

Donor Preference Meets Heterochromatin: Moonlighting Activities of a Recombinational Enhancer in *Saccharomyces cerevisiae*

Anne E. Dodson and Jasper Rine¹

Department of Molecular and Cell Biology, California Institute for Quantitative Biosciences, University of California, Berkeley, California 94720-3220

ORCID IDs: 0000-0001-7719-0496 (A.E.D.); 0000-0003-2297-9814 (J.R.)

ABSTRACT In *Saccharomyces cerevisiae*, a small, intergenic region known as the recombination enhancer regulates donor selection during mating-type switching and also helps shape the conformation of chromosome III. Using an assay that detects transient losses of heterochromatic repression, we found that the recombination enhancer also acts at a distance *in cis* to modify the stability of gene silencing. In a mating-type-specific manner, the recombination enhancer destabilized the heterochromatic repression of a gene located ~17 kbp away. This effect depended on a subregion of the recombination enhancer that is largely sufficient to determine donor preference. Therefore, this subregion affects both recombination and transcription from a distance. These observations identify a rare example of long-range transcriptional regulation in yeast and raise the question of whether other *cis* elements also mediate dual effects on recombination and gene expression.

KEYWORDS silencing; mating-type switching; long-range regulation; Sas2

CIS-ACTING elements regulate several processes in the nucleus, including gene expression, recombination, and DNA replication. Some elements act locally, whereas others act in some eukaryotes at distances ranging from several kilobase pairs to remarkable lengths of 1 or 2 Mbp (Pfeifer *et al.* 1999; Lettice *et al.* 2003; Smemo *et al.* 2014) or even an entire chromosome (Clemson *et al.* 1996; Lee and Jaenisch 1997). In budding yeast, a 2-kbp region referred to as the recombination enhancer (*RE*) acts *in cis* to promote the use of sites located throughout the left arm of chromosome III as donor templates during mating-type interconversion (Wu and Haber 1996). Through a separate, unknown mechanism, the *RE* also regulates the spatial conformation of chromosome III (Belton *et al.* 2015). Hence, this extraordinary locus is enriched for features that mediate long-range effects.

The *RE* functions with other loci on chromosome III to facilitate mating-type switching (Haber 2012). The *MAT α*

and *MAT α* alleles of the mating-type (*MAT*) locus, located on the right arm of chromosome III, specify mating type. In addition, silent copies of the mating-type alleles reside on opposite ends of chromosome III; on the left end, the *HML* locus typically contains a silent copy of the α information, and on the right end, the *HMR* locus typically contains a silent copy of the *a* information. To switch mating types, haploids induce expression of the *HO* site-specific endonuclease, which creates a double-strand break at the *MAT* locus. Then, the homologous recombination machinery orchestrates a gene conversion event that replaces the original *MAT* sequence with a copy of the sequence stored at either *HML* or *HMR*. *HMR* serves as the default donor. In *a* cells, however, the *RE* somehow promotes the use of *HML* as the donor template. Therefore, *a* cells preferentially use *HML α* as a donor, and α cells preferentially use *HMR a* as a donor.

Similar to transcriptional enhancers in metazoans, the *RE* interacts with transcription factors in a cell-type-specific manner. The left portion of the *RE* contains several binding sites recognized by the forkhead transcription factor *Fkh1* (Sun *et al.* 2002). *Fkh1* binds the *RE* in *a* cells but not in α cells, and is important for *RE* activity (Sun *et al.* 2002; Ercan *et al.* 2005; Coic *et al.* 2006b; Li *et al.* 2012). The *RE* also contains an SCB (*Swi4/Swi6* cell cycle box) sequence that

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¹Corresponding author: 374 Stanley Hall, University of California, Berkeley, CA 94720-3220. E-mail: jrine@berkeley.edu

binds the SBF (SCB binding factor) transcription factor (Coic *et al.* 2006b). The SCB contributes to *RE* activity independently of Fkh1 (Coic *et al.* 2006b). In α cells, the $\alpha 2$ protein encoded by *MAT α* functions with Mcm1 to bind to and inactivate the *RE* and also repress the transcription of *a*-specific genes (Johnson 1995; Szeto and Broach 1997; Szeto *et al.* 1997). The $\alpha 2$ -Mcm1 repressor complex binds two sites in the *RE* and positions highly ordered nucleosomes over the locus that presumably inhibit the binding of transcription factors (Weiss and Simpson 1997). Hence, the effect of the *RE* on recombination (and therefore donor preference) is specific to *a* cells.

The *RE* promotes recombination at sites located throughout the entire left arm of chromosome III, which is ~ 110 kbp, and the efficacy of the *RE* is roughly proportional to its proximity to the locus serving as a donor template (Wu and Haber 1995, 1996; Coic *et al.* 2006a). Given that *a* cells and α cells show similar patterns of DNase-I sensitivity throughout this chromosome arm, the *RE* does not dramatically alter the local chromatin structure (Ercan and Simpson 2004). Remarkably, the *RE* does effect a change in the higher-order folding of chromosome III; however, this function is genetically separable from the recombination function (Belton *et al.* 2015; Lassadi *et al.* 2015). The mechanism by which the *RE* acts at a distance remains unresolved.

The *RE* locus is atypical for yeast in that its effects extend across a large region. Other *cis* elements in budding yeast tend to act locally. In contrast to metazoan enhancers, which often activate promoters at a distance of multiple kbp (Smith and Shilatifard 2014), upstream activation sequences in yeast typically reside within the promoter region (Hahn and Young 2011) and are limited in their ability to activate transcription when moved further away from the TATA box (Dobi and Winston 2007; Reavey *et al.* 2015). Although silencer elements have the potential to act distally, boundary elements typically prevent them from doing so in their native context (Fourel *et al.* 1999; Pryde and Louis 1999; Donze and Kamakaka 2001; Meneghini *et al.* 2003). Therefore, long-range effects on gene expression are rarely observed in yeast.

We recently developed an assay to capture transient events of expression and have since utilized this assay to identify factors that modify the dynamics of heterochromatin-mediated gene silencing (Dodson and Rine 2015). Unexpectedly, our analysis revealed that the *RE* mediates a long-range, *cis* effect on the stability of heterochromatic repression. The identification and characterization of this effect, described here, adds a new dimension to our understanding of the *RE* and provides an exceptional example of long-range regulation in *Saccharomyces cerevisiae*.

Materials and Methods

Yeast strains

The strains used in this study were derived from the W303 background and are listed in Supplemental Material, Table S1.

Deletions were made using one-step integration of gene-disruption cassettes (Goldstein and McCusker 1999; Gueldener *et al.* 2002) with the primers listed in Table S2, and were confirmed by PCR of the 5' and 3' junctions. The full *RE* locus was defined as chr III: 28,961–31,213, whereas *RE-left* was defined as chr III: 28,961–29,852 and *RE-right* was defined as chr III: 29,853–31,213. Strains containing the *re^{SCB}* allele (JRY10721 and JRY10722) were made by first replacing the native *RE* with *Kluyveromyces lactis URA3*, followed by replacement of *K. lactis URA3* with the *re^{SCB}* sequence (amplified from pJR3396) through selection on 5-fluoroorotic acid and confirmation by sequencing. ARS305 was defined as chr III: 39,239–39,591 and ARS306 was defined as chr III: 74,458–74,677. All chromosome coordinates refer to version R64-2-1 of the S288C genome sequence in the *Saccharomyces* Genome Database (Engel *et al.* 2014).

The plasmids used in this study are listed in Table S3. For the construction of pJR3389, the *RE* locus (chr III: 28,961–31,213) was amplified from genomic DNA (W303) using the *RE^{EagI}* forward and *RE^{XhoI}* reverse primers and inserted into pRS313 following digestion of both the insert and backbone with *EagI* and *XhoI*. For the construction of pJR3396, the *RE* locus (chr III: 28,961–31,213) was first amplified from genomic DNA (W303) using the *RE^{EagI}* forward and *RE^{XhoI}* reverse primers and inserted into pRS303 following digestion of both the insert and backbone with *EagI* and *XhoI*. Then, the TC-to-GA mutation in the SCB was created using site-directed mutagenesis.

Colony growth and imaging

For tetrad analysis, JRY10656 was streaked out onto YPD containing both G418 (Geneticin; Life Technologies) and Hygromycin B [Sigma (Sigma Chemical), St. Louis, MO] to select for cells expressing red fluorescent protein (RFP), which were then sporulated for 4–5 days on 1% potassium acetate. Tetrads were dissected on Complete Supplement Mixture (CSM) –Trp (Sunrise Science Products) plates and imaged after 3 days of growth at 30°. For all other image analyses, strains were first streaked out onto YPD + G418 (or CSM –His + G418 for strains containing *HIS3*-marked plasmids) to select for RFP-expressing cells. Cells were then grown to log phase in CSM –Trp and plated onto CSM –Trp, 1% agar at a density of ~ 10 cells/plate to allow for uniformly large colonies. Cells containing *HIS3*-marked plasmids (JRY10668–JRY10673) were grown to log phase in CSM –His –Trp and plated onto CSM –His –Trp 1% agar at a density of ~ 10 cells/plate. Colonies were imaged after 6 days of growth at 30°. Images of at least 12 colonies per genotype were acquired with a Zeiss Axio Zoom.V16 microscope equipped with ZEN software (Zeiss), a Zeiss AxioCam MRm camera, and a PlanApo Z 0.5 \times objective. Images were assembled in Photoshop (Adobe Systems).

RNA preparation for quantitative RT-PCR

Cells were grown to log phase in YPD and then harvested for RNA isolation. Total RNA was isolated using hot acidic phenol

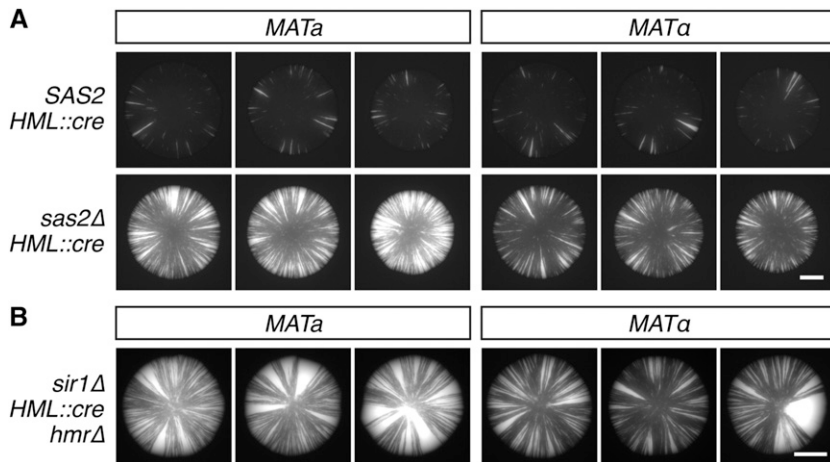


Figure 1 Contributions of *SAS2* and *MAT* to the dynamics of heterochromatic repression. (A) GFP fluorescence of colonies grown from tetrad dissections of a *sas2* hemizygote (JRY10656). All genotypes contain a *cre* gene at *HML* that, upon expression, triggers the permanent activation of GFP expression. See Figure S1 for complete tetrads. Bar, 1 mm. (B) GFP expression of *sir1*Δ mutants containing *HML::cre* and the RFP-GFP cassette. Bar, 2 mm. RFP, red fluorescent protein.

and chloroform (Collart and Oliviero 2001). Following treatment with DNase I (Roche Diagnostics), RNA was purified by phenol–chloroform extraction. Complementary DNA (cDNA) was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) and oligo(dT) primers. Quantitative PCR of cDNA was performed using the primers listed in Table S2, the Thermo Scientific DyNamo HS SYBR Green qPCR Kit (Fisher Scientific), and an Mx3000P machine (Stratagene, La Jolla, CA). Standard curves were generated from the cDNA of a *sir4*Δ mutant (JRY10663). Three independent RNA preparations were performed for each sample. A two-tailed Student's *t*-test was used to determine the raw *P*-values presented in the text.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article. Strains and plasmids are available upon request.

Results

An assay designed to capture transient lapses of silencing recently revealed a dynamic dimension to heterochromatin at the silent mating-type loci *HML* and *HMR* (Dodson and Rine 2015). On rare occasions, a *cre* reporter gene under control of the native $\alpha 2$ promoter at either *HML* α or *HMR* α escapes heterochromatin-mediated gene silencing and triggers a recombination-based switch from RFP expression to GFP expression. By converting transient transcription into permanent changes in genotype and phenotype, the Cre-Reported Altered States of Heterochromatin (CRASH) assay preserves a historical record of silencing loss that manifests as GFP-expressing sectors within an otherwise red colony.

Sas2 stabilized the silencing of *HML*

With the ability to now monitor the dynamics of silencing, we set out to identify factors that modify these dynamics. *Sas2* was a particularly interesting candidate, as it both promotes and antagonizes the silenced state. *Sas2* catalyzes the addi-

tion of an acetyl group on lysine 16 of histone H4 (H4 K16), which *Sir2* must remove to establish and maintain silencing (Tanny *et al.* 1999; Imai *et al.* 2000; Shia *et al.* 2005). Consistent with the antisilencing role of H4 K16 acetylation, deletion of *SAS2* improves silencing at *HMR* and the telomeres (Ehrenhofer-Murray *et al.* 1997; Kimura *et al.* 2002; Suka *et al.* 2002). At *HML*, however, *sas2* mutations delay the *de novo* establishment of silencing and enhance the silencing defect of *sir1* mutants (Reifsnyder *et al.* 1996; Xu *et al.* 2006; Osborne *et al.* 2009).

To determine the effects of *Sas2* on the stability of silencing, we deleted one copy of *SAS2* in a diploid homozygous for *HML::cre* and the RFP-GFP cassette, induced sporulation, dissected tetrads, and then imaged the fluorescence of the resulting colonies. Within each tetrad, two of the four colonies showed sectoring patterns indicative of frequent silencing loss (Figure S1). Genotyping of each tetrad revealed that the high-sectoring phenotype cosegregated consistently with the *sas2*Δ mutation (Figure 1A and Figure S1). Therefore, silencing of *HML* was less stable in colonies derived from the *sas2*Δ spores than in colonies derived from the *SAS2* spores.

Silencing of *HML* was less stable in α cells than in α cells

Curiously, *sas2*Δ colonies seemed to fall into one of two phenotypic classes. Qualitatively, half of the *sas2*Δ colonies exhibited high rates of silencing loss compared to wildtype, whereas the other half exhibited even higher rates of silencing loss (Figure S1). The 1:1 ratio of the two *sas2*Δ phenotypes suggested that a second modifier of silencing (the first being *SAS2*) was segregating in the cross. Surprisingly, *sas2*Δ colonies with the highest levels of sectoring contained the *MAT* α mating-type allele, whereas *sas2*Δ colonies with relatively lower sectoring levels contained the *MAT* α mating-type allele (Figure 1A and Figure S1). This observation strongly suggested that the *MAT* genotype affected the stability of silencing at *HML*.

To test whether the apparent *MAT* effect was due to the *MAT* genotype or rather to a *de novo* mutation that was closely linked to one of the *MAT* copies, we analyzed the

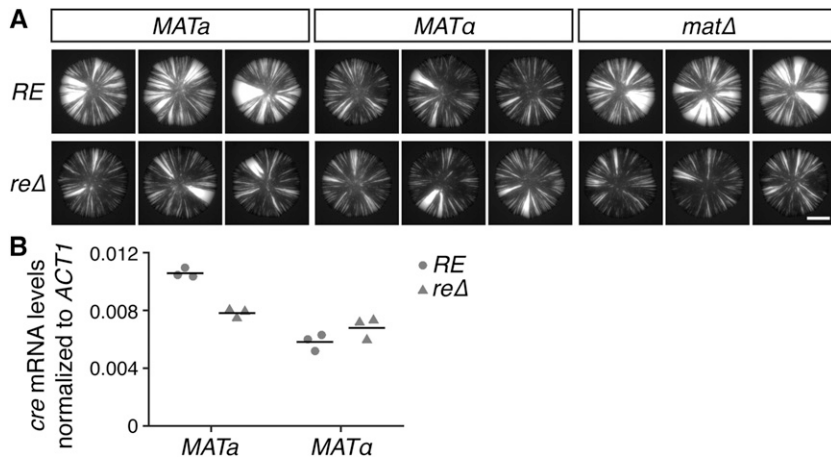


Figure 2 Effects of mating type and the *RE* on *HML::cre* expression. (A) GFP images of colonies. All strains contained the *sas2Δ* mutation. Bar, 2 mm. (B) Quantitative RT-PCR of *cre* mRNA levels normalized to *ACT1* mRNA levels. Horizontal lines represent the means ($n = 3$). All strains contained the *sas2Δ* mutation and exhibited ~100-fold repression of *HML::cre* relative to a mutant lacking Sir4, an essential component of heterochromatin (data not shown). mRNA, messenger RNA; *RE*, recombination enhancer.

two *MAT* genotypes in a different strain background. Consistent with the tetrad analysis of the *sas2Δ* hemizygote, *MATa* cells showed more frequent losses of silencing than *MATα* cells in the absence of Sir1, another protein that contributes to the stability of silencing at *HML* (Figure 1B) (Dodson and Rine 2015). *HMR* was deleted in both *sir1Δ* strains to prevent *HMR* expression from influencing mating-type identity by mimicking the *a/α* cell type. Therefore, the *MAT* genotype affected the stability of silencing at *HML* in both *sas2Δ* and *sir1Δ* mutants, and this effect did not depend on the presence of *HMR*. The phenotype showed incomplete penetrance in both mutants, as a small fraction of *MATa* colonies exhibited sectoring levels comparable to the majority of *MATα* colonies and vice versa (data not shown).

Mating type affected silencing through the *RE* locus

Only a few features distinguish *a* cells from α cells. *MATα* encodes $\alpha 1$, an activator of α -specific gene expression, as well as $\alpha 2$, a repressor of both *a*-specific gene expression and *RE* activity. By contrast, *MATa* encodes *a1*, which functions with $\alpha 2$ in diploids to repress haploid-specific gene expression, as well as *a2*, a protein of unknown function. In principle, any of the *MAT* products or any of the loci regulated by a *MAT* product could be responsible for the effect on silencing.

Given that most mating-type-specific genes encode components of the pheromone signaling pathway and therefore seemed unlikely to affect heterochromatin, we instead turned our attention to the *RE* locus. We deleted the *RE* region (chr III: 28,961–31,213) in both *sas2Δ MATa* and *sas2Δ MATα* strains and analyzed the stability of silencing. Deletion of the *RE* had no detectable effect on silencing in *MATα* cells (Figure 2A). In *MATa* cells, however, deletion of the *RE* reduced the frequency of silencing loss to a level indistinguishable from the levels observed in *MATα RE* and *MATα reΔ* cells. Thus, in the absence of the *RE*, the stability of silencing was similar between *a* and α cells.

Although the strains used in this study do not contain a functional *HO* gene and therefore do not induce mating-type interconversion, the *cre* gene at *HML* could, in principle, be

copied into the transcriptionally active *MAT* locus at a very low rate. If the *RE* were to promote the occurrence of this event as it normally does during mating-type interconversion, then the *RE* could potentially increase the frequency of RFP-to-GFP switches in *a* cells without affecting the stability of silencing. To test this possibility, we removed the sequences shared between *HML* and *MAT* by deleting the entire *MAT* locus, thus blocking all potential for mating-type interconversion. Since *a*-specific genes are constitutively expressed in the absence of $\alpha 2$, *matΔ* cells mate as *a*. Sectoring levels were similar between *MATa* and *matΔ* colonies, suggesting that the majority of RFP-to-GFP switches observed in *MATa* colonies did not arise from a transposition of *cre* into the *MAT* locus (Figure 2A). Furthermore, the *reΔ* mutation reduced sectoring levels in *matΔ* colonies as it did in *MATa* colonies and therefore acted independently of the *MAT* locus (Figure 2A).

The *RE* could conceivably increase the frequency of RFP-to-GFP switches by enhancing recombination between the two *loxP* sites in the RFP-GFP cassette rather than by modifying the expression of *HML::cre*. However, the *RE* promotes recombination only *in cis*, and the RFP-GFP cassette was located on a different chromosome (chromosome V).

To test whether the *RE* directly affected silencing, we measured the levels of *cre* mRNA using quantitative RT-PCR (Figure 2B). Consistent with the sectoring phenotypes in the CRASH assay, *cre* mRNA levels were significantly higher in *MATa RE* cells than in *MATα RE* cells ($P = 2 \times 10^{-4}$). Deletion of the *RE* reduced the levels of *cre* mRNA in *MATa* cells ($P = 4 \times 10^{-4}$), but did not significantly alter *cre* mRNA levels in *MATα* cells ($P = 0.2$). Moreover, *cre* mRNA levels were not significantly different between *MATa* cells and *MATα* cells in the absence of the *RE* ($P = 0.1$). Therefore, mating type directly affected *HML::cre* expression, and this effect depended on the *RE*.

The left region of the *RE* mediated the effect on silencing

The *RE* locus contains several features of interest. A 700-bp sequence in the left portion of the *RE*, often referred to as

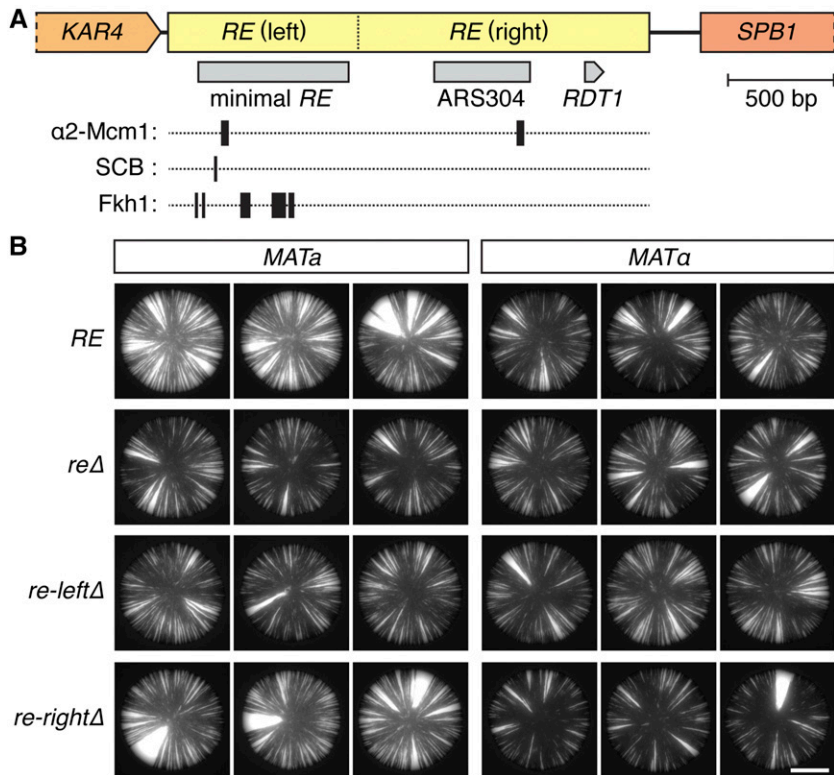


Figure 3 *RE* features and their effects on *HML::cre* expression. (A) Map of the *RE* locus. The left region contains the minimal *RE* and several protein binding sites, whereas the right region contains an ARS element (ARS304) and a putative open reading frame (*RDT1*). (B) GFP images of colonies. All strains contained the *sas2Δ* mutation. Bar, 2 mm. *RE*, recombination enhancer.

the minimal *RE*, is necessary and largely sufficient to promote the usage of *HML* as a donor in *MAT α* cells (Wu and Haber 1996). The minimal *RE* contains an SCB site, multiple *Fkh1* binding sites, and one of the two $\alpha 2$ -*Mcm1* operators located within the *RE* (Figure 3A). The right portion of the *RE* contains the second $\alpha 2$ -*Mcm1* operator, an autonomously replicating sequence (ARS) element, and a putative open reading frame (Figure 3A). In addition, a cells transcribe multiple RNA species from the central and right portions of the *RE* (Szeto *et al.* 1997; Ercan *et al.* 2005). A recent study shows that the conformation of chromosome III differs between *a* and α cells, and that this difference depends on the right portion of the *RE*, but not on the left portion containing the minimal *RE* (Belton *et al.* 2015). Given that the right portion makes only a small contribution to the effect on recombination, the *RE* is roughly divisible into two subregions with separate functions.

To determine which features were required for the mating-type effect on silencing, we made partial deletions that removed either the left (chr III: 28,961–29,852) or right (chr III: 29,853–31,213) portion of the *RE* (Figure 3). Deletion of the left portion, which contained the minimal *RE*, eliminated the difference in sectoring between *MAT α* and *MAT α* colonies (Figure 3B). By contrast, *MAT α* colonies lacking the right *RE* portion still exhibited high levels of sectoring relative to their *MAT α* counterparts (Figure 3B). Therefore, the mating-type effect on silencing depended only on the left region of the *RE* locus. Interestingly, deletion of the right side slightly stabilized silencing in α cells, suggesting that the *RE* affected silencing in more than one way (Figure 3B).

The *RE* did not act through *Fkh1* or the SCB to antagonize silencing

The effect of the *RE* on donor preference depends on the transcription factor binding sites enriched in the left portion of the *RE* (Sun *et al.* 2002; Coic *et al.* 2006b). Since the left portion of the *RE* was responsible for the effect on silencing, we tested whether this effect depended on the transcription factors known to bind this region.

Fkh1, which recognizes several sites within the *RE*, serves a positive role in Sir-mediated silencing of *HMR* (Hollenhorst *et al.* 2000). Consistent with this observation, we found that *Fkh1* also contributes to the stability of silencing at *HML*, as deletion of *FKH1* increased the rate of silencing loss in both *a* and α cells (Figure 4A). In the absence of *Fkh1*, however, silencing was still less stable in *a* cells than in α cells, suggesting that the mating-type effect on silencing did not depend on *Fkh1*. Furthermore, the *RE* antagonized silencing independently of *Fkh1*, as deletion of the *RE* stabilized silencing in *MAT α fkh1Δ* cells (Figure 4A). Thus, unlike the effect of the *RE* on donor preference, the effect of the *RE* on silencing did not require *Fkh1*.

The SBF (*Swi4/Swi6*) complex binds the SCB located in the left portion of the *RE*, and a TC-to-GA mutation in the SCB, referred to hereinafter as *re^{SCB}*, reduces the preference for *HML* as a donor during mating-type switching (Coic *et al.* 2006b). To determine whether the SCB contributes to the effect of the *RE* on silencing, we replaced the native *RE* locus with the *re^{SCB}* allele and assayed loss-of-silencing events. Whereas deletion of the *RE* stabilized

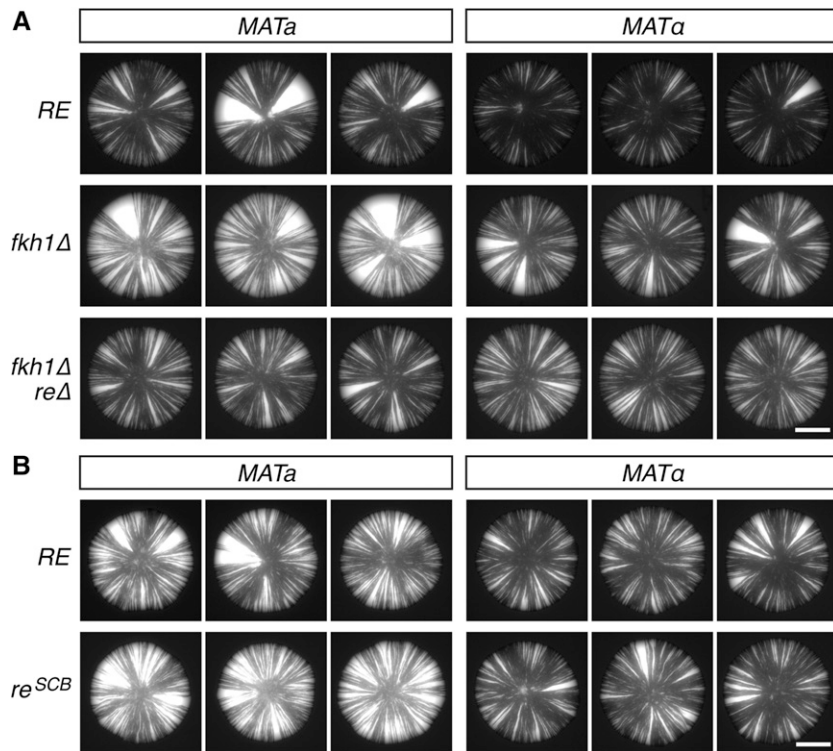


Figure 4 The *RE* destabilized silencing independent of Fkh1 and the SCB. (A and B) GFP expression within colonies of cells containing *HML::cre* and the *sas2Δ* mutation. Bar, 2 mm. The *re^{SCB}* allele in (B) is a TC-to-GA mutation in the SCB sequence within the *RE*. *RE*, recombination enhancer; SCB, Swi4/Swi6 cell cycle box.

silencing in α cells (Figure 2), mutation of the SCB slightly destabilized silencing in α cells (Figure 4B). This observation suggested that the *RE* did not act through the SCB to affect silencing; rather, the *RE* seemed to be even more effective at weakening silencing in the absence of an intact SCB.

The *RE* destabilized the silencing of *HML* in *cis*

Since the *RE* activates recombination *in cis* and resides ~ 17 kbp away from *HML* (Wu and Haber 1996), we tested whether the *RE* affected silencing *in cis* by introducing a plasmid-borne copy of the *RE* into *reΔ* mutants. If the *RE* were to act *in cis*, then the plasmid-borne *RE* should not be sufficient to restore the difference in silencing between α and α cells. As expected, *MATa reΔ* cells containing an empty plasmid showed fewer losses of silencing than *MATa RE* cells (Figure 5A). *MATa reΔ* cells containing *RE* on a plasmid phenocopied the empty-plasmid control and therefore also showed fewer losses of silencing than *MATa* cells containing the *RE* at its native locus (Figure 5A). These observations suggested that the *RE* must be on the same molecule of DNA as *HML* to affect *HML* expression.

The effect of the *RE* on gene expression was not widespread

Approximately 10 genes lie between *HML* and the *RE* (Figure 5B). If the *RE* were to activate a large domain for transcription as it does for recombination, then the *RE* should increase the expression levels of these genes, as well. Since quantitative RT-PCR was sensitive enough to detect a difference in *HML::cre* expression between *MATa RE* cells and *MATa reΔ*

cells ($P = 4 \times 10^{-4}$) (Figure 2B), we used this approach to sample a subset of the other nearby genes. *CHA1* mRNA levels were slightly higher in *RE* cells than in *reΔ* cells, whereas *KRR1* and *PRD1* mRNA levels were not significantly different between *RE* and *reΔ* cells ($P_{CHA1} = 0.03$, $P_{KRR1} = 0.9$, and $P_{PRD1} = 0.7$) (Figure 5B). Collectively, these measurements suggested that the effect of the *RE* on gene expression was locus-specific rather than widespread.

Early-firing replication origins distal to *HML* affected the stability of silencing

After identifying the long-distance, *cis* effect of the *RE* on silencing, we explored whether other distal elements affect silencing, as well. The firing of origins during DNA replication was of particular interest, as the silencers flanking *HML* contain ARS elements that become active origins of replication upon deletion of *ARS305* and *ARS306*, two early-firing origins located 25- and 59 kbp centromere-proximal to *HML*, respectively (Figure 6A) (Vujcic *et al.* 1999; Wang *et al.* 2001). To test whether the timing or location of origin firing affects the stability of silencing, we deleted active origins on the left arm of chromosome III and analyzed the rate of silencing loss at *HML*. Deletion of *ARS305*, the left-most active origin on chromosome III, caused a slight destabilization of silencing in both mating types, as did deletion of both *ARS305* and *ARS306* (Figure 6B). However, neither deletion removed the striking difference in the stability of silencing at *HML* between the two mating types. Therefore, active origins of replication exerted subtle effects on the dynamics of heterochromatin, but were not related to the role of the *RE*.

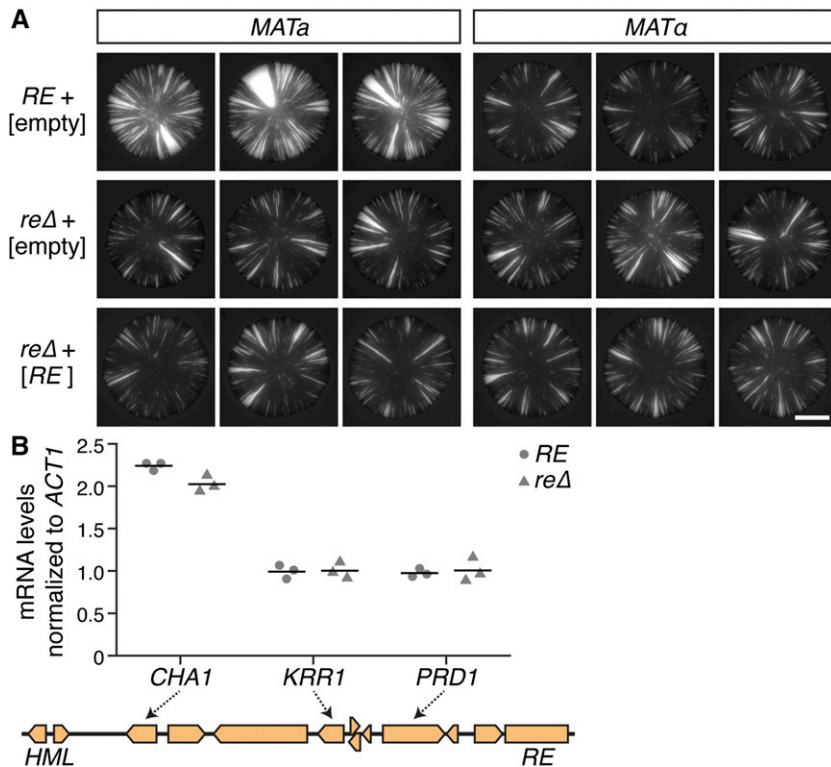


Figure 5 The *RE* affected specific loci *in cis*. (A) Comparison between the native *RE* and a plasmid-borne *RE*. Images show GFP expression within colonies. Brackets indicate plasmid contents. All strains contained *HML::cre* and the *sas2Δ* mutation. Bar, 2 mm. (B) RNA measurements corresponding to genes that lie between *HML* and the *RE*. *CHA1*, *KRR1*, and *PRD1* mRNA levels were normalized to *ACT1* mRNA levels. Horizontal lines represent the means ($n = 3$). All strains contained the *sas2Δ* mutation. mRNA, messenger RNA; *RE*, recombination enhancer.

Discussion

An evaluation of *Sas2* and its contributions to heterochromatin dynamics led to the unexpected identification of a long-range effect on gene expression. We found that the *RE*, a 2-kbp intergenic region located on chromosome III, acted *in cis* at a distance of ~ 17 kbp to antagonize the silencing of *HML*. Partial deletions of the *RE* locus revealed that this effect depended on an 891-bp subregion that contains an unusually high density of transcription factor binding sites. This subregion is also critical for the role of the *RE* in donor preference (Wu and Haber 1996). Similar to the effect of the *RE* on recombination, its effect on gene expression was specific to α cells. Thus, the *RE* locus, originally defined for its role in enhancing recombination, moonlighted as a cell-type-specific modifier of heterochromatin dynamics.

RE: transcriptional enhancer or silencing suppressor?

Whereas the *RE* promotes recombination throughout the entire left arm of chromosome III, the effects of the *RE* on gene expression were locus-specific. That is, the *RE* increased the expression of *HML*, but did not generally affect other genes in the vicinity. At least two explanations could account for this specificity. In the first scenario, the *RE* would somehow interfere with the ability of Sir proteins to silence transcription. *KRR1* and *PRD1* are euchromatic loci and would therefore be immune to any effect mediated by the *RE*. In support of this model, the *RE* showed a very modest effect on the expression of *CHA1*, which sits adjacent to *HML* and is partially repressed by the Sir proteins (Moreira and Holmberg 1998; Ellahi *et al.* 2015). In the second scenario, something

specific about the $\alpha 2$ promoter at *HML* would render it sensitive to the *RE*'s activity. For example, the *RE* could physically interact with the $\alpha 2$ promoter and thereby activate transcription. Of these two models, we favor the one in which the *RE* antagonizes the silencing machinery, particularly because we were unable to detect a reproducible effect of the *RE* on *HML* expression in the absence of silencing (data not shown).

Action at a distance

One noteworthy difference between α cells and α cells is the spatial conformation of chromosome III (Belton *et al.* 2015; Lassadi *et al.* 2015). In comparison to α cells, α cells show more frequent interactions between the left arm of chromosome III, which contains *HML* and the *RE*, and a region on the right arm that extends from the centromere to the *MAT* locus. Upon deletion of the right portion of the *RE* locus, chromosome III adopts a configuration that is indistinguishable between the two mating types (Belton *et al.* 2015). This configuration is distinct from that of both *MAT α RE* cells and *MAT α RE* cells, suggesting that the *RE* regulates chromosome folding in both mating types.

Since the *RE* controls the three-dimensional organization of chromosome III, it is tempting to speculate that the *RE* antagonized silencing by either facilitating or disrupting certain long-range contacts involving *HML*. However, the role of the *RE* in chromosome folding depends largely on the right portion of the *RE* locus (Belton *et al.* 2015), whereas the difference in silencing between α and α cells depended only on the left portion of the *RE*. Deletion of the right portion

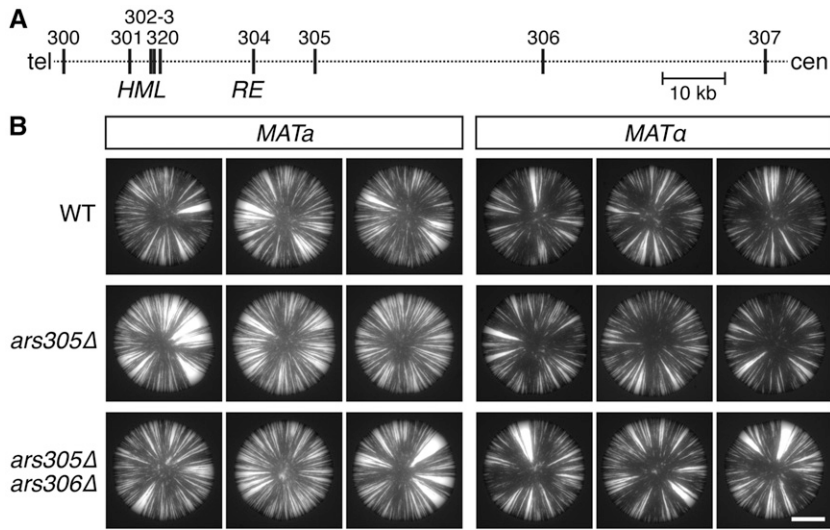


Figure 6 Effects of distal replication origins on the stability of silencing. (A) Map of the left arm of chromosome III showing the ARS elements (vertical lines) located between the telomere (tel) and centromere (cen). (B) GFP expression within colonies of cells containing *HML::cre* and the *sas2Δ* mutation. Bar, 2 mm. ARS, autonomously replicating sequence; WT, wild-type.

slightly stabilized silencing in α cells, but this effect only heightened the overall difference between the two mating types. Therefore, the major effect of the *RE* on silencing could not be attributed to its role in chromosome folding.

Alternatively, the *RE* could relay a message to *HML* by establishing a particular chromatin state that then spreads *in cis* across the left arm of chromosome III. At face value, this model seems to conflict with the observation that the *RE* did not detectably affect the expression of more proximal loci such as *KRR1* and *PRD1*. However, the nature of the chromatin state could be such that it only affects loci subject to silencing. This model predicts that a large, continuous domain on the left arm of chromosome III contains a mating-type-specific chromatin signature. Although no such domain is evident in genome-wide surveys of chromatin modifications to date, a comprehensive comparison between α and α cells remains to be tested.

Given that deletion of *ARS305* and *ARS306* affected the stability of silencing, it is possible that the *RE* destabilized silencing by regulating the firing of replication origins or by altering progression of the replication fork. Interestingly, *Fkh1*, which binds several sites in the left portion of the *RE*, regulates the timing of origin firing (Knott *et al.* 2012). However, *Fkh1* was not required for the *RE* to antagonize silencing, and at this point there is no clear connection between the *RE* and ARS elements with respect to their effects on silencing.

At the intersection of heterochromatin and recombination

The effect of the *RE* on silencing had several aspects in common with the effect of the *RE* on recombination. For example, both effects occur *in cis* and depend specifically on the left portion of the *RE* (Wu and Haber 1996). Therefore, future insight into the mechanism of donor preference may provide additional clues for the mechanism through which the *RE* affects silencing. Determining which factors affect *RE* activity upstream of a double-strand break should be particularly in-

formative given that the *RE* destabilized silencing in the absence of a programmed double-strand break. Interestingly, measurements made by chromatin immunoprecipitation show that the *RE* promotes α -cell-specific enrichment of *yKu70* at *HML* in the absence of *HO* induction (Bystricky *et al.* 2009). Both *yKu70* and *yKu80*, which together form a heterodimer involved in DNA repair, affect silencing and also contribute to donor preference (Ruan *et al.* 2005; Coïc *et al.* 2006b; Patterson and Fox 2008; Vandre *et al.* 2008; Bystricky *et al.* 2009). Given their connections to both silencing and *RE* activity, the Ku proteins could, in principle, contribute to the effect of the *RE* on silencing. The DNA helicase-like protein *Chl1*, another protein that affects both silencing and *RE* activity, is another promising candidate (Weiler *et al.* 1995; Das and Sinha 2005).

Notably, the *RE* did not depend on *Fkh1* or the SCB to antagonize silencing as it does to enhance recombination. Although *Fkh1* and *Swi4/Swi6* both affect Sir-mediated silencing (Laman *et al.* 1995; Sussel *et al.* 1995; Hollenhorst *et al.* 2000), the *RE* did not act through either of these transcription factors to increase the rate of silencing loss. In fact, the destabilization of silencing resulting from mutation of the SCB suggested that the SCB may normally act to dampen the effect of the *RE* on silencing. Perhaps association of the SBF complex with the *RE* interferes with the binding or activity of some other protein that mediates the effect on silencing. Other proteins known to interact with the *RE* include *Fkh2* and *Ndd1* (Sun *et al.* 2002), and there may be additional *RE*-interacting proteins that have yet to be identified.

A newfound appreciation of mating type

Sir proteins preserve the mating-type identity of cells by silencing the transcription of *HML* and *HMR*. We found that mating type, in turn, affected the ability of Sir proteins to silence transcription. Specifically, cells that mated as α lost silencing at *HML* more often than cells that mated as α . This distinction had gone unnoticed until now, presumably because the effects of the *RE* are rare and transient. Deletion

of *SAS2* or *SIR1* sensitized silencing to a level where the difference between α and α cells became apparent, although a close evaluation of wild-type cells showed a similar trend (data not shown). Overall, these observations highlight both the power of segregation analysis and the importance of maintaining consistent *MAT* genotypes when comparing the stability of silencing between strains.

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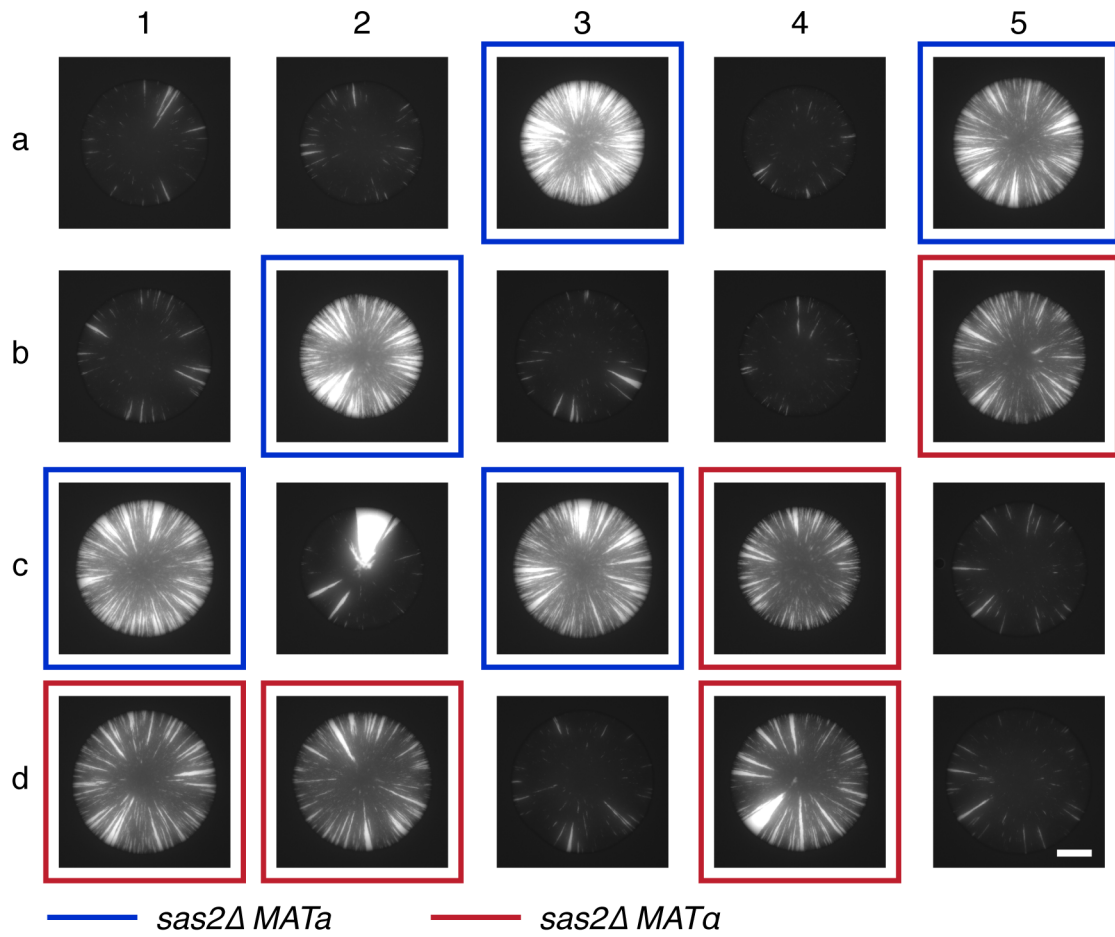


Figure S1. Tetrad analysis of a *sas2* hemizygote containing *HML::cre* and the RFP-GFP cassette (JRY10656). Images show GFP expression within colonies derived from the four spores (a-d) of individual tetrads (1-5). Boxes indicate spores that inherited the *sas2Δ* mutation. Scale bar, 1 mm.

Table S1: Strains used in this study. (.xlsx, 26 KB)

Available for download as a .xlsx file at:

<http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.116.194696/-/DC1/TableS1.xlsx>

Table S2: Oligonucleotides used in this study. (.xlsx, 34 KB)

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Table S3: Plasmids used in this study. (.xlsx, 24 KB)

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