A Fission Yeast Repression Element Cooperates With Centromere-like Sequences and Defines a *mat* Silent Domain Boundary

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ABSTRACT

REII is a *Schizosaccharomyces pombe* repression element located at the centromere-proximal end of the *mat* silent domain. Here we show that inversion of REII enhances silencing on its centromere-proximal side while suppressing silencing on its centromere-distal side. Transplacement of REII to a position 2.5 kb from its native locus extends the region of stringent repression to the new REII site. These results suggest that REII defines a *mat* silent domain boundary by acting preferentially toward its centromere-distal side. To investigate cooperation between REII and a *K*-region sequence that shares homology with the centromeric *dg dh* repeats (*cen2* homology), we targeted combinations of these elements to an ectopic site and monitored expression of an adjacent reporter gene. Centromeric *dh*-like sequences conferred low-level silencing on the adjacent reporter gene, and REII, which did not display silencing activity on its own, enhanced *cen2* homology-mediated silencing. Cooperation was also apparent at the *mat* locus, where deletion of REII impaired repression stability. We propose that REII and the *cen2* homology play different yet complementary roles in silencing establishment and inheritance at the *mat* locus.

CHROMOSOMES of eukaryotic cells are organized into discrete functional domains that delineate independent units of gene activity. The expression state within each unit is controlled autonomously by *cis*-acting elements that recruit transcription factors or chromatin remodeling proteins and by boundary elements that protect internal genes from the long-range effect of external enhancers or silencers (reviewed in Gerasimova and Corces 1996; Geyer 1997; Kamakaka 1997; Kioussis and Festenstein 1997; Sherman and Pillus 1997; Kellum and Elgin 1998; Sun and Elgin 1999; Udvardy 1999). Boundaries between constitutively expressed and repressed domains are not always distinct, and genes in peripheral regions may be subjected to stochastic, but clonally inherited repression. This phenomenon, named position effect variegation (PEV), was first described in Drosophila (Muller 1930). Since then, PEV was observed in other organisms, including budding and fission yeast (Gottschling *et al.* 1990; Allshire *et al.* 1994; Karpen 1994; Eissenberg *et al.* 1995; Henikoff and Matzke 1997; Ayoub *et al.* 1999). A variegated phenotype may also be caused by alternative states of chromatin assembly, affecting the entire length of the silent domain (Pillus and Rine 1989; Grewal and Klar 1996; Thon and Friis 1997; Cavalli and Paro 1998b; Klar *et al.* 1998).

The fission yeast *Schizosaccharomyces pombe* switches its mating type by transposing a copy of unexpressed genes from the respective *mat2-P* or *mat3-M* donor cassettes to the transcriptionally active *mat1* (Beach 1983; Beach and Klar 1984). The donor cassettes flank a 10.9-kb region, named *K*, which is also stringently repressed (Thon and Klar 1992; Thon *et al.* 1994; Grewal and Klar 1997). The silent *mat2-K-mat3* domain is separated from the transcriptionally active *mat1* by the *L* region (Figure 1). Several lines of evidence indicate that repression at the *mat* locus is accomplished by chromatin remodeling: silencing is regional, rather than gene specific (Thon *et al.* 1994; Grewal and Klar 1997; Ayoub *et al.* 1999); mutations in silencing genes increase DNA accessibility in the repressed region to *in vivo* methylation (Sing *et al.* 1998); two silencing genes, *swi6* and *clr4*, encode chromodomain proteins (Lorentz *et al.* 1994; Grewal *et al.* 1998) that, like their Drosophila and mammalian counterparts, are likely to play a role in maintaining epigenetically controlled chromatin states (Platero *et al.* 1995; Cavalli and Paro 1998a); two other silencing genes, *clr3* and *clr6*, encode putative histone deacetylases (Ivanova *et al.* 1998); and transient exposure to trichostatin A, a specific inhibitor of histone deacetylases (Yoshida *et al.* 1990), has a long-lasting derepression effect (Grewal *et al.* 1998; Olsson *et al.* 1999).

Repression is gradually alleviated with increasing the distance from *mat2-P*, leading to variegated expression of reporter genes along a stretch of ~3 kb in the *L* region (Ayoub *et al.* 1999). Separation between the stringently repressed domain and the region of variegated expression appears to be controlled by a *cis*-acting element,

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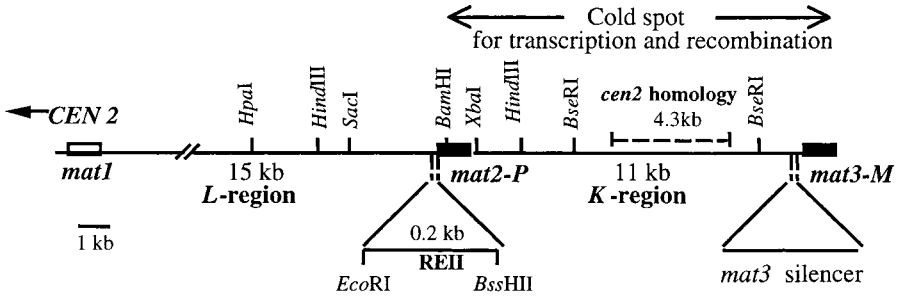


FIGURE 1.—The mating-type region of *S. pombe*. The *mat2-P* and *mat3-M* mating-type donor cassettes are located within a silent domain of ~17 kb and are separated from each other by a repressed region named *K*. The silent *mat2-K-mat3* domain is separated from the transcriptionally active *mat1* by a region of ~15 kb named *L*. *S. pombe* switches its mating type by transposing a copy of the unexpressed genes from the respective *mat2-P* and *mat3-M* cassettes to *mat1*. The locations of the silent donor cassettes (solid boxes), *mat1* (open box), *cen2* homology, REII, *mat3* silencer, and relevant restriction sites are indicated.

named REII, located at the junction between *mat2-P* and the *L* region. REII was first identified as one of four *cis*-acting elements that cooperatively repress plasmid-borne *mat2-P* genes (EKWALL *et al.* 1991). Its deletion from the chromosome has only a subtle effect on the repressed state of *mat2-P*. Yet this deletion markedly enhances *mat2-P* expression in *swi6* or *clr1-clr4* mutants (THON *et al.* 1994, 1999; AYOUB *et al.* 1999). Intriguingly, REII inhibits the propagation of an active state, associated with gene expression in the *L* region, into the silent domain. Thus, in REII deletion mutants, expression of *mat2-P* and an adjacent *ura4*$^+$ gene in the *K* region covariegates uniformly with *ade6*$^+$ expression from the *L* region. Yet in strains with REII intact, genes in the silent domain are stringently repressed, regardless of the expression state in the *L* region (AYOUB *et al.* 1999). The mechanism by which REII ensures separation between the regions of stringent and variegated repression is not yet understood. One possibility is that REII acts as a repression element with a preferred directionality that helps assemble a heterochromatin complex on its centromere-distal side. The other is that REII acts as an insulator that inhibits the spreading of a transcriptionally active chromatin state into the *mat2-K-mat3* domain. These two possibilities are not necessarily mutually exclusive.

Repression at the chromosomal *mat2-K-mat3* region is controlled by at least two additional *cis*-acting elements. One element, located near *mat3-M*, controls the repressed state of *mat3* and of markers at the centromere-distal part of the silent domain (THON *et al.* 1999). The other element, located within the *K* region, affects the expression state along the entire silent domain. Deletion of a 7.5-kb fragment from the *K* region lowers repression frequency, but the alternative epigenetic states are stably inherited in mitosis and meiosis. The *K* region also plays an important role in controlling mating-type switching directionality, as is evident from the observation that in *K*Δ mutants switching competence covariegates with silencing (GREWAL and KLAR 1996; THON and FRIIS 1997). Remarkably, about one-third of the *K* region is homologous to the *dgIIa* and *dhIIa* centromeric repeat (GREWAL and KLAR 1997). Because transgenes in the centromeres are subjected to position effect repression (ALLSHIRE *et al.* 1994), it has been postulated that the *cen2* homology is an active repression element in the *K* region (GREWAL and KLAR 1997).

To investigate the role of REII in promoting silencing at the *mat* silent domain, we examined the effect of its deletion, transplacement, or inversion on the expression state of reporter genes within the silent domain and at its periphery. We also attempted to create a synthetic silent domain at an ectopic site by inserting a reporter gene with various combinations of the centromeric outer repeat homology and REII and by monitoring reporter gene expression. The results indicate that *dh* sequences within the *cen2* homology were sufficient to establish repression at an ectopic site. REII has no detectable silencing activity on its own. Yet it acts with a preferred directionality and cooperates with the *cen2* homology to enhance silencing stability and define the boundary of the silent domain at the *mat* locus.

MATERIALS AND METHODS

Strain construction: All strains used in this study and their genotypes are listed in Table 1. Molecular manipulations of cloned *Hin*dIII or *Hin*dIII-*Bgl*II fragments, carrying *mat2* and parts of the flanking *L* and *K* regions (BEACH and KLAR 1984), were performed on derivatives of Bluescript (ALTING-MEESE and SHORT 1989). To insert an *ade6*$^+$ gene or an REII cassette into the designated *mat* locations, chimera plasmids were linearized by the appropriate restriction endonuclease and ligated to a 3.5-kb *Pvu*II-*Sma*I fragment carrying *ade6*$^+$ (SZANKASI *et al.* 1988) or a 0.23-kb *Eco*RI-*Bss*HII fragment containing an REII cassette (EKWALL *et al.* 1991). Where necessary, single-stranded ends were converted to blunt ends by T4 DNA polymerase-mediated synthesis or digestion. To insert *ade6*$^+$ into the *Hpa*I site in the *L* region, the appropriate 5.5-kb *Sac*I fragment of the *L* region was isolated from the CM740 cosmid (MIZUKAMI *et al.* 1993) and cloned into the *Sac*I site of Bluescript. The Bluescript derivative (pNA1) was linearized by *Hpa*I digestion and ligated to a *Sma*I-*Pvu*II fragment carrying the *ade6*$^+$ gene (pNA2).

Targeted integration into the *mat* region was accomplished by transformation with purified restriction fragments of the appropriate plasmids. To overcome the recombination block at the *mat2-mat3* interval (EGEL 1984), targeted integration to this interval was performed in a *clr1* mutant (PG377).

Molecular manipulations of a cloned 1.8-kb *Hin*dIII fragment carrying the *ura4*$^+$ gene (BACH 1987) were conducted on a Bluescript derivative (pNA3). The *Spe*I site in Bluescript was inactivated before *ura4*$^+$ insertion by *Spe*I digestion, conversion of the single-stranded overhangs to blunt ends and

TABLE 1

***S. pombe* strains used in this study**

Strains[a]	Relevant genotype	Source
AP125	*LΔ(BssHII-EcoRI) K(XbaI)::ura4⁺ ade6-210 ura4-D18*	Ayoub *et al.* (1999)
AP128	*mat1-PΔ17::LEU2 ura4-D18*	This study
AP165	*L(SacI)::ade6⁺ K(XbaI)::ura4⁺ ade6-210 ura4-D18*	Ayoub *et al.* (1999)
AP179	*ura4::ade6⁺-K$_{6.3}$ ade6-210*	This study
AP259	*ura4::ade6⁺-cen$_{3.6}$ ade6-210*	This study
AP262	*REII$_{inv}$ K(XbaI)::ura4⁺ clr1-165 ade6-210 ura4-D18*	This study
AP263	*ura4::ade6⁺-cen$_{3.6(inv)}$ ade6-210*	This study
AP264	*ura4::ade6⁺ ade6-210*	This study
AP270	*ura4*::REII-*ade6⁺-cen$_{3.6}$ ade6-210* [b]	This study
AP277	*ura4::ade6⁺-cen$_{1.4}$ ade6-210*	This study
AP278	*ura4::ade6⁺-cen$_{2.2}$ ade6-210*	This study
AP288	*ura4::ade6⁺-cen$_{0.58}$ ade6-210*	This study
AP293	*L(SacI)::ade6⁺ K(XbaI)::ura4⁺ ade6-DN/N ura4-D18*	This study
AP294	*L(SacI)::ade6⁺ REII$_{inv}$ K(XbaI)::ura4⁺ ade6-DN/N ura4-D18*	This study
AP295	*L(SacI)*::REII-*ade6⁺*Δ(*BssH*II-*Eco*RI) *K(XbaI)::ura4⁺ ade6-DN/N ura4-D18*	This study
AP313	*mat2(Bam*HI)::*ade6⁺ K(XbaI)::ura4⁺ ade6-210 ura4-D18*	Ayoub *et al.* (1999)
AP347	*L(SacI)::ade6⁺* Δ(*Bss*HII-*Eco*RI) *K(XbaI)::ura4⁺ade6-210 ura4-D18*	Ayoub *et al.* (1999)
AP363	*L*Δ (*Bss*HII-*Eco*RI) *mat2(Bam*HI)::*ade6⁺ K(XbaI)::ura4⁺ ade6-210 ura4-D18*	This study
AP377	*L*Δ(*Bss*HII-*Eco*RI) *K(XbaI)::ura4 clr1-165 ade6-210 ura4-D18*	This study
AP379	*ura4*::REII-*ade6⁺ ade6-210*	This study
AP383	*ura4*::REII-*ade6⁺-K$_{6.3}$ ade6-210*	This study
AP384	*ura4*::REII-*ade6⁺-K$_{6.3}$ ade6-210 swi6-116*	This study
AP389	*ura4*::REII$_{inv}$-*ade6⁺-K$_{6.3}$ ade6-210*	This study
AP394	*L(SacI)*::REII-*ade6⁺* Δ(*Bss*HII-*Eco*RI) *K(XbaI)::ura4⁺ ade6-210 ura4-D18*	This study
AP396	*L(SacI)*::REII$_{inv}$-*ade6⁺*Δ(*Bss*HII-*Eco*RI) *K(XbaI)::ura4⁺ade6-210 ura4-D18*	This study
AP407	*L(Hpa*I)::REII-*ade6⁺* Δ(*Bss*HII-*Eco*RI) *K(XbaI)::ura4⁺ ade6-210 ura4-D18*	This study
AP419	*ura4::cen$_{3.6}$*-REII-*ade6⁺ ade6-210*	This study
AP421	*mat2(Bam*HI)::*ade6⁺ K*Δ::*ura4⁺ ade6-210 ura4-D18*	This study
AP803	*ura4::ade6⁺-dg$_{0.56}$-ade6-210*	This study
AP804	*ura4::ade6⁺-cen$_{3.6}$ ade6-210*[b]	This study
SP1172	*ade6-210 ura4-D18*	A. J. Klar
PG377	*clr1-165 ade6-210 ura4-D18*	A. J. Klar
FY370	*h⁺ ade6-DN/N ura4-D18*	R. C. Allshire

[a] All strains, except AP128 and FY370, are *mat1-M-smt0 leu1-32 his2.*
[b] AP270 and AP804 are isogenic except for the orientation of the inserted *ade6⁺* fragment (Figure 7).

religation. *ade6⁺* or combinations of *ade6⁺* with the indicated REII and/or *K* region fragments (Figures 4 and 5) were inserted into the *Stu*I site within *ura4*. The 5.8-kb *cen2* homology fragment was generated by PCR using pSGK (Grewal and Klar 1997) as a template and ATGTCTACTTCAAAACTCGC and CCATGTTCCATTACATATCC as primers. Targeted integration of molecular constructs into the *ura4* locus was accomplished by transformation with purified *Sac*I-*Apa*I fragments of the appropriate plasmids. Integration of molecular constructs at the desired sites was confirmed by Southern hybridization analysis (Southern 1975). Standard genetic crosses (Moreno *et al.* 1991) were used in strain constructions.

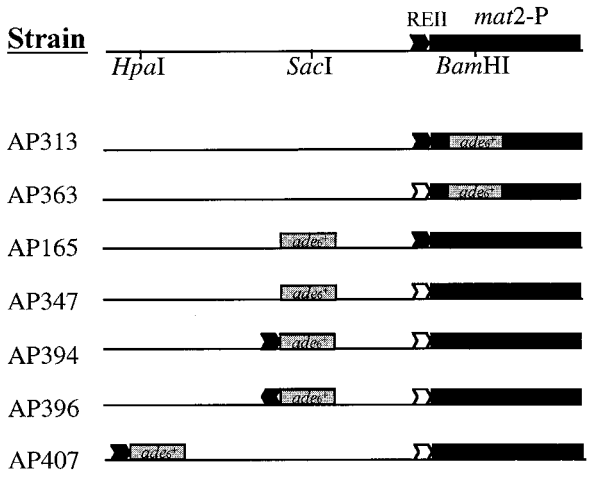
Northern analysis: Total cellular RNA was isolated from 10-ml aliquots of growing cultures (~10^7 cells/ml) in YEA medium, using the TRIZol reagent (GIBCO-BRL, Gaithersburg, MD), according to the manufacturer's protocol. All strains used for Northern analysis had an *ade6-DN/N* mutation (Ekwall *et al.* 1997), and the DNA probe used to detect the *ade6* transcript was a 150-nucleotide (nt) *Nco*I fragment homologous to the *DN/N* deletion. Thus, only transcripts of the reporter gene were detectable. Quantitation of relative transcript levels was by phosphorimaging analysis. Transcript levels were standardized relative to *his1* RNA, detected by hybridization to a 503-nt *his1* fragment, generated by PCR, using pBS-*his1* plasmid (R. Weisman, personal communication) as a template and ACAAGGTCGAGAAGAAAGCG and CCATCCAGGTTCATCCAAAG as primers.

Culture conditions: Strains were grown on rich medium (YEA), adenine-limiting rich medium (YE), or sporulation medium (PM-N) (Moreno *et al.* 1991). All incubations were at 30°. For scoring Ade phenotypes on YE plates, standard incubation periods were 4 days at 30°. More than 500 colonies from each of 5 independent colonies of each strain were examined. One standard deviation values (±) are presented.

Iodine staining: Haploid meiosis phenotype in heterothallic strains was examined by staining colonies on PM-N sporulation medium (Moreno *et al.* 1991) with iodine vapors, since spores, but not vegetative cells, contain a starch component. Plates were incubated for 4 days at 30° before staining (Bresch *et al.* 1968).

RESULTS

To explore the possibility that REII acts as a repression element with a preferred directionality, we determined the effect of its deletion, transplacement, or inversion



Strain	Ade phenotype		
	Red (-)	Pink (+/-)	White (+)
AP313	97.4±1.7	0.9±0.5	2.1±2
AP363	<0.1	11.3 ±4	88.7 ±4
AP165	<0.1	24.4 ± 2	75.5 ± 2
AP347	0.1 ± 0.1	9.0 ±5	90.9 ± 5
AP394	99.3 ±0.2	0.2 ± 0.1	0.6 ± 0.4
AP396	<0.1	3 ± 0.7	97 ± 0.7
AP407	<0.1	<0.1	100 ± 0

FIGURE 2.—The effect of REII deletion or transplacement on the expression state of *ade6*$^+$ at sites in the *mat* region. The diagrams show the locations of the various *ade6*$^+$ insertions and the transplacement or inversions of REII. Cells from patches on YEA plates were plated on low adenine (YE) medium and the percentages of red (Ade$^-$), white (Ade$^+$), and pink (intermediate levels of *ade6* repression) were determined. The locations of *mat2-P*, REII (arrowhead), and relevant restriction sites are indicated in the top diagram. A shaded box indicates an *ade6*$^+$ insertion, an open arrowhead indicates deleted REII, and a reversed arrowhead designates REII inversion.

on the expression state within the silent domain and its periphery. This was achieved by targeting an *ade6*$^+$ reporter gene to sites within *mat2-P* or the *L* region and monitoring *ade6* expression by examining colony color on low adenine medium (YE). On this medium, red and white colonies imply Ade$^-$ and Ade$^+$ phenotypes, respectively (MORENO *et al.* 1991), and pink colonies indicate intermediate levels of *ade6* repression (ALLSHIRE *et al.* 1994). Unless indicated otherwise, all strains used in these experiments carried the *ade6-210* mutation at the endogenous *ade6* locus.

REII enhances silencing stability: An *ade6*$^+$ reporter gene, located within the *mat2-P* cassette (*Bam*HI), is stringently repressed in >97% of the cell lines (AYOUB *et al.* 1999). Deletion of REII alleviated repression (Figure 2, AP363). Most colonies of the REII deletion mutant displayed an Ade$^+$ phenotype and the rest exhibited intermediate levels of repression. Deletion of REII had a lesser effect on *ade6* expression from the *Sac*I site in the *L* region. Normally, ~20% of the colonies of the *L*(*Sac*I)::*ade6*$^+$ strain (AP165) exhibit partial *ade6* repression (AYOUB *et al.* 1999). Deletion of REII (AP347) decreased the proportion of colonies exhibiting any degree of *ade6* repression to ~10%.

Deletion of a 7.5-kb *K*-region fragment alleviates repression at the *mat* silent domain, but the alternative expression states are stably maintained (GREWAL and KLAR 1996; THON and FRIIS 1997). To compare the effect of *K* and REII deletions on the stability of the alternative expression states within *mat2-P*, we constructed the appropriate derivatives of a *mat2-P*(*Bam*HI)::*ade6*$^+$ strain. Cell suspensions from white (Ade$^+$) or red (Ade$^-$) colonies on YE medium were replated on the same medium and incubated at 30°, and the proportion of red, white, and sectored colonies was determined (Table 2). The expressed state in the REII$^+$ *K*$^+$ control strain (AP313) was unstable. Less than 5% of the cells retained the Ade$^+$ phenotype upon replating and the rest yielded red or sectored colonies. Conversion from the expressed to the repressed state in the ΔREII mutant (AP363) was lower than that in the strain with REII intact, but higher than in the *K*Δ mutant (AP421). Consistent with earlier studies (GREWAL and KLAR 1996; THON and FRIIS 1997), deletion of the 7.5-kb *K*-region fragment had little or no effect on the stability of the repressed state within *mat2-P*. Less than 0.5% of the cells from Ade$^-$ colonies yielded Ade$^+$ colonies upon replating and the frequency of sectored colonies was ~1%. Unlike the *K*Δ mutation, the ΔREII mutation impaired the stability of the repressed state. About 6% of the cells from Ade$^-$ colonies yielded white (Ade$^+$) colonies and the frequency of sectored colonies exceeded 40% (Table 2). About half of the sectored colonies had multiple white (Ade$^+$) sectors. To estimate the rate of change per cell division, we determined the frequency of half-sectored colonies (ALLSHIRE *et al.* 1995). This frequency was 2.4% for the ΔREII strain and <0.1% for the isogenic strains with REII intact or with a *K*Δ mutation. These results indicate a role for REII in assuring silencing stability.

Transplacement of REII toward *mat1* extends the silent domain: The decline in repression stringency on the centromere-proximal side of REII (AYOUB *et al.* 1999) and the effect of REII deletion on reporter gene repression within *mat2-P* (Figure 2) suggest that REII is a repression element that defines the boundary of stringent repression. To test this proposition, we asked whether transplacement of REII toward *mat1* would extend the region of stringent repression. Transplacement of REII to the centromere-proximal side of *ade6*$^+$ at the *Sac*I site in the *L* region (Figure 1) enhanced *ade6*$^+$ repression. The frequency of colonies exhibiting any degree of *ade6*$^+$ repression increased from ~20% in the wild-type strain (Figure 2, AP165) or 10% in the ΔREII mutant (AP347) to >99% in the transplacement mutant (AP394). Furthermore, unlike in the ΔREII strain or in the strain with REII intact, where intermediate levels of

TABLE 2

The effect of REII deletion on the stability of the alternative expression states in *mat2-P*

			Phenotype of starting colony							
			Red				White			
Strain	REII	*K*	Red	Sectored	1/2 Sect.	White	Red	Sectored	1/2 Sect.	White
AP313	WT	WT	95.7 ± 2.8	3.9 ± 2.7	<0.1	0.4 ± 0.2	65.7 ± 11.9	33.2 ± 4.9	4.1 ± 1.5	4.2 ± 1.2
AP363	Del.	WT	51.0 ± 0.7	42.8 ± 1.1	2.4 ± 0.7	5.7 ± 1.9	<0.1	17.4 ± 4.0	0.28 ± 0.07	82.0 ± 4.0
AP421	WT	Del.	98.6 ± 0.6	0.9 ± 0.5	<0.1	0.4 ± 0.4	<0.1	<0.1	n.r.	100

Cells of strains with *ade6*$^+$ insertions in *mat2-P* (*Bam*HI) and REII or the *K*-region fragment intact (WT) or deleted (Del.) were grown on low adenine medium (YE) at 30°. Cells from red or white colonies ($n = 5$) were replated on the same medium and the percentage of red, white, or sectored colonies was determined. The percentage of half-sectored colonies (1/2 sect.) represents an estimate of the rate of change per cell division (Allshire *et al.* 1995). WT, wild type; Del., deleted; n.r., not relevant.

repression were observed, repression at the *Sac*I site of the transplacement mutant was stringent (Figure 2). Silencing at the extended region of repression was alleviated by any of the *swi6* and *clr1-clr4* mutations (data not shown), thus indicating a role for chromatin remodeling proteins in REII-mediated repression at the *L* region. Further translocation of REII to a distance of 6.0 kb from its native locus (*Hpa*I) did not extend the silent domain. The Ade$^+$ phenotype of a strain with an *ade6*$^+$ insertion at the *Hpa*I site was not affected by transplacement of REII to the centromere-proximal side of the reporter gene (Figure 2, AP407). These results suggest that REII has no repression activity on its own. However, if located close enough to the silent domain it cooperates with an internal *cis*-acting element(s) to enhance repression on its centromere-distal side. Attempts to confirm this hypothesis and identify elements that cooperate with REII are described below.

Orientation dependence of REII activity: If REII acts as a repression element with a preferred directionality, its inversion should enhance silencing on its centromere-proximal side. To test this prediction, we constructed a strain with an inverted REII at its native locus and an *ade6*$^+$ insertion at the *Sac*I site in the *L* region (Figure 1). We then compared the expression states of *ade6*$^+$ in this strain to that in an isogenic strain with REII in the original orientation. The differences in the stability of the alternative *ade6* expression states between the strains with REII in the original and inverted orientation (Table 3) indicate that inversion of REII enhances repression on its centromere-proximal side.

To rule out alternative interpretations of the colony color assay, we conducted Northern hybridization experiments with an internal *ade6*$^+$ sequence as a probe (Figure 3). All strains used in this experiment had an *ade6-DN/N* deletion (Ekwall *et al.* 1997), and the hybridization probe was homologous to the fragment that was deleted from *ade6* at its native locus. Thus, only transcripts of the reporter gene at the *L* region generated a hybridizable RNA product. Results of the Northern hybridization experiments clearly indicate that inversion of REII enhances repression on its centromere-proximal side. The levels of *ade6* expression from the *Sac*I site were lower in cultures of the inversion mutant than in cultures of the wild-type strain. The corrected ratios of these values were ~0.1 for cultures originated from Ade$^-$ or Ade$^+$ colonies.

If a transplaced REII at the *Sac*I site acts with a preferred directionality, its inversion should alleviate *ade6* repression on its centromere-distal side. We tested this prediction by determining the effect of REII orientation on the expression state of an adjacent *ade6*$^+$ gene in REII transplacement mutants. REII enhanced *ade6*$^+$ repression at the *Sac*I site in the *L* region if placed on its centromere-proximal side in the same orientation as in its native location (Figure 2, AP394). However, when placed in an inverted orientation (AP396), its silencing enhancement activity was markedly reduced.

TABLE 3

Inversion of REII enhances repression on its centromere-proximal side

		Phenotype of starting colony					
		Pink			White		
Strain	REII	Red	Pink	White	Red	Pink	White
AP293	WT	<0.1	81.9 ± 3.9	18.1 ± 3.6	<0.1	29.2 ± 5.3	70.8 ± 5.3
AP294	Inv.	90.1 ± 1.6	2.0 ± 1.3	7.9 ± 2.0	3.8 ± 2.3	43.4 ± 8.9	52.8 ± 7.9

Cells with an *ade6*$^+$ insertion in the *Sac*I site at the *L* region (Figure 1) and the indicated REII genotypes were plated on YE medium. Cells from pink or white colonies (n = 5) were replated on the same medium and the percentages of the indicated colony types were determined. WT, wild type; Inv., inverted.

We next asked whether inversion of REII at its native locus would affect the expression state of *mat2-P*. Deletion of REII has only a subtle effect on *mat2-P* repression in wild-type strains, but a combination of a ΔREII mutation with any one of the *swi6* or *clr1-clr4* mutations has a synergistic derepression effect (Thon *et al.* 1994; Ayoub *et al.* 1999). Therefore, to enhance the sensitivity of the assay, we examined the effect of REII inversion on the expression state of *mat2-P* in *clr1* mutants as well as in wild-type cells. *mat2-P* expression was monitored by assaying for haploid meiosis in heterothallic *mat1-M* strains. In these strains derepression of *mat2-P* results in simultaneous expression of *P* and *M* genes in the same haploid cell, and this leads to haploid meiosis (Kelly *et al.* 1988). Haploid meiosis was monitored by microscopic examination and iodine staining of colonies on sporulation medium (Bresch *et al.* 1968). Deletion or inversion of REII had no detectable effect on the repressed state of *mat2-P* in *clr1*$^+$ cells. Nevertheless, either one of these REII mutations markedly enhanced *mat2-P* expression in *clr1* mutants (Figure 4).

Taken together, these observations are consistent with the hypothesis that REII is a repression element that defines the boundary of the silent domain by acting with a preferred directionality toward its centromere-distal side.

Creation of a silent domain at an ectopic site: The results of the preceding experiments suggest that REII has no silencing activity on its own. Yet it seems to cooperate with an internal *cis*-acting element to enhance silencing at the centromere-proximal end of the silent domain. To explore this possibility, we attempted to assemble a synthetic silent domain at an ectopic site by combining the activities of REII and *K*-region DNA fragments. Molecular constructs consisting of an *ade6*$^+$

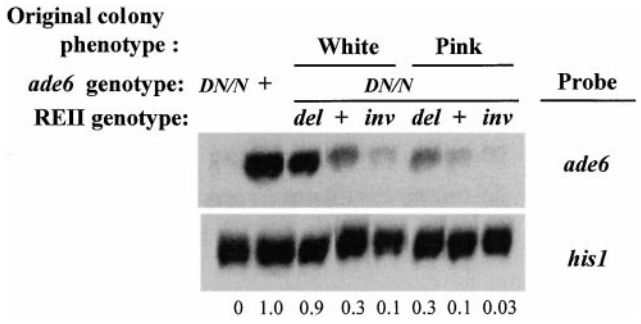


Figure 3.—Inversion of REII enhances silencing on its centromere-proximal side. *ade6* and *his1* probes were used in Northern blot hybridization analysis of RNA preparations from cells with an *ade6*$^+$ insertion at the *Sac*I site in the *L* region and the indicated REII genotypes. Cultures were of cells from colonies on YE medium, showing Ade$^+$ (white) or partially repressed (pink) phenotypes. The first two control lanes are of RNA preparations from an *ade6-DN/N* mutant (FY370) and an *ade6*$^+$ strain (AP128). The REII$^+$ strain is AP293, the REII inversion mutant is AP294, and the REII deletion mutant is AP295. The indicated values under each lane are arbitrary values of the respective *ade6* hybridization signal divided by the signal in the control *ade6*$^+$ lane and corrected for the ratio of the *his1* signals.

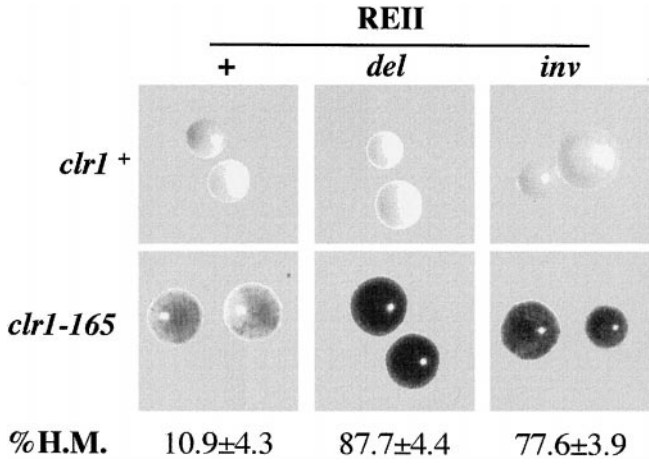
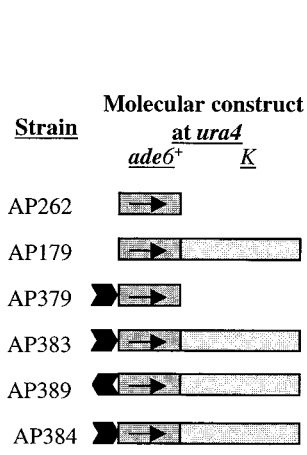


Figure 4.—Inversion of REII suppresses *mat2-P* silencing. Colonies of stable *mat1-M* strains with the indicated REII and *clr1* genotypes were grown on sporulation medium (PM-N), exposed to iodine vapor, and photographed. The percentage of *clr1-165* cells showing a haploid meiosis phenotype (H.M.) was determined by microscopic examination of at least five fields. Each field was of an independent colony and 80–100 cells were scored in each field. Photographed colonies are of the following strains: SP1172 (*clr1*$^+$ REII$^+$), AP125 (*clr1*$^+$ ΔREII), AP262 (*clr1*$^+$ REII$_{inv}$), PG377 (*clr1-165* REII$^+$), AP377 (*clr1-165* ΔREII), and AP262 (*clr1-165* REII$_{inv}$). See Table 1 for detailed genotypes.



Strain	Molecular construct at *ura4* (*ade6*$^+$ — *K*)	*swi6*	Original colony phenotype: Red — Red	Red — Pink/sectored	Red — white	White — Red	White — Pink/sectored	White — white
AP262		+	n.r.	n.r.	n.r.	<0.1	0.1	100
AP179		+	<0.1	15.6±8.2	84.4±8.2	<0.1	0.1	100
AP379		+	n.r.	n.r.	n.r.	<0.1	0.1	100
AP383		+	84.9±3.9	13.3±3.7	1.8±0.2	1.5±0.5	13.3±8.0	85.2±8.5
AP389		+	15.5±5.0	66.2±13.0	18.3±8.0	0.2±0.2	11.6±5.0	88.0±5.2
AP384		*165*	n.r.	n.r.	n.r	<0.1	<0.1	100

FIGURE 5.—Cooperative effect of a *K*-region fragment and REII enhances repression at an ectopic site. The figure includes structures of the various molecular constructs inserted in the *ura4* locus, as well as the name of the strain and its *swi6* genotype. Cells of Ade$^-$ (red) or Ade$^+$ (white) colonies were replated on YE medium and the percentage of colonies showing the indicated phenotypes was determined by the colony color assay. *clr1-clr4* derivatives of AP383 (not shown) had a similar phenotype as *swi6* derivative (AP384). An arrow marks the orientation of the inserted *ade6* gene. The polarity of REII (arrowhead) with respect to the *K* region in AP383 is the same as at the native *mat* locus. The polarity of REII in AP389 is inverted. n.r., not relevant.

reporter gene and various combinations of REII and a 6.3-kb *K*-region DNA fragment (*Bse*RI-*Bse*RI; Figure 1) were targeted to the *ura4* locus on chromosome 3. The expression state of *ade6*$^+$ in the respective strains was monitored by the colony color assay. Consistent with the data in Figure 2 (AP407), expression of *ade6*$^+$ from the *ura4* locus was not affected by an adjacent REII cassette (Figure 5, AP379). On the other hand, the *K*-region fragment conferred low repression frequency at the ectopic site (AP179). A small proportion of the *ura4*::*ade6*$^+$-*K* colonies (~0.1%) displayed a partial Ade$^-$ phenotype (pink colonies on YE medium) and ~15% of the cells in these colonies maintained this phenotype upon replating. Replating of cells from white (Ade$^+$) colonies yielded a very low proportion of red or sectored colonies.

To test for cooperation between REII and the *K*-region fragment, we targeted a molecular construct containing an *ade6* reporter gene flanked by the two elements to the *ura4* locus (AP383). The expression state of *ade6* in the *ura4*::REII-*ade6*$^+$-*K* strain was compared to that in the isogenic strains with *K*-*ade6*$^+$ or *ade6*$^+$-REII insertions. REII markedly enhanced *K*-region-mediated repression. About 15% of the *ura4*::REII-*ade6*$^+$-*K* cells from Ade$^+$ colonies yielded red or sectored colonies, and the majority of the cells from Ade$^-$ colonies retained a repressed or partially repressed state upon replating. As in its native location, REII activity had a preferred directionality at the ectopic site. Inversion of the REII cassette with respect to the reporter gene (AP389) lowered its silencing-enhancing capacity.

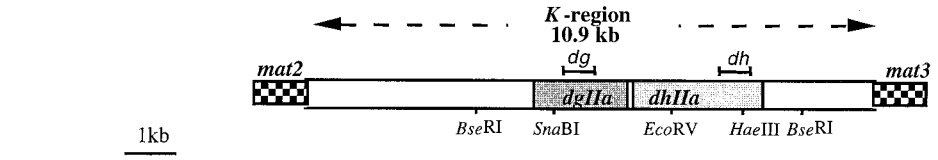
To confirm that *ade6* repression at the *ura4* locus occurred by a chromatin remodeling mechanism, we examined silencing dependence on *swi6* and *clr1-clr4* genes. *ade6* repression in the *ura4*::REII-*ade6*$^+$-*K* strain was totally alleviated by mutations in any of the tested silencing genes (AP384 is an example). These results indicate that cooperation of the *K*-region fragment with REII establishes and stably maintains a repressed chromatin state at an ectopic site.

Silencing activity of *dh*-like sequences within the *cen2* homology: The *cen2* homology within the *K* region has been postulated to promote heterochromatin assembly at the *mat* locus (GREWAL and KLAR 1997). To test this hypothesis, we replaced the 6.3-kb *K*-region fragment in molecular constructs at the *ura4* locus with a *cen2* homology sequence and monitored *ade6* expression in the respective strains. A 3.6-kb *Sna*BI-*Hae*III fragment (Figure 6, AP804), containing 84% of the *cen2* homology, acted as a weak silencer outside the *mat* region context. Cells from white colonies rarely grew to red or pink colonies and ~50% of the cells from red colonies maintained a partially repressed phenotype upon replating. As expected, *cen2*-mediated silencing depended on the activity of *swi6* and *clr1-clr4* silencing genes (data not shown). These observations imply that sequences within the *cen2* homology are sufficient to establish a heterochromatin structure at an ectopic site.

We tested for cooperation between the 3.6-kb *cen2* homology and REII. A molecular construct consisting of an *ade6*$^+$ reporter gene flanked by REII and the *cen2* homology was targeted to the *ura4* locus, and the expression state of *ade6* was determined by the colony color assay. REII enhanced *cen2* homology-mediated repression. More than 95% of the cells from Ade$^-$ colonies retained a fully or partially repressed state upon replating, and ~6% of the cells from Ade$^+$ colonies regained it (Figure 6, AP270). These results demonstrate that *cen2* homology-mediated silencing is enhanced by cooperation with REII.

In all the molecular constructs of Figure 5 and in the first two constructs of Figure 6 (AP804 and AP270) *ade6*$^+$ was inserted with its promoter distal to the *cen2* homology. Inversion of *ade6*$^+$ (AP259) had a minor effect on the stability of the alternative expression states. This inversion enhanced the stability of the repressed state and lowered the stability of the derepressed state.

To determine whether the *cen2* homology acts with a preferred directionality, we inverted the 3.6-kb fragment in the molecular construct at the ectopic site and



Strain	Molecular construct at *ura4*	Original colony phenotype: Red			White		
	K region — *Ade6*$^+$	Red	Pink/sectored	White	Red	Pink/sectored	White
AP804	SnaBI 3.6kb HaeIII	0.3±0.4	55.2±11.5	44.5±11.7	<0.1	<0.1	99.9±0.06
AP270	SnaBI 3.6kb HaeIII	69.2±6.4	27.1±6.9	3.7±2.6	<0.1	6.3±2.9	93.7±2.9
AP259	SnaBI 3.6kb HaeIII	24.9±6.1	41.3±6.8	33.8±3.3	<0.1	1.8±0.8	98.2±0.8
AP263	HaeIII 3.6kb SnaBI	11.6±1.8	42.3±5.7	46.1±5.7	<0.1	0.22±0.2	99.8±0.2
AP278	SnaBI 2.2kb EcoRV	8.4±3.0	41.4±10.8	50.2±12.8	<0.1	0.8±0.7	99.2±0.7
AP287	EcoRV 1.4kb HaeIII	20.1±3.7	40.8±9.4	39.1±11.1	<0.1	5.2±2.9	94.8±2.9
AP288	0.58kb *dh*	29.7±2.57	45.1±6.7	25.18±6.2	<0.1	4.3±1.3	95.7±1.3
AP803	0.56kb *dg*	n.r.	n.r.	n.r.	<0.1	<0.1	100±0.0
AP419	SnaBI 3.6kb HaeIII	<0.1	37.0±5.6	63.0±5.6	<0.1	<0.1	100±0.0

FIGURE 6.—Silencer activity of *cen2* homology sequences. The indicated restriction fragments of the *cen2* homology and PCR-generated *dg* and *dh* fragments (top diagram) were incorporated into the molecular constructs at the *ura4* locus. The distribution of Ade phenotypes of colonies originated by cells from red (Ade$^-$) or white (Ade$^+$) colonies on YE plates was determined by the colony color assay as in Figure 5. n.r., not relevant.

determined the stability of the alternative expression states. Inversion of the *cen2* homology with respect to the reporter gene (AP263) did not abolish its activity. Yet silencing activity in one orientation was higher than in the other.

Studies of centromere activities indicate functional redundancy among sequences of the centromeric outer repeats (BAUM *et al.* 1994; NGAN and CLARKE 1997). To test for redundancy in silencing-promoting activity, we compared the ability of four *cen2* homology fragments, ranging in length from 0.56 to 2.2 kb, to confer silencing at the ectopic site (Figure 6). The three fragments sharing homology with the *dhII* repeat (AP278, AP287, AP288) conferred chromatin-mediated repression on an adjacent *ade6*$^+$ gene. Yet the 0.56-kb *dgII*-like sequence (AP803) had no detectable silencing activity. Assuming that the proportion of cells from red colonies maintaining the Ade$^-$ phenotype upon replating reflects repression stability, the data imply that repression stability in strains with *dhII* homology at the ectopic site was similar to that in the strain with the longer *cen2* homology insertion. However, significant differences in the proportions of cells from white colonies yielding pink colonies upon replating were observed. Fragments of 0.58 kb (AP288) and 1.4 kb (AP287) that share 99% homology with the centromeric *dhII* repeats were more effective than the entire 3.6-kb *cen2* homology fragment, whereas a 2.2-kb fragment (AP278), consisting mainly of *dgII* sequences, was somewhat less effective.

We asked whether the configuration of cooperating elements with respect to the reporter gene affects cooperation efficiency. Specifically, is a reporter gene flanked by the two elements more stringently repressed than a reporter gene with both elements on its same side? To address this question, we placed REII and the *cen2* homology on the same side of *ade6*$^+$ and targeted the molecular construct to the *ura4* locus. REII silencing-enhancing activity at the ectopic site was abolished when placed together with the *cen2* homology on the same side of the *ade6*$^+$ gene. Less than 0.1% of the cells from Ade$^+$ colonies acquired the Ade$^-$ phenotype and only 37% of the cells from Ade$^-$ colonies maintained partial *ade6* repression upon replating (Figure 6, AP419). This suggests that cooperation between the two elements outside their native chromosomal context affects only the region between the elements. However, the possibility that the orientation of the *ade6*$^+$ reporter gene with respect to the *cen2* homology or to that surrounding DNA sequences affects silencing cannot be ruled out.

DISCUSSION

Silencing along the chromosomal *mat2-K-mat3* domain is mediated by several *trans*-acting proteins and

by at least three *cis*-acting elements: the *cen2* homology, REII, and the *mat3* silencer (Grewal and Klar 1996; Thon and Friis 1997; Ayoub *et al.* 1999; Thon *et al.* 1999). Here we show that sequences within the *cen2* homology promote silencing at an ectopic site through a mechanism that depends on chromatin-modifying proteins and that REII cooperates with the *cen2* homology to enhance silencing. We also show that REII enhances the stability of the repressed state at the *mat* locus and acts with a preferred directionality to define the boundary of stringent repression at the junction between *mat2-P* and the *L* region.

By acting with a preferred directionality, REII defines the boundary of stringent repression: A *cis*-acting element may define the boundary of a silent domain by acting as an insulator that blocks the propagation of enhancer or silencer activities (Sun and Elgin 1999; Udvardy 1999) or by organizing repressive chromatin in a unidirectional manner (Gdula *et al.* 1996; Bi *et al.* 1999). The following evidence suggests that REII defines the boundary of stringent repression at the junction between *mat2-P* and the *L* region by promoting silencing to its centromere-distal side: (a) repression is stringently controlled on the centromere-distal side of REII and is gradually alleviated on its centromere-proximal side (Ayoub *et al.* 1999); (b) inversion of REII enhances silencing on its centromere-proximal side while suppressing silencing on its centromere-distal side; (c) transplacement of REII to a site within the *L* region extends the region of stringent repression, and this extension depends on a direct orientation of REII and the activity of chromatin modifying proteins. Thus, the location and orientation of REII define the boundary of stringent repression at the centromere-proximal end of the *mat* silent domain. This conclusion is consistent with the observation that REII ensures repression in the silent domain, regardless of the expression state in the *L* region (Ayoub *et al.* 1999).

The reason that silencing enhancement capacity of a translocated REII is diminished as its distance from *mat2-P* increases from 2.5 (*Sac*I) to 6.0 kb (*Hpa*I; Figure 2) is not yet understood. One possibility is that REII activity depends on cooperation with an internal repression element, like the *cen2* homology, and this cooperation can take place only within a limited chromosomal distance. Another possibility is that as the distance between the two elements increases, the length of the silent domain becomes limited by the availability of heterochromatin components. A third possibility is that a *cis*-acting element, located between the *Hpa*I and *Sac*I sites, interferes with the cooperation between REII and the *cen2* homology. Further analysis of the *L* region should help distinguish between these possibilities.

***Cis*-acting repression elements at the *mat* locus:** Deletion of any one of the three *cis*-acting elements that promote silencing at the *mat* locus alleviates repression of reporter genes within the silent domain and leads to a variegated phenotype. However, different deletion mutants display different modes of variegation. Whereas *K*Δ mutants adopt one of two stable states of reporter gene expression (Grewal and Klar 1996; Thon and Friis 1997; and this study), the repressed state in strains with deletions of REII or the *mat3* silencer is highly unstable (Ayoub *et al.* 1999; Thon *et al.* 1999; and this study). The stability of the alternative expression states in *K*Δ mutants implies that an element within the *K* region is involved in repression establishment, but repression inheritance is independent of this element activity (Grewal and Klar 1996; Thon and Friis 1997). Consistent with this hypothesis we show here that different sequences of the *cen2* homology promote the establishment of chromatin-mediated repression at an ectopic site. However, the repressed state conferred by the *cen2* homology alone is relatively unstable. Remarkably, REII, which does not display independent silencing activity outside the *mat* silent domain, enhances the stability of *cen2*-mediated repression at the ectopic site. The effect of the REII deletion on the stability of the repressed state within *mat2-P* suggests that it is likely to play a similar role at the *mat* locus.

REII activity is limited to the centromere-proximal end of the silent domain and a functionally similar element, the *mat3* silencer, enhances repression at the centromere-distal end of the domain (Thon *et al.* 1999). The proposition that REII and the *mat3* silencer are functionally similar is consistent with our recent observations that the *mat3* silencer has no detectable silencing activity on its own. However, like REII, it cooperates with the *cen2* homology to enhance silencing stability at the ectopic site (I. Goldshmidt and A. Cohen, unpublished results).

Altogether, these observations are consistent with the notion that the *cen2* homology plays a key role in the assembly of a repressive chromatin structure at the *mat* locus. Furthermore, cooperation of this element with REII on its centromere-proximal side, and with the *mat3* silencer on its centromere-distal side, enhances repression along the entire length of the *mat2-K-mat3* silent domain. The phenotypes of the various deletion mutants and of strains with different combinations of the three repression elements at the ectopic site suggest that the *cen2* homology mediates the establishment of the repressed state, while REII and the *mat3* silencer contribute to silencing stability.

Silencing activity of centromeric DNA: The intriguing discovery that one-third of the *K* region shares 96% homology with the centromeric *dgII dhII* repeats suggests that shared *cis*-acting elements may play similar roles in heterochromatin assembly at the *mat* and *cen* loci. The *cen2* homology in the *K* region may also promote silencing at the *mat* locus through homologous DNA-DNA interactions with its centromeric counterparts (Grewal and Klar 1997). Such interactions have been postulated before to promote regional silencing by playing a functional role in nuclear organization (Henikoff 1997; Henikoff and Matzke 1997). If mainly

homologous interactions are involved, the length of the homology is likely to affect silencing proficiency. This is clearly not the case in the experiments described above. A 0.56-kb *dgII* homology has no detectable silencing-promoting activity. On the other hand, silencing-promoting activity of the 0.58-kb *dhII* homology is higher than that of the 3.6-kb or 2.2-kb *cen2* homologies and similar to that of the 1.4-kb *dhII* homology. Thus, it is unlikely that merely homologous interactions are involved in *cen2*-homology-mediated silencing.The observation that each one of the nonoverlapping fragments of the *cen2* homology is sufficient to promote silencing at the ectopic site is consistent with the notion that heterochromatization is promoted by redundant DNA elements distributed along centromeric sequences. The 580-bp fragment that promotes silencing at the ectopic site contains a 310-bp sequence that shares >99% homology with *dh* repeats in all three centromeres. Further analysis of the 580-bp fragment should define the minimal requirements for *dh*-like sequences to establish a repressed state and may reveal specific *cis*-acting sequences through which silencing proteins may exert their function at the *cen* and *mat* loci.

Cooperation at a distance between *cis*-acting elements: REII has no detectable repression activity on its own. Yet it cooperates with the *cen2* homology to enhance silencing at the ectopic site. Cooperation between *cis*-acting elements may involve either one or a combination of the following mechanisms: transient or stable interaction between proteins, associated with the cooperating elements to create a loop structure that defines a silent domain (Pirrotta and Rastelli 1994; Henikoff 1996); enhancement of local concentration of weak binding sites for chromatin remodeling proteins; or functional complementation by the proteins associated with the two elements. The indications that REII defines the boundary of stringent repression and that cooperation between REII and the *cen2* homology at the ectopic site affect only the region between the elements are consistent with the "looped domain" hypothesis. However, the observation that inversion of REII at the *mat* locus enhances silencing on its centromere-proximal side, while suppressing silencing on its centromere-distal side, argues against this possibility. This observation is more easily explained by unidirectional propagation of a silencing-enhancing complex from REII toward the *cen2* homology and a cumulative effect of the complexes propagating from the two elements toward each other. It is likely that a similar mode of cooperation between the *cen2* homology and the *mat3* silencer promotes silencing at the centromere-distal end of the silent domain. Because genetic experiments suggest that REII and the *cen2* homology play different yet complementary roles in silencing, we favor the "functional complementation" hypothesis. We therefore speculate that the two elements recruit partially overlapping or different sets of proteins that propagate toward each other and that the cumulative effect of the two complexes promotes stringent repression at the centromere-proximal end of the silenced domain.

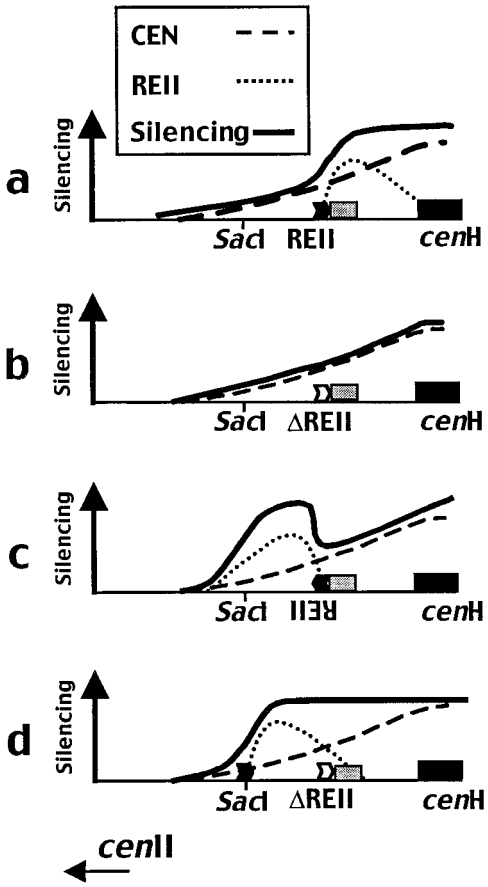


Figure 7.—Model for cooperation of the *cen2* homology (*cen*H) with REII in promoting silencing and in defining the centromere-proximal limit of stringent repression at the *mat* silent domain. This model assumes that DNA elements within the *cen2* homology serve as nucleation points for heterochromatin components that propagate toward *mat2-P* and *mat3-M*. (a) Normally, the gradual decrease in heterochromatin density as the distance from the *cen2* homology increases is compensated by a complementary structure propagating in a unidirectional manner from REII. Thus, repression is more stringently controlled on the centromere-distal side of REII than on its centromere-proximal side (Ayoub *et al.* 1999). (b) Deletion of REII alleviates repression at the centromere-proximal end of the silent domain but not at its centromere-distal end, where cooperation of the *cen2* homology and the *mat3* silencer controls repression. (c) An inverted REII enhances repression on its centromere-proximal side while suppressing repression on its centromere-distal side. (d) Cooperation of a translocated REII with the *cen2* homology extends the silent domain to the new REII locus. Presumably, cooperation of the *cen2* homology with the *mat3* silencer enhances repression at the centromere-distal end of the silent domain (not shown). REII is designated by a solid arrowhead, REII deletion by an open arrowhead, *mat2* by a shaded box, silencing activity of REII by a dotted line, and silencing activity of *cen2* homology by a broken line. A solid line illustrates the degree of silencing along the centromere-proximal end of the silent domain.

The speculative model in Figure 7 is based on results

of this study—the observations that REII activity is limited to the centromere-proximal end of the silent domain (Thon *et al.* 1999) and that repression is gradually alleviated as the distance from REII toward *mat1* increases (Ayoub *et al.* 1999). We assume that DNA elements within *cen2* homology, like their counterparts at the centromere outer repeats, serve as nucleation points for heterochromatin components that propagate in a bidirectional manner toward *mat2-P* and *mat3-M*. Normally, the gradual decrease in heterochromatin density as the distance from the *cen2* homology increases is compensated by the complementary structures that propagate from REII (Figure 7a). This explains why repression is stringently controlled within the *mat2-K-mat3* domain and is gradually alleviated with distance at the centromere-proximal side of REII (Ayoub *et al.* 1999). The gradual decline in *cen2*-mediated repression leads to variegated expression of reporter genes within *mat2-P* in REII deletion mutants (Figure 7b). Because REII activity propagates in a unidirectional manner, inversion of REII enhances repression on its centromere-proximal side while suppressing repression on its centromere-distal side (Figure 7c). Likewise, transplacement of REII to sites within the region of variegated repression (*Sad*) extends the region of stringent repression to the new REII locus (Figure 7d).

Possible roles for REII in epigenetically modulated silencing inheritance: Stable chromosomal inheritance of the repressed state in *K*Δ mutants is regulated by an epigenetic mechanism (Grewal and Klar 1996; Thon and Friis 1997). Thus, heterochromatin at the *mat* locus not only promotes mating-type switching and silencing of the donor cassettes but also templates its own reformation every cell cycle. The nature of the epigenetic marking or the mechanism that initially marks the affected region is not yet understood. Our current study reveals that the repressed state conferred by the *cen2* homology alone is relatively unstable, but stability is enhanced by cooperation of the *cen2* homology with REII. Thus, REII is likely to be involved in the formation or maintenance of the putative epigenetic marking. REII may enhance the stability of an epigenetic state through either one of two nonmutually exclusive mechanisms: (a) cooperation between REII and *cen2* homology may promote the assembly of a higher order chromatin structure that is physically different and more stably inherited than the structure assembled by the *cen2* homology alone or (b) REII, like *S. cerevisiae HML-E* silencer (Holmes and Broach 1996), may help provide the genomic memory that assures persistence of the repressed state from one generation to the next. For example, a protein complex may remain associated with REII during replication and serve as a nucleus for the reassembly of a complete heterochromatin structure on each sister chromatid after replication. Resolution between these hypotheses must await a comparative analysis of the chromatin structures assembled at the *mat* locus in the wild-type cells and REII mutants and the monitoring of the chromatin state following *in vivo* deletion of REII.

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