *REP1* and *REP2* had no effect on the expression of *ADE2* when at its normal chromosomal location (not shown).

number was reduced (not shown). As a control for the *scc1-73* experiment, we isolated a revertant of this strain that had regained wild-type growth at 37°. We observed that this strain also reverted the mating defect (Figure 7).

DISCUSSION

We have defined a new form of silencing in yeast mediated by the 2μ circle *REP3* sequence. This sequence was previously defined as a *cis*-acting element required for stable mitotic segregation of the yeast 2μ plasmid. The behavior of plasmids bearing the *REP3* sequence suggests that by binding the Rep1 and Rep2 proteins *REP3* mediates attachment to a nuclear structure. A similar proposal has been put forth to explain the stable segregation and anchoring mediated by the Sir4- and Rap1-silencing proteins (ANSARI and GARTENBERG 1997; ANDRULIS *et al.* 2002). Here we have shown that, like sequences bound by Rap1 or Sir4, the *REP3* sequence can mediate transcriptional repression, raising the possibility that anchoring ability is linked to the ability to silence gene expression. Despite these similarities, the plasmid-anchoring mechanisms mediated by Sir4, Rap1, and the Rep proteins appear to be distinct. Rap1 and Sir4 are found at the nuclear periphery at discrete locations that include the Sir2, Sir3, and Ku proteins (GOTTA *et al.* 1996; LAROCHE *et al.* 1998). The simple model that these foci provide attachment points for plasmids bound by Rap1 and Sir4 is likely not true; instead, attachment appears to be mediated by the Sir4-binding protein Esc1, a nuclear peripheral protein with a localization distinct from the well-characterized Sir/Rap1 foci (ANDRULIS *et al.* 2002). While the Rep1 and Rep2 proteins are also found in foci that may be preferentially localized to the nuclear periphery (SCOTT-DREW and MURRAY 1998), Rep protein clustering is not coincident with Sir protein foci (SCOTT-DREW *et al.* 2002). We also show in this study that deletion of *YKU70*, a mutation that eliminates Sir/Rap1 protein clustering (LAROCHE *et al.* 1998), does not diminish *REP3* silencing. In addition, while deletion of the *ESC1* gene eliminates Sir4-mediated plasmid segregation, it does not affect the ability of *REP3* to act as an anchor (ANDRULIS *et al.* 2002) or mediate segregation of *REP3* plasmids (Table 2). Finally, the Mlp and Yku proteins have been shown to contribute to telomere localization and telomere silencing (LAROCHE *et al.* 1998 ; GALY *et al.* 2000; FEUERBACH *et al.* 2002); here we observed no significant effects of deleting genes for these proteins on *REP3* plasmid segregation, and these gene deletions did not diminish *REP3* silencing. Therefore, known mediators of Sir4-mediated plasmid segregation and Sir-protein clustering do not significantly affect *REP3* silencing.

The observation that deletion of *ESC1* has only subtle effects on telomeric silencing led to a proposal that Esc1p could represent one of multiple redundant pathways that function to localize silencing complexes (ANDRULIS *et al.* 2002). Our observation that the *REP3* sequence mediates Sir-dependent transcriptional repression raises the possibility that it may utilize one of these proposed alternate pathways. This model could accommodate the observation that Rep protein overexpression antagonizes conventional silencing; if Rep proteins and silencing factors share an anchoring site, alterations in Rep protein concentration could alter accessibility of the site to silencing factors. Experiments demonstrating that recruiting telomere or *HMR*-linked reporters to the nuclear membrane using a lipid-dock-

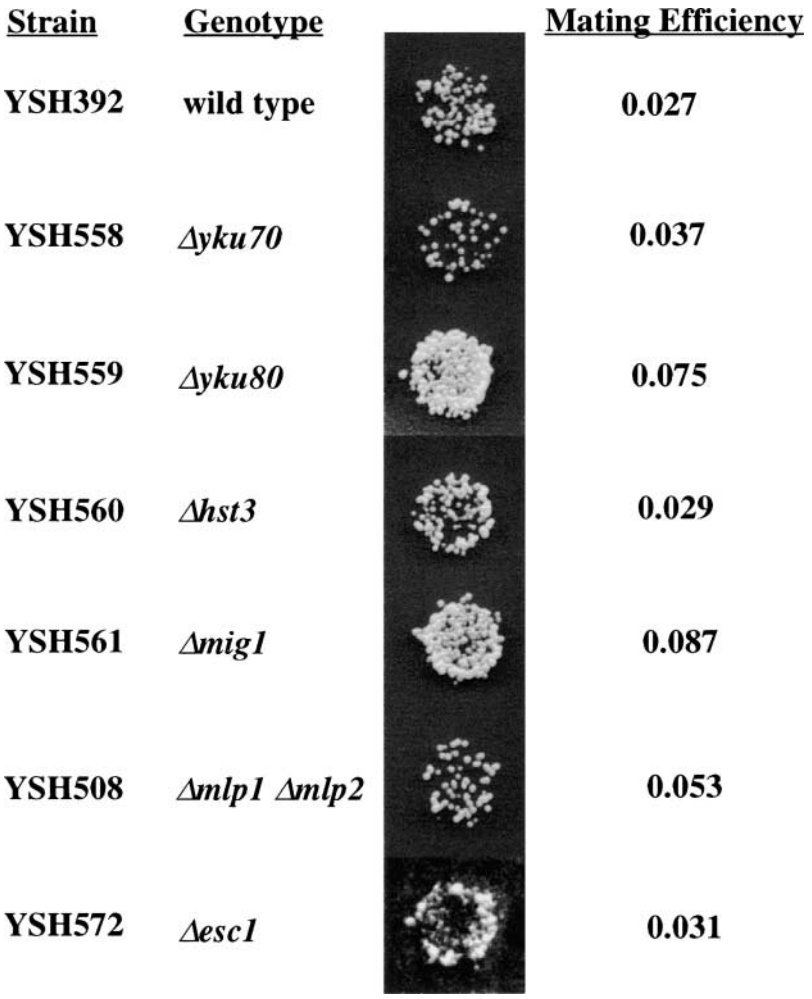

FIGURE 6.—*Trans*-factors and *REP3* silencing. Qualitative and quantitative mating assays were performed on strains differing only at the indicated loci.

ing strategy (ANDRULIS *et al.* 1998, FEUERBACH *et al.* 2002) suggest that fairly nonspecific localization can aid silencing. However, thus far there is no direct evidence that the Rep system anchors plasmids to a peripheral nuclear position, and the mechanism for *REP3*'s ability to mediate plasmid segregation is not clear. MEHTA *et al.* (2002) have proposed a mechanism for segregation of the 2μ circle on the basis of the ability of the *REP3* sequence to attract the cohesin complex and to associate with chromosomes. However, the contribution of these properties to *REP3*'s anchoring and silencing abilities is also unclear. The 2μ circle does not appear to associate with the mitotic spindle (SCOTT-DREW *et al.* 2002), nor is it associated with cohesin-binding sites on chromosomes (SCOTT-DREW *et al.* 2002). Here we observe that a mutation in the *MCD1/SCC1* cohesin gene affects *REP3* silencing, but does not alter 2μ copy number.

REP3 silencing may occur via a mechanism that is independent of its anchoring and partitioning functions. For instance, *REP3* may directly or indirectly re-

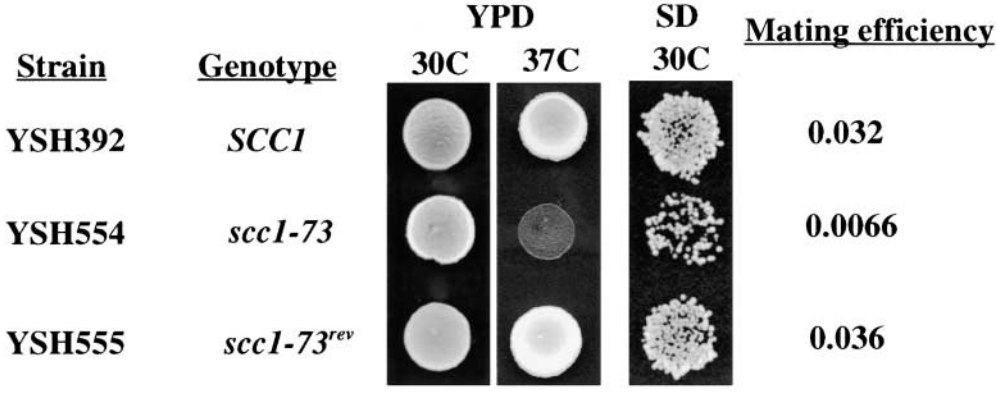

FIGURE 7.—*MCD1/SCC1* influences *REP3* silencing. The temperature-dependent growth and mating efficiencies of strains differing only at the *MCD1/SCC1* locus are shown. The $SCC1^{REV}$ strain (YSH555) is a revertant of strain YSH554 that has growth characteristics indistinguishable from the parent (YSH392) strain. YPD columns show growth of the three strains on nonselective media at the indicated temperatures. The SD column shows the results of a qualitative mating test performed at 30°.

cruit the Sir proteins. The *REP3* sequence we used does not contain consensus sequences for known silencer-binding factors, which recruit the Sir proteins to their known sites of action. Sir proteins could be attracted via interactions with Rep1, Rep2, or other *REP3*-associated proteins. We also note that the *HML*α promoter sequences contain a Rap1-binding site that has been shown to contribute to silencing in some contexts (Cheng and Gartenberg 2000); thus, a minimal ability to attract Sir proteins may be important for the silencing we have observed, a phenomenon also observed using the lipid-docking approach (Andrulis *et al.* 1998). Similar context effects may explain why the *REP3* sequence was not found to mediate significant silencing of a plasmid-based reporter gene (Grunweller and Ehrenhofer-Murray 2002). Genes found on the 2μ circle are subject to complex transcriptional regulation (Som *et al.* 1988; Veit and Fangman 1988; Grunweller and Ehrenhofer-Murray 2002). In particular, the *FLP1*, *REP1*, and *RAF* genes of the 2μ circle are transcriptionally repressed by a mechanism that depends on the Rep1 and Rep2 proteins (Som *et al.* 1988; Veit and Fangman 1988). Thus, *REP3* may participate in controlling gene expression in its natural context.

One set of proteins known to associate with *REP3* via interactions with Rep1 and Rep2 is the cohesin complex (Mehta *et al.* 2002). We show here that mutations in the Mcd1/Scc1 cohesin reduce *REP3* silencing. Cohesins have been identified in two prior contexts related to Sir-dependent silencing. First, the Smc1 and Smc3 cohesins were found to be important for establishing a boundary to silencing at the *HMR* locus (Donze *et al.* 1999). Second, depletion of the Mcd1/Scc1 cohesin allowed establishment of silencing to occur earlier in the cell cycle (Lau *et al.* 2002). Therefore, cohesins may influence the association of Sir proteins with target sequences. Our results are consistent with cohesins positively influencing the association of Sir proteins, leading to increased silencing of *REP3*-associated sequences.

We thank Karen Malatesta, Marc Gartenberg, Kim Nasmyth, and James Broach for providing strains and plasmids; Adrienne Woike, Xiaobin Li, and Marie Knight for assistance in strain construction and plasmid stability assays; and members of the Holmes lab for helpful discussions. This study was supported by grant RPG-98-351-01-MGO from the American Cancer Society and grant MCB-0096561 from the National Science Foundation to S.G.H.

LITERATURE CITED

Andrulis, E. D., A. M. Neiman, D. C. Zappulla and R. Sternglanz, 1998 Perinuclear localization of chromatin facilitates transcriptional silencing. Nature **394:** 592–595.

Andrulis, E. D., D. C. Zappulla, A. Ansari, S. Perrod, C. V. Laiosa *et al.*, 2002 Esc1, a nuclear periphery protein required for Sir4-based plasmid anchoring and partitioning. Mol. Cell. Biol. **22:** 8292–8301.

Ansari, A., and M. R. Gartenberg, 1997 The yeast silent information regulator Sir4p anchors and partitions plasmids. Mol. Cell. Biol. **17:** 7061–7068.

Brachmann, C. B., A. Davies, G. J. Cost, E. Caputo, J. Li *et al.*, 1998 Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast **14:** 115–132.

Braunstein, M., A. B. Rose, S. G. Holmes, C. D. Allis and J. R. Broach, 1993 Transcriptional silencing in yeast is associated with reduced nucleosome acetylation. Genes Dev. **7:** 592–604.

Broach, J. R., and F. C. Volkert, 1991 Circular DNA plasmids of yeast, pp. 297–331 in *The Molecular and Cellular Biology of the Yeast Saccharomyces*, edited by J. R. Broach, E. W. Jones and J. R. Pringle. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Carmen, A. A., L. Milne and M. Grunstein, 2002 Acetylation of the yeast histone H4 N terminus regulates its binding to heterochromatin protein SIR3. J. Biol. Chem. **277:** 4778–4781.

Cheng, T., and M. R. Gartenberg, 2000 Yeast heterochromatin is a dynamic structure that requires silencers continuously. Genes Dev. **14:** 452–463.

Chi, M.-H., and D. Shore, 1996 SUM1-1, a dominant suppressor of SIR mutations in Saccharomyces cerevisiae, increases transcriptional silencing at telomeres and HM mating-type loci and decreases chromosome stability. Mol. Cell. Biol. **16:** 4281–4294.

Chien, C. T., S. Buck, R. Sternglanz and D. Shore, 1993 Targeting of SIR1 protein establishes transcriptional silencing at HM loci and telomeres in yeast. Cell **75:** 531–541.

Cockell, M., F. Palladino, T. Laroche, G. Kyrion, C. Liu *et al.*, 1995 The carboxy termini of Sir4 and Rap1 affect Sir3 localization: evidence for a multicomponent complex required for yeast telomeric silencing. J. Cell Biol. **129:** 909–924.

Donze, D., C. R. Adams, J. Rine and R. T. Kamakaka, 1999 The boundaries of the silenced HMR domain in Saccharomyces cerevisiae. Genes Dev. **13:** 698–708.

Dula, M. L., and S. G. Holmes, 2000 MGA2 and SPT23 are modifiers of transcriptional silencing in yeast. Genetics **156:** 933–941.

Feuerbach, F., V. Galy, E. Trelles-Sticken, M. Fromont-Racine, A. Jacquier *et al.*, 2002 Nuclear architecture and spatial positioning help establish transcriptional states of telomeres in yeast. Nat. Cell Biol. **4:** 214–221.

Galy, V., J. C. Olivo-Marin, H. Scherthan, V. Doye, N. Rascalou *et al.*, 2000 Nuclear pore complexes in the organization of silent telomeric chromatin. Nature **403:** 108–112.

Gartenberg, M. R., and J. C. Wang, 1993 Identification of barriers to rotation of DNA segments in yeast from the topology of DNA rings excised by an inducible site-specific recombinase. Proc. Natl. Acad. Sci. USA **90:** 10514–10518.

Gotta, M., T. Laroche, A. Formenton, L. Maillet, H. Scherthan *et al.*, 1996 The clustering of telomeres and colocalization with Rap1, Sir3, and Sir4 proteins in wild-type Saccharomyces cerevisiae. J. Cell Biol. **134:** 1349–1363.

Gotta, M., S. Strahl-Bolsinger, H. Renauld, T. Laroche, B. K. Kennedy *et al.*, 1997 Localization of Sir2p: the nucleolus as a compartment for silent information regulators. EMBO J. **16:** 3243–3255.

Gravel, S., M. Larrivee, P. Labrecque and R. J. Wellinger, 1998 Yeast Ku as a regulator of chromosomal DNA end structure. Science **280:** 741–744.

Grunweller, A., and A. E. Ehrenhofer-Murray, 2002 A novel yeast silencer: the 2μ origin of *Saccharomyces cerevisiae* has HST3-, MIG1- and SIR-dependent silencing activity. Genetics **162:** 59–71.

Hecht, A., T. Laroche, S. Strahl-Bolsinger, S. M. Gasser and M. Grunstein, 1995 Histone H3 and H4 N-termini interact with SIR3 and SIR4 proteins: a molecular model for the formation of heterochromatin in yeast. Cell **80:** 583–592.

Hediger, F., K. Dubrana and S. M. Gasser, 2002a Myosin-like proteins 1 and 2 are not required for silencing or telomere anchoring, but act in the Tel1 pathway of telomere length control. J. Struct. Biol. **140:** 79–91.

Hediger, F., F. R. Neumann, G. Van Houwe, K. Dubrana and S. M. Gasser, 2002b Live imaging of telomeres: yKu and Sir proteins define redundant telomere-anchoring pathways in yeast. Curr. Biol. **12:** 2076–2089.

Holmes, S. G., A. B. Rose, K. Steuerle, E. Saez, S. Sayegh *et al.*, 1997 Hyperactivation of the silencer proteins Sir2p and Sir3p causes chromosome loss. Genetics **145:** 605–614.

Horton, R. M., H. D. Hunt, S. N. Ho, J. K. Pullen and L. R. Pease, 1989 Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene **77:** 61–68.

Huang, Y., 2002 Transcriptional silencing in Saccharomyces cerevisiae and Schizosaccharomyces pombe. Nucleic Acids Res. **30:** 1465–1482.

Imai, S., C. M. Armstrong, M. Kaeberlein and L. Guarente, 2000 Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature **403:** 795–800.

Jayaram, M., A. Sutton and J. R. Broach, 1985 Properties of REP3: a cis-acting locus required for stable propagation of the Saccharomyces cerevisiae plasmid 2 microns circle. Mol. Cell. Biol. **5:** 2466–2475.

Kikuchi, Y., 1983 Yeast plasmid requires a cis-acting locus and two plasmid proteins for its stable maintenance. Cell **35:** 487–493.

Kimmerly, W., A. Buchman, R. Kornberg and J. Rine, 1988 Roles of two DNA-binding factors in replication, segregation and transcriptional repression mediated by a yeast silencer. EMBO J. **7:** 2241–2253.

Landry, J., A. Sutton, S. T. Tafrov, R. C. Heller, J. Stebbins *et al.*, 2000 The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases. Proc. Natl. Acad. Sci. USA **97:** 5807–5811.

Laroche, T., S. G. Martin, M. Gotta, H. C. Gorham, F. E. Pryde *et al.*, 1998 Mutation of yeast Ku genes disrupts the subnuclear organization of telomeres. Curr. Biol. **8:** 653–656.

Lau, A., H. Blitzblau and S. P. Bell, 2002 Cell-cycle control of the establishment of mating-type silencing in S. cerevisiae. Genes Dev. **16:** 2935–2945.

Longtine, M. S., S. Enomoto, S. L. Finstad and J. Berman, 1992 Yeast telomere repeat sequence (TRS) improves circular plasmid segregation, and TRS plasmid segregation involves the RAP1 gene product. Mol. Cell. Biol. **12:** 1997–2009.

Mahoney, D. J., and J. R. Broach, 1989 The *HML* mating-type cassette of *Saccharomyces cerevisiae* is regulated by two separate but functionally equivalent silencers. Mol. Cell. Biol. **9:** 4621–4630.

Martin, S. G., T. Laroche, N. Suka, M. Grunstein and S. M. Gasser, 1999 Relocalization of telomeric Ku and SIR proteins in response to DNA strand breaks in yeast. Cell **97:** 621–633.

Mehta, S., X. M. Yang, C. S. Chan, M. J. Dobson, M. Jayaram *et al.*, 2002 The 2 micron plasmid purloins the yeast cohesin complex: A mechanism for coupling plasmid partitioning and chromosome segregation? J. Cell Biol. **158:** 625–637.

Michaelis, C., R. Ciosk and K. Nasmyth, 1997 Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. Cell **91:** 35–45.

Mirabella, A., and M. R. Gartenberg, 1997 Yeast telomeric sequences function as chromosomal anchorage points in vivo. EMBO J. **16:** 523–533.

Palladino, F., T. Laroche, E. Gilson, A. Axelrod, L. Pillus *et al.*, 1993 SIR3 and SIR4 proteins are required for the positioning and integrity of yeast telomeres. Cell **75:** 543–555.

Rusche, L. N., A. L. Kirchmaier and J. Rine, 2003 The establishment, inheritance, and function of silenced chromatin in Saccharomyces cerevisiae. Annu. Rev. Biochem. **27:** 27.

Scott-Drew, S., and J. A. Murray, 1998 Localisation and interaction of the protein components of the yeast 2μ circle plasmid partitioning system suggest a mechanism for plasmid inheritance. J. Cell Sci. **111:** 1779–1789.

Scott-Drew, S., C. M. Wong and J. A. Murray, 2002 DNA plasmid transmission in yeast is associated with specific sub-nuclear localisation during cell division. Cell Biol. Int. **26:** 393–405.

Shei, G. J., and J. R. Broach, 1995 Yeast silencers can act as orientation-dependent gene inactivation centers that respond to environmental signals. Mol. Cell. Biol. **15:** 3496–3506.

Sikorski, R., and P. Hieter, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae.* Genetics **122:** 19–27.

Smith, J. S., C. B. Brachmann, I. Celic, M. A. Kenna, S. Muhammad *et al.*, 2000 A phylogenetically conserved NAD+-dependent protein deacetylase activity in the Sir2 protein family. Proc. Natl. Acad. Sci. USA **97:** 6658–6663.

Som, T., K. A. Armstrong, F. C. Volkert and J. R. Broach, 1988 Autoregulation of 2 micron circle gene expression provides a model for maintenance of stable plasmid copy levels. Cell **52:** 27–37.

Tham, W. H., J. S. Wyithe, P. K. Ferrigno, P. A. Silver and V. A. Zakian, 2001 Localization of yeast telomeres to the nuclear periphery is separable from transcriptional repression and telomere stability functions. Mol. Cell **8:** 189–199.

Veit, B. E., and W. L. Fangman, 1988 Copy number and partition of the Saccharomyces cerevisiae 2 micron plasmid controlled by transcription regulators. Mol. Cell. Biol. **8:** 4949–4957.

Velmurugan, S., Y. T. Ahn, X. M. Yang, X. L. Wu and M. Jayaram, 1998 The 2 micrometer plasmid stability system: analyses of the interactions among plasmid- and host-encoded components. Mol. Cell. Biol. **18:** 7466–7477.

Velmurugan, S., X. Yang, C. S.-M. Chan, M. Dobson and M. Jayaram, 2000 Partitioning of the 2 micrometer circle plasmid of Saccharomyces cerevisiae: functional coordination with chromosome segregation and plasmid-encoded Rep protein distribution. J. Cell Biol. **149:** 553–566.

Volkert, F. C., and J. R. Broach, 1986 Site-specific recombination promotes plasmid amplification in yeast. Cell **46:** 541–550.

Wach, A., A. Brachat, R. Pohlmann and P. Philippsen, 1994 New heterologous modules for classical or PCR-based gene disruptions in Saccharomyces cerevisiae. Yeast **10:** 1793–1808.

Wu, L. C., P. A. Fisher and J. R. Broach, 1987 A yeast plasmid partitioning protein is a karyoskeletal component. J. Biol. Chem. **262:** 883–891.

Communicating editor: L. Pillus