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UAS_{rpg} can function as a heterochromatin boundary element in yeast

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The *HM* loci in *Saccharomyces cerevisiae* constitute region-specific but gene-nonspecific repression domains, as a number of heterologous genes transcribed by RNA polymerase II or III are silenced when placed at these loci. The promoters of the *Ashbya gossypii TEF* gene and the *S. cerevisiae TEF1* and *TEF2* genes, however, are resistant to transcriptional silencing by the *HM* silencers in yeast. Moreover, when interposed between the *HML* α genes and the *E* silencer, certain segments of these promoters block the repression effect of the silencer on the α genes. All of these fragments contain UAS_{rpg} (upstream activation sequence of ribosome protein genes) composed of multiple binding sites for Rap1. In fact, a 149-bp segment consisting essentially of only three tandem Rap1-binding sites from the UAS_{rpg} of yeast *TEF2* exhibits silencer-blocking activity. This element also exhibits insulating activity and orientation dependence characteristic of known chromatin boundary elements. Finally, the element blocks the physical spread of heterochromatin initiated at a silencer. This segment provides the first example of chromatin domain boundary or insulator elements in yeast.

[*Key Words*: Chromatin boundary elements; promoters; silencers; DNA topology; Rap1; transcriptional silencing]

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The expression state of a eukaryotic gene depends on its location in the chromosome. This position effect results from the organization of the eukaryotic genome into discrete functional domains, defined in part by local differences in chromatin structure. The extent of each domain appears to be defined and maintained by boundary, or insulator, elements. Examples of boundary elements in chromosomes of metazoans include the insulator of the *gypsy* retrotransposon, the scs and scs′ elements flanking the 87A1 hsp70 locus in *Drosophila*, and the chicken β-globin boundary elements (Kellum and Schedl 1991, 1992; Geyer and Corces 1992; Chung et al. 1993). Boundary elements have been defined functionally by their ability both to block trancriptional activation of a promoter by a nearby enhancer and to protect transgenes from positive (activating) or negative (repressing) position effects.

Mating type determination and telomere position effect provide examples of position dependent gene expression in the yeast *Saccharomyces cerevisiae*. The mating type of a haploid *S. cerevisiae* strain is determined by the allele (**a** or α) present at the *MAT* locus near the centromere of chromosome III. Copies of the mating type genes, including intact structural genes and their promoters, also reside at the homothalic mating loci *HML* and *HMR*, respectively. Whereas genes at *MAT* locus are freely expressed, genes at the *HM* loci are transcriptionally repressed and only serve as donors of information during a mating type switching event.

Similar to position effect in higher eukaryotes, transcriptional silencing at the *HM* loci and at telomeres in yeast derives from a heterochromatin-like structure (for review, see Braunstein et al. 1997). First, DNA in transcriptionally silent chromatin in yeast is relatively inaccessible to various modifying agents (Terleth et al. 1989; Gottschling 1992; Singh and Klar 1992; Loo and Rine 1994). Second, similar to DNA in heterochromatin in higher cells, transcriptionally silenced regions in yeast replicate late in S phase (Reynolds et al. 1989). Third, nucleosomes from the silent *HM* loci and telomeres have reduced acetylation compared to nucleosomes from active regions of the genome (Braunstein et al. 1993) and the pattern of acetylation of histone H4 at *HM* loci is identical to that in centric heterochromatin in *Drosophila* (Braunstein et al. 1996). Fourth, DNA within transcriptionally silenced *HM* loci is more negatively supercoiled than that in active *HM* loci (Bi and Broach 1997; Cheng et al. 1998), indicating that silent chromatin is more compact. Finally, high-resolution chromatin mapping of *HML*α and *MAT*α revealed that the silent *HML*α locus has a uniquely organized chromatin structure (Weiss and Simpson 1998).

The combined actions of *cis*-acting DNA elements and *trans*-acting factors establish and maintain transcriptional silencing at the *HM* loci (for review, see Laurenson and Rine 1992). The *cis*-acting sites, known as the *E* and *I* silencers, are small negative regulatory sequences

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flanking each of the *HM* loci (Abraham et al. 1984; Feldman et al. 1984) and are both necessary and sufficient for silencing (Brand et al. 1985; Mahoney and Broach 1989; Shei and Broach 1995). They are composed of various combinations of binding sites for proteins Rap1, Abf1, and the origin recognition complex (ORC). The *trans*-acting factors required for silencing include histones, silencer binding proteins, and the four *SIR* proteins—Sir1 through Sir4.

The proteins involved in silencing form extensive homotypic and heterotypic interactions. Both Sir3 and Sir4 can homodimerize and heterodimerize and both can bind to Sir2, Rap1 (Moretti et al. 1994; Strahl-Bolsinger et al. 1997), and histones H3 and H4 in vitro (Johnson et al. 1990; Hecht et al. 1995). Sir3 also can bind to histones H2A and H2B (Hecht et al. 1996). Both Sir4 and ORC interact with Sir1 (Triolo and Sternglanz 1996). These interactions prompted the current model for silencing, in which silencers recruit Sir1 and Sir3/Sir4 through their direct interactions with ORC and Rap1, respectively. Sir1 and Sir3/Sir4 in turn recruit Sir2 to the silencer. This complex at the silencer then seeds an array of complexes comprised of Sir2, Sir3, and Sir4 that spreads outward into the adjacent chromatin. In this fashion, Sir1 functions only in initiating silencing (Triolo and Sternglanz 1996), whereas Sir2, Sir3, and Sir4 form an extended complex as an integral part of the silent chromatin (Hecht et al. 1996).

Transcriptional silencing at the *HM* loci has been considered region-specific but gene-nonspecific, as translocation of the mating type genes resident at silent loci to different sites de-represses them and insertion of heterologous genes into the *HM* loci results in their repression. *LEU2*, *URA3*, *ADE2*, and *TRP1*, transcribed by RNA polymerase II (Pol II), and *SUP3* and *SUP4*-o, transcribed by RNA polymerase III, are all repressible by *HML* or *HMR* silencers (Brand et al. 1985; Schnell and Rine 1986; Mahoney and Broach 1989; Sussel and Shore 1991; Sussel et al. 1993). The fact that Ty*5* retrotransposons inserted within *HML* can be transcriptionally activated by the pheromone response pathway, and that the heat shock promoter inserted next to *HMR* can be induced by heat shock, questions the generality of silencer-mediated repression (Lee and Gross 1993; Ke et al. 1997). In both of these cases, though, the silencing apparatus represses basal transcription.

In this report, we show that the promoters of *S. cerevisiae TEF2* and *TEF1* genes as well as that of the *TEF* gene of filamentous fungus *Ashbya gossypii* are resistant to silencing when inserted at *HM* loci. Moreover, these promoters have silencer-blocking activity that does not require transcription per se. In these promoters, the UAS_{rpg} (upstream activation site for ribosomal protein genes) sequence consisting of binding sites for Rap1 is necessary and sufficient for the silencer-blocking activity. UAS_{rpg}-containing elements from these promoters also block the spread of the unique chromatin structure correlated with silencing. These elements present the first examples of chromatin boundary/insulator elements in yeast.

Results

Promoters of the A. gossypii *and* S. cerevisiae TEF *genes are resistant to transcriptional silencing*

In an attempt to compare features of transcriptional silencing at *HML*α and *HMR***a**, we inserted the heterologous *kanMX* module at *HML* and the *ADE2* gene at *HMR* (Fig. 1A) in the same strain. The *kanMX* module consists of the kan^R open reading frame (ORF) of *Escherichia coli* transposon *Tn903* fused to the transcriptional control sequences of the *TEF* gene from filamentous fungus *A. gossypii* (Fig. 1A). This hybrid *kanMX* module confers geneticin (G418) resistance to yeast (Wach et al. 1994). Whereas the *ADE2* gene at *HMR* in this SIR^+ strain was repressed (cells did not grow on SC – Ade medium; Fig. 1B), the *kanMX* module was actively transcribed (YXB15 cells grew on YPD + Geneticin medium) even though it was flanked by the *E* and *I* silencers of *HML* (Fig. 1B). Therefore, the *kanMX* gene was resistant to silencing whereas the *ADE2* gene was sensitive to silencing. One explanation for the apparent resistance of the *kanMX* module to Sir-mediated silencing is that silencing of the gene does in fact occur but that the repressed level of expression still yields sufficient product to confer a geneticin resistance phenotype. To test this possibility, we used Northern analysis to examine directly the transcript levels of the *kanMX* module inserted in the *HML* locus in a Sir^+ versus Sir^- strain. The results of this analysis, in Figure 1C, show that the level of transcription of the *kanMX* module differs by <20% in the SIR^+ versus the sir^- background. Therefore, the *kanMX* module is resistant to Sir-mediated transcriptional silencing.

To determine whether the sensitivity of the two different marker genes to silencing was a function of the different *HM* loci, we examined strains containing the *ADE2* gene inserted into *HML* at the same site as that used for the *kanMX* module. As evident from the results in Figure 2, the *ADE2* gene resident at *HML* was subject to Sir-dependent repression. The SIR^+ version of the strain was auxotrophic for adenine (Fig. 2, strains YXB19-I and YXB19-II) whereas the isogenic sir^- strain was prototrophic (data not shown). Because a hybrid gene consisting of the *A. gossypii TEF* promoter (P_{AgTEF}) fused to the *ADE2*-coding region inserted into the *HML* locus was resistant to silencing (Fig. 2, strains YXB21-I and YXB21-II), the difference in sensitivity of *ADE2* versus *kanMX* to Sir-mediated repression can be ascribed to their respective promoters. Therefore, the ability of the *kanMX* module to overcome silencing is the property of P_{AgTEF}.

The *TEF* gene in *A. gossypii* is the only gene in this organism coding for translation elongation factor-1α (EF-1α) (Steiner and Philippsen 1994). *S. cerevisiae*, on the other hand, has two redundant genes, *TEF1* and *TEF2*, encoding EF-1α (Nagata et al. 1984; Schirmaier and Philippsen 1984; Cottrelle et al. 1985). To test if the *S. cerevisiae TEF* promoters are also resistant to transcriptional silencing, we fused the promoter of the *TEF2* gene (P_{TEF2}) with the coding region and 3′ flanking region of *ADE2* and inserted this chimeric module at the *HML*

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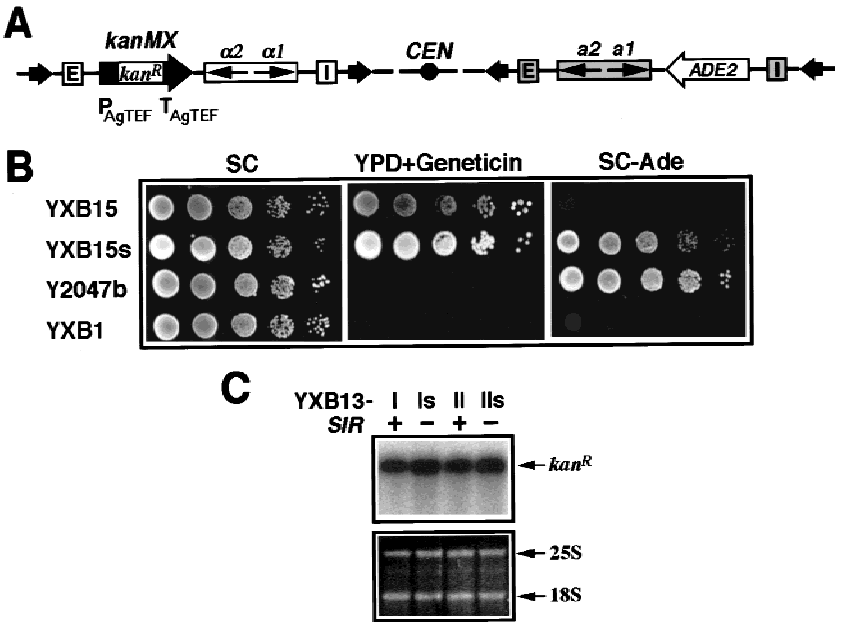

Figure 1. The *kanMX* module is refractory to *SIR*-mediated transcriptional silencing. (*A*) Modified *HM* loci in strain YXB15. The *kanMX* module (P_{AgTEF}–*kan*^{R}–T_{AgTEF}) and *ADE2* gene were inserted at *HML* and *HMR*, respectively. (Open bars) *HML* silencers. (Shaded bars) *HMR* silencers. (Arrows in open bars) *HML*α genes. (Arrows in shaded bar) *HMR***a** genes. (Solid arrows) *FRT* sites. See Materials and Methods for details. (*B*) Growth phenotypes of YXB15 (*HML*α::*KanMX HMR***a**::*ADE2 ade2-1 SIR*$^+$), YXB15s (*HML*α::*KanMX HMR***a**::*ADE2 ade2-1 sir3*), Y2047b (Δ*E–HML*α*–SUP4–o–*Δ*I-HMR***a** *ade2-1 SIR*$^+$) and YXB1 (*HML*α *HMR***a** *ade2-1 SIR*$^+$). Cells were grown to late log phase and serial dilutions (10-fold) were spotted on test plates and allowed to grow for 3 days. Geneticin (G418) was used at 250 μg/ml. (*C*) Northern blot analysis of *kanMX* expression. Cells of each strain were grown to late log phase before being harvested and their total RNA extracted as described (Kaiser et al. 1994). Ten micrograms of total RNA was loaded in each lane. The gel was stained with ethidium bromide to reveal the 25S and 18S rRNAs as loading controls. The *kan*^{R} mRNA was detected by Northern blotting and hybridization with a radioactive probe made from the ORF sequence of *kan*^{R}. Note that strain YXB13-I was the parental strain of YXB15 (Materials and Methods; Fig. 2).

locus (Fig. 2, strain YXB22). This P_{TEF2}–*ADE2* construct was fully expressed. Strain YXB22 grew on SC–Ade medium and strain YXB22 colonies were white on YPD and indistinguishable from strain YXB22s (*sir*$^-$) colonies (Fig. 2; data not shown). A similar result was obtained with the yeast *TEF1* promoter (data not shown). Therefore, like their *A. gossypii* counterpart, the *S. cerevisiae TEF* promoters are resistant to transcriptional silencing.

The *HML E* and *I* silencers and the *HMR E* and *I* silencers are not equivalent in their strength in transcriptional silencing. By several criteria, the relative order of repression activity is *HMR E* > *HML E* > *HML I* > *HMR I* (Shei and Broach 1995; Z. Zhang and A.R. Buchman, pers. comm.). Therefore, a gene resistant to *HML* silencing may not necessarily be resistant to *HMR* silencing. To address whether the *TEF* promoters were also resis-

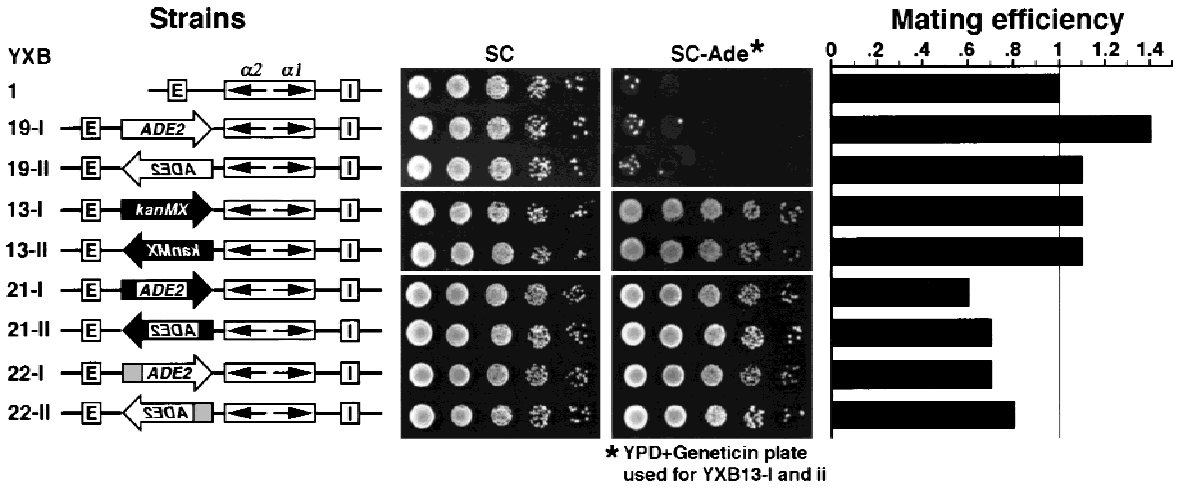

Figure 2. The *A. gossypii* and yeast *TEF* promoters confer resistance to transcriptional silencing. (*Left*) Modified *HML* loci in the strains tested. YXB19 contains unmodified *ADE2* gene (open block arrows) at *HML*. YXB13 has the *kanMX* module (solid block arrows) at *HML*. YXB21 and YXB22 have chimeric genes P_{AgTEF}–*ADE2*–T_{AgTEF} (solid regions of the block arrows represent *AgTEF* sequences and open regions represent the *ADE2* coding region) and P_{TEF2}–*ADE2* (shaded region of the block arrows represents the TEF2 promoter and open regions represent the *ADE2*-coding region) at *HML*, respectively. (*Middle*) Growth phenotypes examined as described in legend to Fig. 1. (*Right*) Mating efficiency as measured by quantitative mating (Materials and Methods). The mating efficiency of YXB1 was taken as one.

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tant to *HMR* silencing, we inserted the chimeric constructs P_{TEF1}–*ADE2* and P_{TEF2}–*ADE2* into *HMR* and showed that they were fully expressed in a *SIR*$^+$ background (data not shown). Therefore the *TEF* promoters are resistant to silencing by even the strongest silencer in yeast.

Active and silenced genes can coexist at HML

We have demonstrated that genes driven by the *TEF* promoters are transcriptionally active when inserted at *HML*. This could be attributable either to *TEF*-induced abrogation of silencing across the entire locus or to resistance of the *TEF* promoters to the transcriptional repression imposed by the silencing apparatus. To distinguish between these possibilities, we investigated whether the presence of these activated genes at *HML* affected silencing of the α*1* and α*2* genes resident at the same locus. This was accomplished by testing the mating ability of the strains of interest, as the mating efficiency of a *MAT***a** strain is inversely proportional to the expression state of the *HML*α genes (Herskowitz 1988). As shown in Figure 2, neither the repressed *ADE2* gene (in strain YXB19) nor the active *kanMX* module at *HML* (strain YXB13) had any effect on mating and the P_{AgTEF}–*ADE2* (in strain YXB21) and P_{TEF2}–*ADE2* (in strain YXB22) genes caused only a slight decrease in mating efficiency. These results suggest that although the *TEF* promoters were active at *HML*, their presence had little, if any, effect on the silencing of the adjacent α*1* and α*2* genes.

To confirm that the silencing apparatus is intact at *HML* containing the P_{TEF}-driven genes, we examined the effect of the *SIR* genes on the topology of the locus. The heterochromatin induced by the silencing apparatus imposes a different topology on the DNA across the *HML* locus than does the chromatin associated with the active form of the locus. This difference can be detected as an increase in the negative superhelical density of a circular DNA molecule obtained by in vivo excision of the *HM* locus from the chromosome of a *SIR*$^+$ versus a *sir*$^-$ strain (Bi and Broach 1997; Cheng et al. 1998). Therefore, a difference in the superhelical density of such a molecule excised from a *SIR*$^+$ versus a sir$^-$ strain would indicate that the locus was packaged in heterochromatin in the *SIR*$^+$ background.

Our analysis of the topology of the *HM* loci containing various inserted genes is shown in Figure 3. Consistent with previous results (Bi and Broach 1997; Cheng et al. 1998), circular molecules spanning the unmodified *HML* locus excised from the chromosome of a *SIR*$^+$ versus a *sir*$^-$ strain exhibited a difference in superhelical density of ~2, with the *SIR*$^+$ species being more negatively supercoiled (Fig. 3, cf. lanes YXB1 and YXB1s). Similarly, we found that circles spanning the *HMR* locus carrying the *ADE2* gene and circles spanning the *HML* locus carrying the *kanMX* module were also more negatively supercoiled in the *SIR*$^+$ versus a *sir*$^-$ strain (Fig. 3, strains YXB15 and YXB15s). Similar results were obtained with the *HML* locus containing either P_{TEF2}–*ADE2* or P_{AgTEF}–*ADE2* (data not shown). Because the extent of Sir-dependent superhelical density change is a function both of length and composition of the DNA, we cannot determine from the precise linking number change whether the entire locus containing the *kanMX* module, or only a part of the locus, is packaged in heterochromatin. Nonetheless, these results clearly demonstrate that the presence of the *kanMX* module does not eliminate Sir-dependent silencing at *HML*.

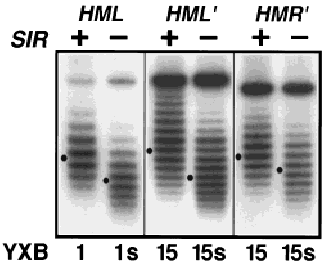

Figure 3. Insertion of a silencing-resistant gene at *HML* does not abolish the *SIR*-dependent silent chromatin structure. Cells of the indicated strains were grown in YPR medium to log phase before galactose was added and the cultures were incubated for 2.5 hr. DNA isolated from cells was fractionated by agarose gel electrophoresis in the presence of 30 μg/ml chloroquine. Under this condition, the more negatively supercoiled circles migrate more slowly in the gel. *HML* and *HMR* circles were revealed by Southern blotting. The Gaussian center of each distribution of topoisomers is indicated (●). (*HML'* and *HMR'*) The modified *HML* and *HMR* loci in strain YXB15, respectively (Fig. 1A).

A silencing-resistant gene has silencer-blocking activity

We showed above that the silencing-resistant promoter inserted at *HML* did not eliminate repression of the adjacent α*1* and α*2* genes. This could indicate that the presence of a silencing-resistant gene between *E* and α-mating genes does not affect the ability of *E* to silence the α-mating genes. Alternatively, the silencing-resistant gene may block *E* from exerting its silencing effect on α-mating genes, with silencing of the α mating genes in such a situation caused solely by the action of the *I* silencer. This later hypothesis is not unreasonable, as the *HML E* and *HML I* silencers are each capable alone of establishing and maintaining silencing of the α-mating genes at *HML* (Mahoney and Broach 1989). To distinguish between these two possibilities, we examined expression of the α*1*/α*2* genes at *HML* in strains in which the P_{TEF2}–*ADE2* module was inserted between *E* and the α genes in a locus that lacked the *HML I* silencer (Fig. 4, strains YXB25-I and YXB25-II). Because this assay determines the ability of a test sequence to block the activity of the silencer on its normal target when the test gene sits between them, such an assay provides one indication of the boundary activity of the inserted sequence.

Consistent with earlier observations (Mahoney and Broach 1989), the mating efficiency of a strain in which *HML I* is deleted (strain YXB26) was comparable with

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the strain with an intact *HML* locus (strain YXB1), confirming that the *E* silencer alone is sufficient for silencing the α-mating genes at *HML*. Insertion of the P_{TEF2}–*ADE2* gene between *E* and the α-mating genes reduced mating efficiency by 100- to 1000-fold (Fig. 4, strains YXB25-I and YXB25-II) below that of strain YXB26, indicating essentially full derepression of the α-mating genes in these strains. Insertion of the P_{AgTEF}–*ADE2* gene had a similar effect (data not shown). These results demonstrate that the silencing-resistant genes exhibit silencer-blocking activity.

UAS_{rpg} from the TEF *genes can function as heterochromatin boundary elements*

To identify the region of P_{TEF2}–*ADE2* responsible for silencer-blocking activity and to examine whether active transcription of the gene is required for activity, we interposed various fragments of P_{TEF2}–*ADE2* between the *E* silencer and the α-mating genes in *HMLΔI* and then tested their effects on silencing of the α-mating genes. As shown in Figure 5A, all of the UAS_{rpg}-containing fragments of yeast P_{TEF2} reduced mating efficiency of cells to some extent (cf. strains YXB29, YXB48, YXB59, and YXB60 with strain YXB26). In particular, a 104-bp fragment containing the *TEF2* UAS_{rpg} (strain YXB59) was sufficient to reduce the mating efficiency by 90% and the 54-bp UAS_{rpg} per se reduced mating efficiency by 70% in one orientation (strain YXB60-II). In contrast, fragments lacking UAS_{rpg} had little, if any, effect on mating efficiency (cf. strains YXB49, YXB28, and YXB27 with strain YXB26). Consistent with the above results for yeast P_{TEF2}, a TATA-less 284-bp fragment of the *A. gossypii TEF* promoter containing UAS_{rpg}-like sequences was sufficient to reduce the mating efficiency of cells by 10-fold (Fig. 5B, compare strain YXB31 with strain YXB26). A 119-bp fragment of the 284-bp fragment excluding the UAS_{rpg} had little effect on silencing (Fig. 5B, strain YXB47). These data suggest that even in the absence of active transcription, UAS_{rpg}-containing elements from *TEF* promoters have silencer-blocking activity.

In contrast to the *TEF* gene promoters, the promoter from *ADE2* did not exhibit silencer blocking activity (Fig. 5B, cf. strains YXB37 and YXB38 with strain YXB26). A 600-bp fragment of the *ADE2* ORF (+10 to +611), even when present in two or three tandem copies, also failed to exhibit silencer blocking activity (strains YXB39, YXB41, and YXB42). Despite the fact that the promoter region of the α-mating genes lies 3.8 kb distal to the *E* silencer in strain YXB42, silencing is maintained, confirming that silencing initiated at *E* can spread over a long distance. This indicates that the silencer-blocking activity observed for the small (<350 bp) *TEF* promoter elements is not a distance effect. Finally, the magnitude of the effect obtained with the UAS elements compared with those obtained with the intact genes suggest that other factors in addition to the UAS elements may contribute to the magnitude of the disruption of *E*-mediated silencing. The UAS_{rpg} element, however, is the only sequence tested from P_{TEF2}–*ADE2* or P_{AgTEF} that alone elicits significant silencer blocking activity. Furthermore, because the magnitude of the effect of UAS_{rpg} is comparable with that of other boundary elements previously described, we conclude that UAS_{rpg} is a viable candidate as a yeast boundary element.

The UAS_{rpg} sequence of *TEF2* consists of three variants (designated R1, R2, and R3 for convenience) of the consensus sequence for Rap1 binding. The R1-containing 114-bp fragment of P_{TEF2} (Fig. 5A, strain YXB28) did not block silencing significantly. The R2 sequence alone, which binds tightly to Rap1 in vitro (Buchman et al. 1988), also had no silencer-blocking activity (Fig. 5A, strain YXB53). Tandem copies of two or five R2 sequences also failed to exhibit silencer-blocking boundary activity (Fig. 5A, strain YXB54; data not shown). Therefore, the particular combination of the R1, R2, and R3 binding sites for Rap1 in the *TEF2* promoter or the particular orientation or spacing of Rap1 sites appears critical for silencer blocking activity.

A second criterion for a chromatin boundary element is that its activity be orientation-dependent in the following sense. A boundary element can render a reporter gene impervious to an enhancer or silencer only when it lies between the silencer or enhancer and the reporter gene. We found that the putative boundary elements identified in this study fulfilled this criterion. As shown in Figure 6A, insertion of the 149-bp fragment spanning the UAS_{rpg} from *TEF2* between *E* and the *HMLα* genes at *HMLΔI* resulted in activation of the α genes (strains YXB48-I and YXB48-II), whereas insertion of the same sequence on the *E*-distal side of the *HMLα* genes failed to elicit their activation (strains YXB58-1 and YXB58-II).

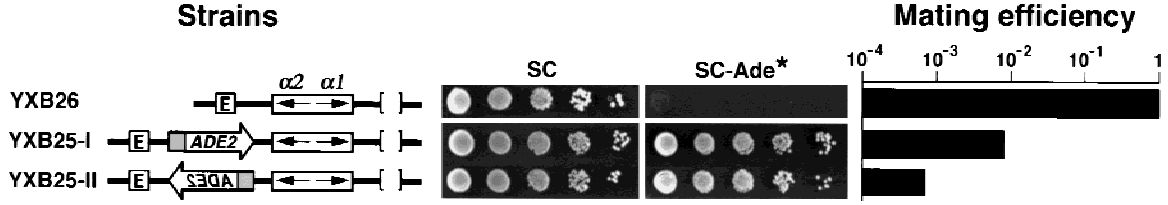

Figure 4. Silencing-resistant genes exhibit silencer-blocking activity. (*Left*) Modified *HML* loci in the strains tested. The *HML I* silencer was deleted from each strain. The P_{TEF2}–*ADE2* module was inserted between the *E* silencer and the *HMLα* genes in YXB25. (*Middle*) Growth phenotypes examined as described in legend to Fig. 1. (*Right*) Mating efficiency as measured by quantitative mating. The mating efficiency of YXB26 was taken as one.

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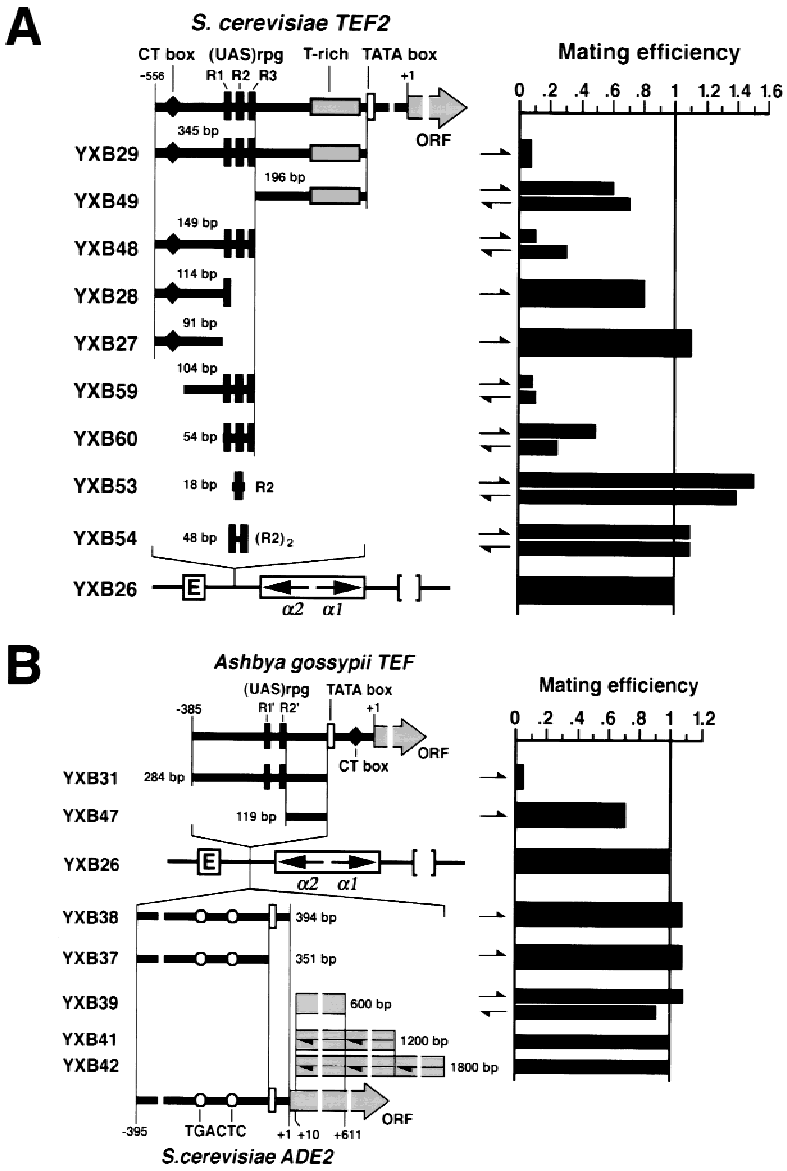

Figure 5. UAS_{rpg} exhibits silencer-blocking activity. (*A*) Various fragments from the yeast *TEF2* promoter were inserted at the *Spe*I site between *HML E* silencer and the α genes at *HMLΔI* (*left*); their effect on α gene expression was analyzed by quantitative mating (*right*). The direction of an insertion is indicated by a half arrow. In the diagram of the TEF2 promoter the following are indicated: (solid bars) Rap1-binding sites; (open bar) TATA box; (♦) CT box (Gcr1-binding site); (shaded bar) T-rich region. (*B*) Similar analyses of *A. gossypii TEF* promoter and the yeast *ADE2* gene. (○) TGACTC sequence. Other symbols are as in *A*.

We concluded from these results that the *TEF2* UAS element did not function simply as a general activator of the α-mating genes when inserted in *HMLΔI* but rather blocked the ability of the *E* silencer to repress the genes. Therefore, the *TEF2* UAS exhibits the orientation properties of a boundary element.

Finally, boundary elements have also been defined functionally by their insulator activity. That is, two copies of a bona fide boundary element bracketing a reporter gene can insulate expression of that reporter gene from effects imposed by the surrounding chromosomal environment. Therefore, we tested whether two copies of the *TEF2* UAS bracketing the α-mating genes would protect them from silencing by both silencers of *HML*. Copies of the 149-bp element of P_{TEF2} were inserted on either side of the α-mating genes at *HML*, in all four combinations of relative directions of the elements (Fig. 6B, strains YXB57-A to YXB57-D). The 149-bp element caused a significant derepression of the α-mating genes in strains YXB57-B and YXB57-D, but only mild derepression in strains YXB57-A and YXB57-C. Although we do not understand the dependence of insulating activity on the direction of the element, these results confirm that the 149-bp element of P_{TEF2} possesses all the properties of a boundary element.

To confirm the insulator activity of the P_{TEF2} UAS, we asked whether this element could insulate a gene other than the α-mating genes from silencing imposed by *HML*. To do so, we examined the expression of a *URA3* gene inserted into the *HML* locus in place of the α-mating genes in an otherwise *ura3*$^-$ strain. As observed previously and as shown in Figure 6C, expression of the *URA3* gene inserted in *HML* is repressed, as witnessed by the resistance of strains YXB61-I and YXB61-II to 5-fluoro-orotic acid (FOA). We then examined the expression of a *URA3* gene inserted at the same site but

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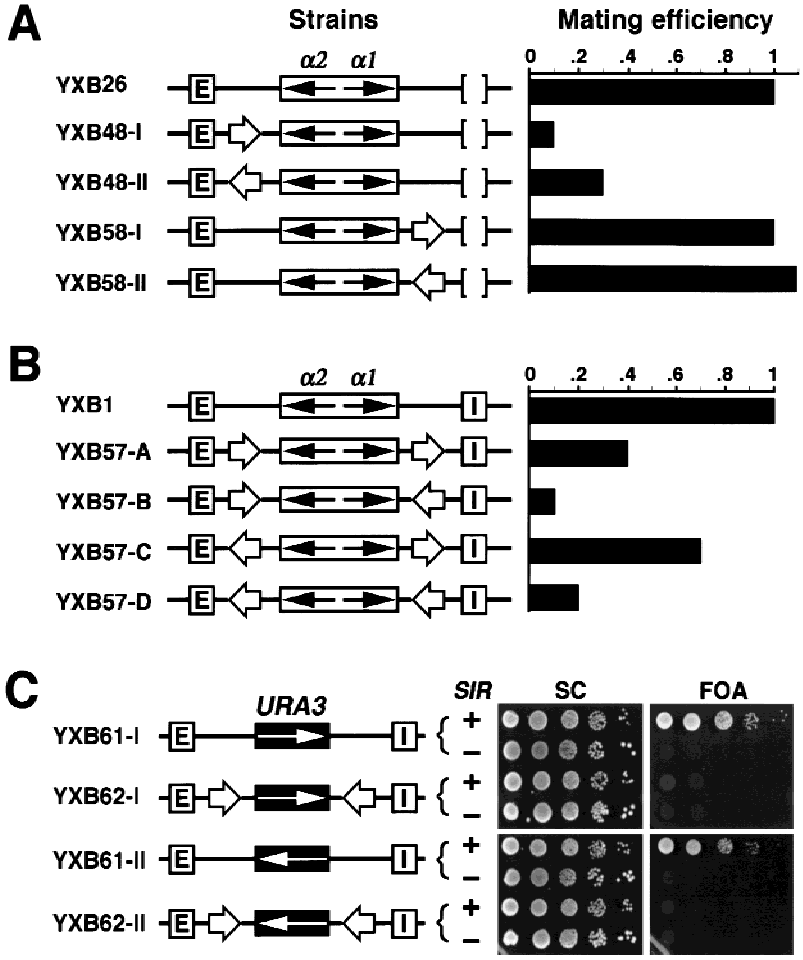

Figure 6. UAS_{rpg} shows position-dependent silencer-blocking activity and insulator activity. (*A*) UAS_{rpg} shows position-dependent silencer-blocking activity. The 149-bp P_{TEF2} fragment (open arrow; Fig. 5A) was inserted *E*-proximal (YXB48-I and YXB48-II) or *E*-distal (YXB58-I and YXB58-II) to the α genes at *HMLΔI*, respectively. The effect of the insertion on α gene expression was examined by quantitative mating. The mating efficiency of YXB26 was taken as one. (*B*) UAS_{rpg} shows insulator activity. In strains YXB57-A to YXB57-D, *HML* α genes were bracketed by a pair of the 149-bp element of *TEF2* (open arrow), which were in turn flanked by the *E* and *I* silencers. The mating efficiency of YXB1 was taken as one. (*C*) UAS_{rpg} can insulate the *URA3* gene from Sir-mediated repression. In strains YXB61-I and YXB61-II, the *HML*α genes were replaced by the *URA3* gene as detailed in Materials and Methods. A pair of the 149-bp fragment of *TEF2* were inserted at sites bracketing *URA3* in strains YXB61-I and YXB61-II, resulting in strains YXB62-I and YXB62-II. The growth phenotypes of the YXB61 and YXB62 strains and their *sir*⁻ derivatives on medium containing 5-FOA are shown at *right*.

bracketed by copies of the P_{TEF2} UAS. As evident from the results in Figure 6C, strains containing such a construct are completely sensitive to FOA (strains YXB62-I and YXB62-II). Therefore, the P_{TEF2} UAS is capable of insulating not only the α-mating genes but also the *URA3* gene from the repression by the *HML* silencers.

UAS_{rpg} blocks the spread of the Sir-dependent chromatin structure initiated at a silencer

We have shown that fragments spanning UAS_{rpg} block the ability of the *E* silencer to repress the α genes at *HML* when inserted between the silencer and the α-gene promoters. We wanted to determine whether it did so by blocking the spread of Sir-dependent heterochromatin initiated at the silencer. To explore this possibility, we constructed strains in which we could simultaneously examine the topology of different segments of the *HML* locus. To do so, we constructed strains carrying the *HML* loci diagrammed in Figure 7A. Each of these loci contain three copies of the *FLP* recombination target site (*FRT*) in direct orientation, two bracketing the entire locus and one lying between the *E* silencer and the *α1*/*α2* genes, at the site we use for inserting the boundary element (insertion of the *FRT* between *E* and the *α1*/*α2* genes does not affect *E*-mediated silencing of those genes; Holmes and Broach 1996; data not shown). As a result of this configuration, induction of *FLP* yields excision of two circles. One circle corresponds to the region encompassing the *E* silencer up to the point of insertion of the boundary element; the other circle corresponds to the region distal to the site of insertion of the boundary element and encompasses the *α1*/*α2* genes. Because the excised circles are of significantly different sizes, we could independently examine the migration of both of them in the same track on chloroquin agarose gels.

The results of this analysis are shown in Figure 7. As expected from our previous observations, both circles (A1 and B1) excised from the two halves of the otherwise wild-type *HML* locus were more negatively supercoiled in a *SIR*⁺ (strain YXB63) than in a *sir*⁻ background (strain YXB63s). Therefore, in a wild-type locus, the Sir-dependent chromatin structure extends over both the silencer region and the *α1*/*α2* genes. The same Sir-dependent pattern was observed with circles (A2 and B2) excised from strains YXB72 and YXB72s, which contain an *HML* locus identical to that in strains YXB63 and YXB63s except for deletion of the *I* silencer. Therefore, consistent with expression studies, the *E* silencer alone is sufficient to impose a Sir-dependent chromatin structure across the *α1*/

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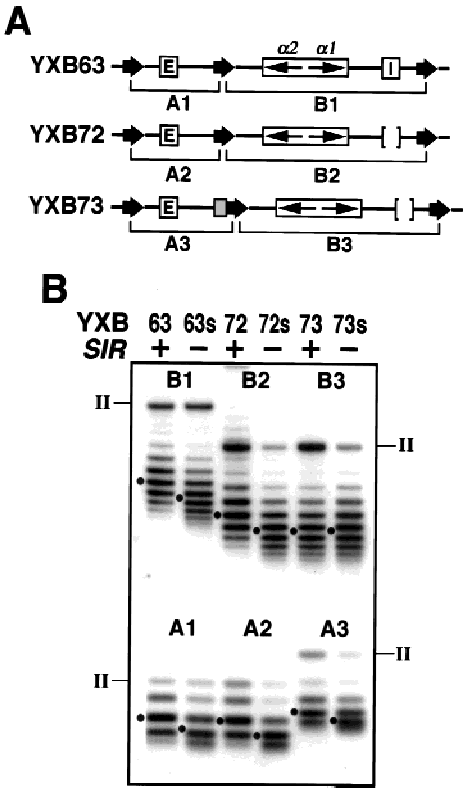

Figure 7. UAS_{rpg} blocks the spread of *SIR*-dependent silent chromatin structure initiated at a silencer. (*A*) Diagram of the *HML* locus in strains in which separate segments of the locus can be simultaneously excised as circles for topological examination. (Thick arrows) *FRT* sites; (shaded box) the 149-bp fragment of P_{TEF2} containing UAS_{rpg}. (*B*) Topological states of separate parts of *HML*. DNA circles from the *HML* locus of each strain diagramed in *A* were analyzed as described in Materials and Methods. The topoisomers of the two circles in each strain are significantly different in size (~1 vs. ~3 kb) so that they can be fractionated without overlap in the same track of the gel. The nicked (from II) circles are indicated. The Gaussian center of each distribution of topoisomers is indicated (●).

α*2* genes. Insertion of the UAS_{rpg} boundary element markedly altered this pattern. In a locus containing the boundary element between the *E* silencer and the α*1*/α*2* genes, the region upstream of the boundary element showed the same Sir-specific change in topology as was found for the locus lacking the boundary (circle A3 in strains YXB73 and YXB73s compared with circle A2 in strains YXB72 and YXB72s). In contrast, the topology of DNA across the α-mating genes, that is, downstream of the boundary element (circle B3), was identical in the SIR^+ and sir^- strains (YXB73 and YXB73s). Further, the topology of the DNA across the mating type genes downstream of the boundary element in the SIR^+ strain was identical to that of the mating type genes in the absence of a boundary but in a sir^- strain (cf. B2 with B3 in lanes 72s and 73). Therefore, the chromatin structure downstream of the boundary element corresponds to that of the derepressed locus. From this we conclude that the UAS_{rpg} blocks the spread of Sir-dependent heterochromatin initiated at the silencer.

Discussion

Genes resistant to transcriptional silencing in yeast

We have shown in this study that promoters of the *TEF* genes from budding yeast *S. cerevisiae* or filamentous fungus *A. gossypii* are refractory to transcriptional silencing. In *S. cerevisiae*, both *TEF1* and *TEF2* code for translation elongation factor 1α (EF-1α), one of the most abundant proteins in eukaryotic cells. The *TEF* genes belong to a large family of genes (ribosomal protein genes or RPG) encoding components of the translation machinery, which are coordinately regulated (for review, see Planta and Raue 1988; Shore 1994). Most yeast RPGs contain an upstream activation site (UAS_{rpg}) consisting of at least one, and usually two or more, Rap1-binding sites located ~250–450 bp upstream of the ATG start codon. UAS_{rpg}s mediate transcriptional activation of RPGs. In addition, a T-rich stretch often lies downstream of UAS_{rpg} and can enhance RPG transcription (see Fig. 5A). Like its *S. cerevisiae* counterparts, *AgTEF* also has a UAS_{rpg} consisting of two Rap1 binding sites in its promoter and is resistant to silencing in yeast. All three promoters contain a binding site for Gcr1, a factor originally identified as a glycolysis regulator required for efficient transcription of glycolytic genes (Santangelo and Tornow 1990). We predict, but have not tested, that all of the ribosome protein genes and other housekeeping genes that have UAS_{rpg} in their promoters would be resistant to transcriptional silencing.

How do *TEF* promoters escape transcriptional silencing? Aparicio and Gottschling (1994) showed that overexpression of the transcriptional activator Ppr1 could reverse silencing of a *URA3* gene subject to telomere position effect. They provided evidence consistent with a model in which an activator complex and the silencing apparatus compete for assembly at the promoter site. Whichever complex formed first in a particular cell cycle would determine the expression state of the locus during that cell cycle. The resistance of *TEF* promoters can be understood in the context of this model, by assuming that an activator complex remains stably bound to the UAS_{rpg} throughout the cell cycle or that the avidity of the complex for UAS_{rpg} is sufficiently high that it always out competes the silencer apparatus for formation at the *TEF* promoter. Which elements of the P_{TEF} confer silencer resistance? The T-rich sequence in yeast *TEF* promoters likely does not contribute to silencing resistance as it is absent from *AgTEF*. Gcr1 binds to Rap1 and its activity at promoters is UAS_{rpg}-dependent, indicating that Gcr1 acts through Rap1. Gcr1 binds to CTTCC (CT-box) sequences present in promoters of many glycolytic genes but DNA binding is not important for Gcr1p function as deletion of the CT-box in the DNA and/or removal of the DNA-binding domain from Gcr1p do not affect its ability to support efficient transcription. Therefore, silencing resistance, like boundary activity as described below, likely resides in the Rap1-binding sites.

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Rap1, also known as Grf1, TBA, or TUF, is an essential nuclear protein present at exceedingly high abundance (10^4 molecules per nucleus) for a sequence-specific DNA-binding protein (for review, see Shore 1994). The 13-bp consensus sequence for Rap1 binding lies not only in the enhancer domains (UASs) of numerous genes but also in the silencers of the *HM* loci and in telomeric ($C_{1-3}A$) repeats. Rap1 is a global regulator that affects transcriptional activation, transcriptional repression, telomere length, circular plasmid segregation and meiotic recombination. Rap1 performs these diverse functions by interacting with different factors in proper contexts. For instance, as a transcriptional repressor, Rap1 binds to both *HML E* and *HML I* and *HMR E* silencers and recruits Sir3 and/or Sir4 to silencers through direct interaction. Similarly, Rap1 interacts with Rif1 and various telomere-specific proteins in executing its role in regulating telomere length. Defining how Rap1 elicits resistance to repression, however, poses some difficulty, especially because in other contexts the insertion of a single Rap1-binding site enhanced, rather than abrogated, repression of a reporter gene inserted into an attenuated *HML* locus (Boscheron et al. 1996). In addition, the UAS element necessary for expression of *α1* and *α2* contains a Rap1-binding site (Giesman et al. 1991) that rather than render the genes resistant to silencing at *HML*, makes them exquisitely sensitive to transcriptional silencing. Therefore, whereas Rap1 is critical to the resistance of certain promoters to transcriptional silencing, other as yet undefined factors or contexts determine whether the Rap1 binding site will enhance silencing or render a promoter resistant to silencing.

Rap1-binding sites can constitute silencer-blocking chromatin boundary element in yeast

In this study we have shown that the silencing-resistant *TEF* genes from yeast or *A. gossypii* can serve as boundaries to contain the spread of heterochromatin. The UAS_{rpg} of P_{TEF2} is both necessary and sufficient for silencer-blocking activity and the UAS_{rpg}-containing elements excluding the TATA-box from the *AgTEF* promoter can also function as boundary elements. These elements block silencing when present between the silencer and the reporter gene but not when present downstream of the reporter gene, indicating these elements do not simply reverse silencing by acting as transcriptional enhancers. Finally, the UAS_{rpg} of P_{TEF2} can insulate both the mating type genes and the *URA3* gene from the repressive effects of a heterochromatin domain. Therefore, we have shown that the UAS_{rpg} of P_{TEF2} exhibits all the properties of chromatin boundary elements identified in metazoans, and moreover, that the UAS_{rpg} of P_{TEF2} functions as a physical barrier to the spread of Sir-dependent heterochromatin.

As in silencer resistance, the Rap1-binding sites are critical for chromatin boundary activity. Boundary activity, however, likely requires the coordinated actions of more than one Rap1-binding sites aligned in a proper organization. In P_{TEF2}, three Rap1p-binding sites are positioned in direct orientation and the distances between them are 6 and 3 bp, respectively; in P_{TEF1}, two Rap1p sites in direct orientations are separated by 4 bp; and in P_{AgTEF}, two Rap1p sites in direct orientation are separated by 17 bp. Whereas the UAS_{rpg} (R1-R2-R3) of *TEF2* possesses orientation-dependent silencer-blocking activity, R1 or R2 alone has no silencer-blocking activity. Furthermore, two directly repeated copies of R2 separated by 11 bp showed no silencer-blocking activity, even though the two Rap1 sites (separated by 17 bp) in *AgTEF* had silencer-blocking activity. These data suggest that the organization (orientation and spacing) of Rap1-binding sites is important for their silencer-blocking activity. In this context, it is noteworthy that Rap1 binding can induce a 50–60° bend in DNA 5′ to the recognition sequence (Vignais and Sentenac 1989; Vignais et al. 1990; Gilson et al. 1993; Muller et al. 1994). For multiple juxtaposed Rap1-binding sites, the overall effect of Rap1 binding on DNA structure would depend on their orientations and spacing. Despite this observation, Buck and Shore (1995) showed that a carboxy-terminal domain of Rap1 fused to the GAL4 DNA-binding domain could mediate silencing when targeted to a mutated silencer with the Rap1 site replaced by the GAL4 site. Therefore, binding of Rap1 to DNA per se is not required for silencing. It will be interesting to test the effect of such a construct on boundary activity.

The best-characterized chromatin boundary elements include the specialized chromatin structure (scs and scs′) at the boundaries of the *Drosophila* 87A *hsp70* genes, the insulator element in the of *Drosophila gypsy* retrotransposon, and the sequences associated with the 5′ constitutive hypersensitive site in the chicken β-globin locus (Corces and Gerasimova 1997). The scs′ element contains the binding site for the BEAF-32 protein, and the *gypsy* insulator is composed of 12 binding sites for su(Hw) (suppressor of Hairy-wing). Like Rap1 in yeast, BEAF-32 binds to many sites in the *Drosophila* genome (specifically, to interband regions that separate polytene bands of *Drosophila* third-instar larval chromosomes) and may have general structural and functional roles throughout the genome. In addition, like Rap1, BEAF-32 binds to at least one promoter, that of the *aurora* gene (Glover et al. 1995). The su(Hw) proteins also bind to many (~200) sites on polytene chromosomes from third-instar larvae, which are not sites of *gypsy* elements, and may also have a role in the higher order organization of the *Drosophila* genome. A second component of the *gypsy* insulator is the mod(mdg4) protein (modifier for mdg4), which interacts with su(Hw). In the absence of mod(mdg4), the *gypsy* insulator becomes a silencer that mediate bidirectional repression of nearby genes via heterochromatin formation. This is caused by the presence of su(Hw) alone at the insulator. Interestingly, su(Hw) can also function as a transcriptional activator (Corces and Geyer 1991). Therefore, similar to Rap1 in yeast, su(Hw) can function in gene repression and activation as well as serve as a component of chromatin boundaries in a context-dependent manner.

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A model for boundary activity

In Figure 8 we present a model that would account for the observations described in this report. Our model postulates that Rap1 bound to sites within the UAS_{rpg} excludes formation of several nucleosomes across the region. This would be consistent with our observation that the UAS_{rpg} on its own alters local chromatin structure, independent of its effect on silencing (X. Bi and J.R. Broach, unpubl.). In addition, if propagation of the silencing complex along chromatin requires sequential interaction of the Sir complex with contiguous nucleosomes, as has been suggested (Hecht et al. 1996; Braunstein et al. 1997), then the nucleosomal 'hole' in the chromatin would block the outward migration of the silencing complex. This nucleosome exclusion activity could account for the function of UAS_{rpg} as an enhancer, in that excluding nucleosomes could render the neighboring DNA accessible to other transcription factors and to assembly of the machinery necessary for initiation of transcription. In addition, the silencer blocking activity of UAS_{rpg} would render the highly active genes in which it acts refractory to any repressive effects emanating from adjacent genes.

We have shown that UAS_{rpg} possess chromatin boundary activity. This element, however, is not present at the boundary between the *HML* heterochromatin region and the adjacent euchromatic region. In fact, testing individual DNA segment in the region surrounding *HML* in the boundary assay described in this report failed to identify any chromatin boundary elements flanking *HML* (X. Bi, M. Braunstein, G. Shei, and J.R. Broach, unpubl.). Therefore, whereas the yeast genome contains sequences that will block the spread of heterochromatin, the yeast cell can use a different mechanism to restrict the spread of heterochromatin and limit its effects to specific domains.

Materials and methods

Plasmid and yeast strain constructions

The *kanMX* module consists of the *A. gossypii TEF* promoter (P_{AgTEF}, the *Spe*I–*Nco*I fragment), the ORF of the *E. coli kan*^{R} gene of Tn*903* (the *Nco*I–*Sca*I fragment) and the terminator sequence of *A. gossypii TEF* (T_{AgTEF}, the *Sca*I–*Not*I fragment) (Wach et al. 1994; see Fig. 1A). The *Not*I–*kanMX*–*Not*I fragment of pFA6–*kanMX* (Wach et al. 1994) was inserted into plasmid pRS416 (Sikorski and Hieter 1991) at the *Not*I site to make pRS–*kanMX*. The *Xba*I–*kanMX*–*Spe*I fragment of pRS–*kanMX* was inserted at the *Spe*I site of pYXB1 (Bi and Broach 1997) in two orientations, resulting in pYXB13-I and pYXB13-II, respectively (see Fig. 2). Plasmid pYXB1 contains the *Bam*HI fragment of yeast chromosome III containing *HML*α (in pUC19) with two *FLP1* recombination target sites (*FRT*; Broach and Volkert 1991) in direct orientation inserted at the *Bsu*36I and *Sna*BI sites bracketing *HML*α. Plasmid pUC18–*HMR* was made by inserting the *Hin*dIII fragment of yeast chromosome III containing *HMR***a** into pUC18 at the *Hin*dIII site. Plasmid pUC18–Δ*hmr::SUP4–o* was derived from pUC18–*HMR* by replacing its *Nru*I–*HMR*α–*Xho*I fragment with the *Eco*RV–*SUP4–o–Xho*I fragment from pMB21 (Braunstein 1996). Two *FRT* sites were inserted into pUC18–*HMR* at the *Xho*I and *Sna*BI sites bracketing *HMR***a** to make p*FHMRF*.

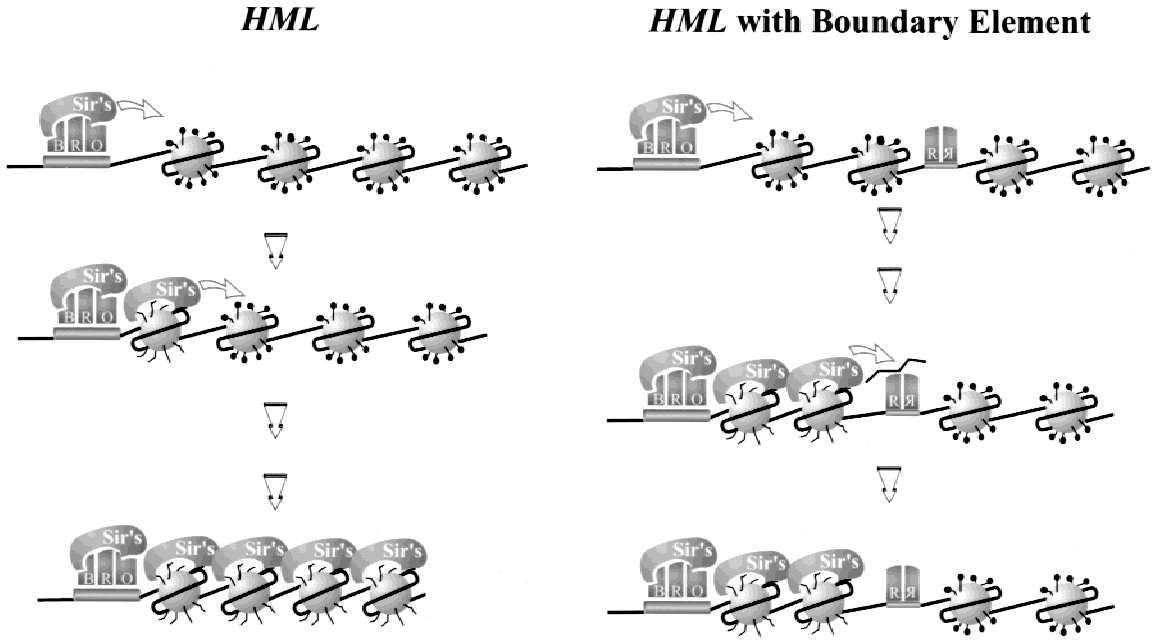

Figure 8. A model for the heterochromatin boundary activity of UAS_{rpg}. Transcriptionally silenced chromatin initiates from a silencer and emanates outward as a result of the spreading of the *SIR* complex along contiguous nucleosomes, leading to an extended complex of Sir proteins across hypoacetylated nucleosomes (*left*; see introductory section and Discussion for detailed descriptions). Binding of multiple Rap1 molecules to UAS_{rpg} excludes formation of several nucleosomes across the region and this nucleosomal 'hole' in the chromatin blocks the migration of the *SIR* complex (*right*). See Discussion for a detailed description. (B) Abf1; (R) Rap1; (O) ORC; (Sir's) complex of Sir2, Sir3, and Sir4.

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Each plasmid described below was made by inserting a PCR-amplified fragment of interest (with appropriate restriction sites added to both ends) into a properly digested vector. The insert was then confirmed by DNA sequencing. Plasmid pYXB15 was made by inserting the *ADE2* gene (coordinates –550 to +1990, with the ORF being +1 to +1716), with two *Bam*HI sites added to both ends, at the *Bgl*II site of p*FHMRF*. pYXB19 was constructed by inserting *ADE2* (coordinates –300 to +1990, ORF being +1 to +1716) at the *Spe*I site of pYXB1. The ORF of *ADE2* (+1 to +1716) was used to replace the *Nco*I–*kan*^{R}–*Sca*I fragment of pYXB13, generating pYXB21. Plasmid pYXB21-H was derived from pYXB21 by replacing its *Spe*I–P_{AgTEF}–*Nco*I fragment with P_{TEF2} (promoter region of *S. cerevisiae TEF2* gene, coordinates –556 to –1). Plasmid pYXB22 was constructed from pYXB21-H by substituting the *Bgl*II–*Blp*I fragment (consisting of part of *ADE2* ORF and the T_{AgTEF} sequence) with the *Bgl*II–*Blp*I fragment of pYXB19, effectively resulting in the replacement of T_{AgTEF} with T_{ADE2} (+1717 to +1994). Plasmid pYXB26 was derived from pYXB1 by deleting a 462-bp fragment (coordinates 4889–5173 of the *Bam*HI–*HML*–*Bam*HI fragment) containing the *HML I* silencer. Plasmid pYXB25 was derived from pYXB22 by deleting the *I* silencer of *HML* as just described for the construction of pYXB26. Plasmid pYXB29 was derived from pYXB26 by inserting the 345-bp fragment of P_{TEF2} shown in Figure 5A at the *Spe*I site. The following plasmids were similarly derived from pYXB26. Plasmids pYXB49, 48, 28, 27, 59, 60, 53, and 54 were constructed using various fragments of P_{TEF2}, respectively (see Fig. 5A). pYXB31, 47 were made using fragments from P_{AgTEF}, respectively (Fig. 5B). pYXB37, 38, 39, 41, and 42 were made using fragments of *ADE2*, respectively (Fig. 5B). The 149-bp fragment of P_{TEF2} (Fig. 5A) was inserted at the *Bst*1107I site of pYXB26 resulting in plasmid pYXB58 (Fig. 6A). Two copies of the 149-bp fragment were inserted at the *Spe*I and the *Bst*1107I sites of pYXB1, respectively, in all four possible combinations of orientations to make plasmids pYXB57-A, pYXB57-B, pYXB57-C, and pYXB57-D (Fig. 6B). Plasmid pUC26 contains the *Bam*HI fragment of yeast chromosome III containing *HML* in pUC19. A 294-bp sequence (coordinates 3319–3613) of the *Bam*HI fragment, containing the promoters for the *HMLα1* and *α2* genes, was replaced by a 1.1-kb *Hin*dIII–*URA3*–*Hin*dIII fragment of pDM22 (Mahoney and Broach 1989), generating pYXB61. Plasmid pYXB62 was derived from pYXB61 by inserting two copies of the 149-bp fragment of P_{TEF2} (Fig. 5A) at the *Spe*I and *Bst*1107I sites flanking *URA3* in opposite orientations (Fig. 6C). Plasmid pYXB63 was derived from pYXB1 by inserting an additional *FRT* site at the *Spe*I site of *HML* (Fig. 7A). Plasmid pYXB72 and 73 (Fig. 7A) were similarly derived from pYXB26 and pYXB48-I, respectively. For all the above plasmids harboring modified *HML* sequences (except pYXB26 and pYXB57), -I or -II denotes that a fragment is inserted at *HML* in the same or opposite orientation as that of the *α1* gene (the direction of a fragment being the 5′ to 3′ orientation of the coding strand of the gene it resides in). See Figures 1, 2, and 4–7 for illustrations. Plasmid pMB21 (Braunstein 1996) is an integrating plasmid containing *SIR3*, *SUP4–o*, and *TRP1* genes.

Yeast strains with modifications at *HML* were derived from Y2047b (*MAT***a** *HMR***a** *HMLα E*Δ*79-113::SUP4–o I*Δ*242 LEU2–GAL10–FLP1 ura3-52 ade2-1 lys1-1 his5-1 can1-100* [cir^0]; Holmes and Broach 1996). The *SUP4–o* allele, which can suppress the *can1-100* mutation is present at *HML*(*ΔEΔI*) in Y2047b, rendering it sensitive to canavanine-killing. The *HML*-containing *Bam*HI fragment of pYXB13-I was used to transform Y2047b to canavanine resistant (due to the loss of *SUP4–o*), resulting in strain YXB13-I (Fig. 2). All the other strains except YXB14, YXB15, YXB61, and YXB62 were made similarly using the *Bam*HI fragments of corresponding plasmids (see Figs. 1, 2, and 4–7). The *Hin*dIII–Δ*hmr::SUP4–o–Hin*dIII fragment of pUC18–Δ*hmr::SUP4–o* was used to transform YXB13-I to lysine prototrophy (attributable to the suppression of *lys1-1* mutation by *SUP4–o*) to make strain YXB14. Strain YXB14 was then transformed to canavanine resistant with the *Hin*dIII fragment of pYXB15, resulting in strain YXB15 (Fig. 1). The *SIR3* gene in some of the above strains was disrupted by *URA3* as described previously (Mahoney and Broach 1989) resulting in the s strains (YXB13-Is, YXB13-IIs, etc.). YXB61s was constructed by transforming DMY2 (*MAT***a** *ura3-52 ade2-1 lys1-1 leu2-3,112 his5-1 can1-100 sir3::LEU2*; Mahoney and Broach 1989) to Ura$^+$ using the *Eco*RI–*hml*Δ*α1α2::URA3–Pvu*II fragment of pYXB61. YXB62s was similarly constructed using the *Eco*RI–*Pvu*II fragment of pYXB62. YXB61s and YXB62s were then transformed to Lys$^+$ using pMB21 digested by *Xba*I whose site is within the *TRP1* gene, resulting in YXB61 and YXB62, respectively, which are SIR$^+$. The relevant genotype of each strain was confirmed by Southern blotting. Growth of yeast strains was done according to standard procedures (Kaiser et al. 1994) unless otherwise specified.

Quantitative mating assay

Quantitative mating was performed as described by Sprague (1991). Strains were grown to a density of 0.5–1.5 × 10^7 per ml. About 3 × 10^6 cells were mixed with ~10^7 cells of the tester strain DC17α and collected on a nitrocellulose filter. In addition, cells of each haploid strain were collected on separate filters. After incubation on YPD for 5 hr, cells from the mating-mix filter were plated on SD medium to select for diploids. Cells of single strains were also plated on SD medium to check for revertants. Cells of the strain being tested were plated on –Leu plate. Mating efficiency is calculated as the number of colonies (from the mating-mix) on SD medium divided by that of the strain being tested (on –Leu medium).

Analysis of DNA circles excised from the HM loci by FLP1

Yeast strains were grown in YPR (1% yeast extract, 2% Bactopeptone, and 2% raffinose) to early log phase (OD_{600} = 0.6). Galactose was then added to the culture to induce the expression of *FLP1*. After 2.5 hr of incubation, cells were collected by centrifugation. Nucleic acid was then isolated using the glass bead method (Kaiser et al. 1994) and fractionated on agarose gels in 0.5× TPE (45 mM Tris, 45 mM phosphate, 1 mM EDTA at pH 8.0) supplemented with chloroquine at a concentration of 30 μg/ml. DNA circles were detected by Southern blotting.

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