

Long-Range Communication between the Silencers of *HMR*[∇]

Lourdes Valenzuela,¹ Namrita Dhillon,¹ Rudra N. Dubey,²
Marc R. Gartenberg,² and Rohinton T. Kamakaka^{1*}

Department of Pharmacology, 683 Hoes Lane, UMDNJ-Robert Wood Johnson Medical School, Piscataway, New Jersey 08854,² and
Department of MCD Biology, Sinsheimer Labs, 1156 High Street, University of California, Santa Cruz, California 95064¹

Received 5 September 2007/Returned for modification 2 October 2007/Accepted 29 December 2007

Gene regulation involves long-range communication between silencers, enhancers, and promoters. In *Saccharomyces cerevisiae*, silencers flank transcriptionally repressed genes to mediate regional silencing. Silencers recruit the Sir proteins, which then spread along chromatin to encompass the entire silenced domain. In this report we have employed a boundary trap assay, an enhancer activity assay, chromatin immunoprecipitations, and chromosome conformation capture analyses to demonstrate that the two *HMR* silencer elements are in close proximity and functionally communicate with one another in vivo. We further show that silencing is necessary for these long-range interactions, and we present models for Sir-mediated silencing based upon these results.

Gene activation and gene repression are central to the proper development and differentiation of organisms. DNA elements such as promoters, enhancers, and silencers play a central role in eukaryotic gene regulation. These elements are separated from each other by several kilobase pairs of DNA but are able to communicate with one another to regulate the activation or repression of genes. The exact mechanism by which distally located elements communicate with one another is not clear and is one of the key questions in gene regulation. Long-range communication between distantly located elements in chromosomes is thought to occur by one of two principal mechanisms (8). One class of models postulate that a signal emanating from a distal regulatory element spreads along the DNA fiber until it encounters a proximal regulatory element. A second class of models postulate that distal and proximal regulatory elements interact with one another directly, with the intervening DNA forming a loop. Both mechanisms must function within the context of the global chromosome structure, which appears to be composed of large chromosome loops that attach to a proteinaceous superstructure (11). The nucleus appears to be divided further into distinct chromatin compartments, with heterochromatic domains being present in regions near the nuclear periphery while euchromatic domains are found mainly in the interior of the nucleus, although a significant portion of euchromatin is located near nuclear pores.

It has been suggested that the functionally and structurally defined chromatin domains may be coincident (36). Enhancers and locus control regions (LCRs) are long-range regulatory elements that activate promoters in a distance- and orientation-independent manner, and recent studies indicate that enhancers and LCRs often cluster together in three-dimensional space to form an “active chromatin hub” (23, 49, 58, 59).

Similarly, in yeast the promoters and terminators of genes are in close proximity to one another (3, 48) and tethered to the nuclear pore (10, 52). The consequence of this spatial organization is that the DNA between these regulatory elements is looped out. It is thought that the formation of these nuclear substructures aids in transcription activation.

Silencers are negative regulatory elements composed of binding sites for various factors that act collectively in the establishment and stable inheritance of a repressed state. Like enhancers, silencers repress promoters in a distance- and orientation-independent manner (35). Silencers flank the silenced *HML* and *HMR* mating-type loci in yeast, while at telomeres the terminal repeated TG1-3 sequences serve as silencers. These silencers recruit the Sir proteins, Sir2p, Sir3p, and Sir4p, which then spread across several kilobase pairs of DNA via interactions with histones. Thus, our current understanding of Sir-mediated repression is that it is an example of long-range effects mediated via transmission along the DNA fiber rather than direct long-range interactions between the silencers (60).

DNA elements that restrict the action of long-distance regulatory elements, such as silencers and enhancers, are generically called insulators. Insulators located between an enhancer and a promoter (called enhancer blockers) disrupt enhancer-promoter communication and prevent the enhancer from activating that promoter, while insulators located between a silencer and a promoter (called barriers) block the silencer from repressing the promoter. Numerous models have been proposed to explain how insulators function to block long-range communication. Some models postulate that insulators act as decoys, forming nonproductive interactions with distal regulatory complexes, or sequester these complexes in specific regions of the nucleus, while other models suggest that insulators function locally by disrupting the propagation of a specific chromatin domain (60).

In this paper we present evidence demonstrating that while silencers function via recruitment and transmission of Sir proteins along the DNA, they also directly communicate with each other. Functional analyses of silencer-mediated repression suggest that silencer elements communicate with one another in

* Corresponding author. Mailing address: Department of MCD Biology, Sinsheimer Labs, 1156 High Street, University of California, Santa Cruz, California 95064. Phone: (831) 459-3391. Fax: (831) 459-3139. E-mail: rohinton@biology.ucsc.edu.

[∇] Published ahead of print on 14 January 2008.

mediating repression in the nucleus. Our studies also show that DNA fragments containing silencers (separated by several kilobase pairs of DNA) are in close spatial proximity in the nucleus and likely form chromatin loops. Interestingly, this long-range communication was lost in mutants of the Sir proteins. Our results suggest that silenced domains are formed by the spreading of repressor proteins from silencers that interact with one another enabling, compaction of the chromatin fiber and stable repression. These results are similar to the long-range interactions between LCRs and promoters and suggest conservation in the mechanism by which genes are activated and repressed.

MATERIALS AND METHODS

The genotypes of the strains, the oligonucleotides, and the exact sequences of the various integrations generated and used in this study will be provided upon request.

Yeast strains. Yeast genomic integrations were performed by homologous recombination and gene replacement, using PCR products or DNA fragments derived from plasmids. Yeast transformations used the lithium acetate method (34). PCR amplifications were carried out with Expand high-fidelity DNA polymerase, and integrations were confirmed by PCR and sequencing analysis. *SIR2*, *SIR3*, *PPR1*, and *ADE2* genes were deleted from the start to the stop codon and replaced with *HIS3* or *kanMX* markers. Deletion of *MAT α* was obtained by replacing *MAT α 2* and *MAT α 1* sequences (SGD coordinates 199731 to 200964) with the *kanMX* cassette.

The *ADE2* gene flanked by Gal4p binding sites (*Gbs*), present at *HML*, in strain KIY54 (32) was PCR amplified with appropriate primers and integrated at the *HMR* locus in a *sir2 Δ* strain (JRY4576) or in an *HMR Δ sir4 Δ* strain (ROY926). Sequencing of the PCR products indicated that a single Gal4p binding site, which is contrary to published results (32), flanked *ADE2*. The PCR product was integrated in the *HMRa2* coding region (SGD coordinates 293212 to 293410) with the *ADE2* promoter close to the *HMR-E* silencer. Strains with the integrated *ADE2* gene (ROY2729 [*MAT α HMR::Gbs-ADE2-Gbs sir2 Δ*] and ROY2914 [*MAT α HMR-E-Gbs-ADE2-Gbs-HMR Δ sir4 Δ*]) were crossed with a W303 wild-type strain to obtain ROY2770 and ROY3001, respectively.

The *HMRa1* coding region in ROY2729 and ROY2914 was replaced by the *URA3* coding region by homologous recombination, and transformants were crossed with an *ade2 Δ ::kanMX* strain to obtain ROY3182 and ROY3194, respectively.

Plasmid pJR1270 contains an EcoRI-HindIII fragment with the *HMR* locus where the *HMR-I* silencer has been deleted. This fragment contains two SpeI sites. The plasmid was partially digested with SpeI, end filled, and religated to obtain pRO698, which contains only one SpeI site 290 bp upstream of the *ARS* element present at *HMR-E*. A pair of oligonucleotides with four *Gbs* flanked by SpeI sites were annealed, digested, and cloned into the SpeI site in pRO698 to produce pRO700. The EcoRI-BglIII fragment from pRO700 was used to replace the *HMR* region in strain ROY2585 (*HMRa2::URA3 sir2 Δ*), to give strain ROY3285 (*Gbs-HMR sir2 Δ*).

The EcoRI-HindIII fragment in pRO700 was used to replace the *HMR* region in ROY2585 to produce ROY3283 (*Gbs-HMR Δ sir2 Δ*). The *URA3* cassette was integrated between *HMR-I* or *HMR Δ* and the tRNA gene (SGD coordinates 295070 to 295281) in strains JRY4566 (W303 *sir2 Δ*), ROY3285, ROY3550 (*HMR Δ sir2 Δ*), and ROY3283, and the transformants were crossed with a *ppr1 Δ ::kanMX* strain to obtain ROY3495 to -3489, -3683, -3699, -3680, and -3697. ROY3495 and -3497 were crossed with a Gal4-TAP-tagged strain to obtain ROY4371 and -4372, respectively.

The tRNA gene was deleted and the *URA3* gene integrated in strain JRY4566 and ROY3285 by PCR-mediated gene replacement. Transformants were then crossed with a *ppr1 Δ* strain to obtain ROY3688, -3703, -3686, and -3701.

Plasmid pJR1571 contains an EcoRI-HindIII fragment comprising the *HMR* locus. Oligonucleotides with three new Sau3A sites were used to PCR amplify the XbaI-EcoNI fragment of the *MATa2* gene. The PCR product was cloned into the XbaI-EcoNI fragment of *HMRa2* in plasmid pJR1571. The EcoRI-HindIII fragment of the new construct (pOS154) was used to replace the *HMR* locus in strain ROY2800 (*HMRa2::URA3*), and transformants were crossed with *mat α Δ ::kanMX*, *sir3 Δ ::HIS3*, and *hml Δ ::TRP1* strains to obtain ROY4064 and ROY4065.

Plasmids. Plasmids RO590 and RO635 contained the full-length *SAS2* or *NUP2* coding regions fused in frame to the Gal4 DNA binding domain (*GBD*), with transcription driven by the *ADHI* promoter in the pGBK-RC-*TRP1* base plasmid (pGBD) (47).

Serial dilutions. Yeast cells were grown overnight at 30°C in 5 ml YPAD or Hartwell's complete (HC) medium without tryptophan to allow maintenance of the plasmids. Cells were diluted to an A_{600} of 1.0 unit/ml in HC-trp medium and serially diluted 5- or 10-fold. Using a cell spotter, approximately 3 μ l of each serial dilution was placed onto properly supplemented HC plates to assay for *ADE2* and *URA3* expression, or onto properly supplemented YMD plates previously spread with 1.0 A_{600} unit of mating lawn (strain JRY19a) diluted in 300 μ l of YPD, for the mating assays. For the mating assays, selection for plasmids was maintained. The plates were incubated at 30°C and photographed. Cells grown in limiting amounts of adenine were kept at 4°C for an additional 2 days for development of the color prior to photography.

ChIP. Quantitative chromatin immunoprecipitation (ChIP) analysis was performed as previously described (46), with minor modifications. The program for the PCR was as follows: 95°C for 3 min (1 cycle), and 95°C for 1 s, 52°C for 30 s, and 72°C for 1 min (45 cycles). The fold enrichment was calculated using the formula $2^{CT(IP)} - CT(input)$ as described previously (40a) and was normalized to the telomeric probe. For Rap1p immunoprecipitation, polyclonal antibodies (Y300; Santa Cruz Biotechnology, Inc.) were used, while antibodies against H4K16Ac were purchased from Upstate.

3C. The chromosome conformation capture (3C) analyses of yeast strains were performed exactly as described previously (18) with a few specific changes. Each strain in each experiment was cross-linked for 0, 5, 10, and 20 min, and each sample was independently processed and analyzed. The restriction enzyme used for the digestion was Sau3A, and the digestion buffers were as recommended by the manufacturer of the enzyme. All primers used were tested with un-cross-linked/ligated DNA, and only primers with equal amplification efficiencies were used for the 3C analyses.

Fluorescence analysis. Two diploid strains were constructed to visualize the relative positions of *HMR* and the nucleolus. The strains differ only by the absence of the tRNA barrier at *HMR*. The *lac* operator array is telomere proximal to *HMR-I*. Both strains contain Sik1p fused to red fluorescent protein (RFP) (a nucleolar marker), express *lac*-green fluorescent protein (*lac*-GFP), and contain a galactose inducible R recombinase.

To examine the relative positions of *HMR* with respect to the nucleolus, the cells were first grown in SC-trp medium containing dextrose. Cells were fixed 2 h with paraformaldehyde and mounted on microscope slides containing agar plugs. Parallel Z stacks of cells were obtained using both rhodamine and GFP filters to visualize the nucleolus and *HMR*, respectively (17 sequential images separated by 0.2 μ m). The two landmarks were considered colocalized if the corresponding fluorescence signals fully overlapped within the same plane or in adjacent planes. The landmarks were considered to touch if contact (but not overlap) was seen between them within a plane or adjacent planes. The landmarks were considered to be fully separated if no contact was observed or if image planes lacking fluorescence separated fluorescent foci in different planes. Multiple fields of cells were examined for each of the two trials. Cell morphology was used to estimate the cell cycle stage of each cell examined. However, the same general trends were observed in G₁, S, and G₂ phases, so these data were pooled (G₁ and S phase cells were well represented, whereas there were considerably fewer G₂ cells).

RESULTS

Silencers flank silenced genes at *HML* and *HMR*. At *HML*, silencing initiates from both silencers, but at *HMR* silencing initiates only at the *HMR-E* silencer. Previous work from our lab showed that tethering proteins with barrier activity near *HMR-E* blocked the spread of silencing from the *HMR-E* silencer if the *HMR-I* silencer was absent (21). However the barrier could be bypassed if a second silencer (*HMR-I*) was positioned downstream of the barrier (references 21 and 47 and data not shown). One explanation for this phenomenon is that silencing nucleates at *HMR-I*, as well as *HMR-E*. However, functional data clearly indicates that *HMR-I* only augments the activity of *HMR-E* and does not possess an autonomous silencing activity (1, 7, 50).

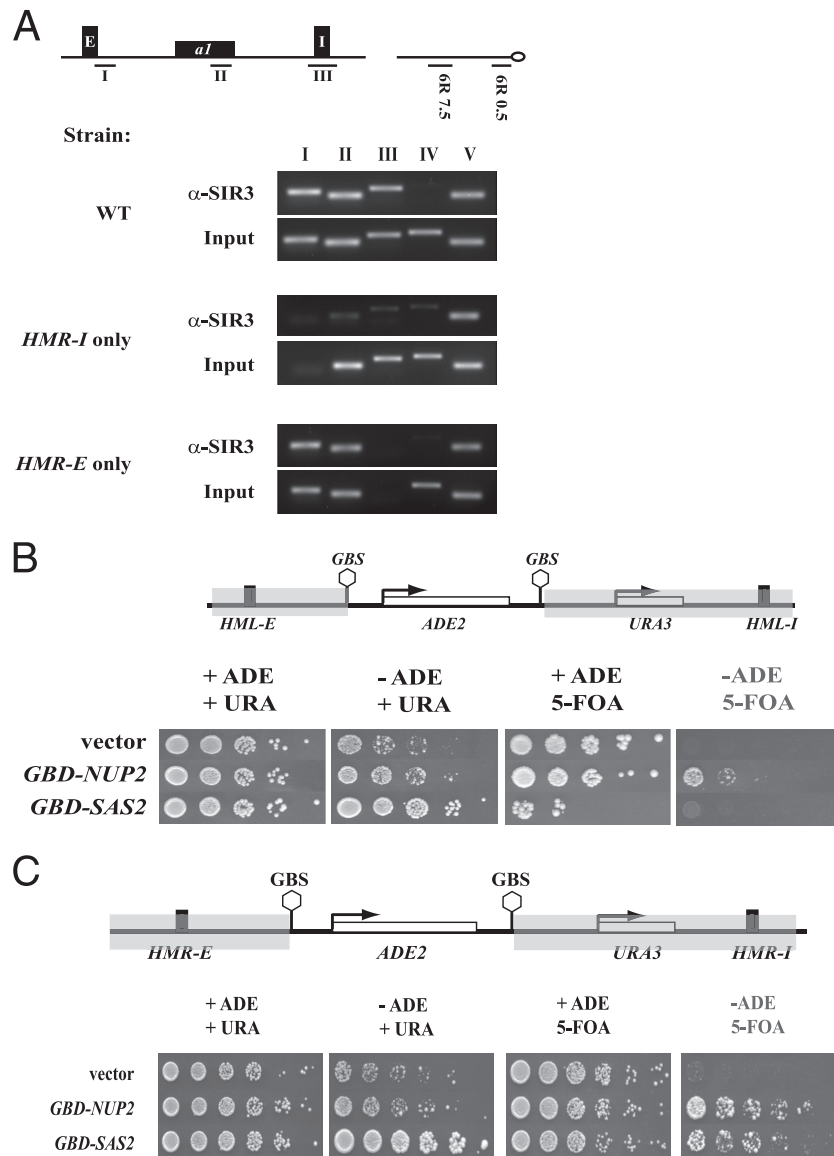


FIG. 1. Boundary trap assays at *HMR* and *HML*. (A) *HMR-I* does not recruit Sir3p. ChIP was used to map the presence of Sir3p in strains with mutant silencers at *HMR*, and the immunoprecipitated samples were analyzed by PCR. The locations of the PCR probes are shown in the schematic diagram. WT, wild type. (B) At *HML*, only Nup2 allows discontinuous silencing. Strains with a boundary trap construct at *HML* were transformed with *TRP1*-containing plasmids constitutively expressing the chimeric protein Gbd-Sas2p (pRO590) or Gbd-Nup2p (pRO635) or the vector. Cells were grown in liquid YM medium (HC-trp), and expression of the *ADE2* and *URA3* genes was monitored by serial dilutions on HC-trp plates lacking or containing adenine, uracil, and 5-FOA as indicated. The plates were photographed after 2 days. The panel labeled in gray allows differentiation between “true barrier” proteins and “desilencing” proteins. (C) At *HMR*, both Nup2 and Sas2p allow discontinuous silencing. Strains with a boundary trap construct at *HMR* were transformed with *TRP1*-containing vector or with *TRP1* plasmids constitutively expressing Gbd-Nup2p or Gbd-Sas2p. Cells were grown overnight in liquid HC-trp, and serial dilutions were spotted on appropriate plates. Cells were spotted on HC-trp plates lacking adenine or containing 30 μ g per ml of adenine and allowed to grow at 30°C prior to photography. To assay for stable repression of *URA3*, cells were spotted onto HC-trp plates containing 5-FOA and lacking adenine or containing 30 μ g per ml of adenine and allowed to grow at 30°C prior to photography. The panel labeled in gray allows differentiation between “true barrier” proteins and “desilencing” proteins.

Since our analyses suggested that silencing at *HMR* might be initiating at *HMR-I*, we reasoned that if *HMR-I* was a silencer, then it should be able to recruit at least some Sir proteins in the absence of the *HMR-E* silencer. To directly test this possibility, we used ChIP to examine the binding of Sir3p near *HMR-E* and *HMR-I* in a variety of *HMR* variants. As expected, Sir3p localized to the two silencers in the wild-type strain (Fig. 1A).

Deleting *HMR-I* did not affect Sir3p levels at the *HMR-E* silencer, but deleting *HMR-E* resulted in loss of Sir3p localization from the *HMR* locus but not the telomeres. In the absence of *HMR-E*, the levels of Sir3p at *HMR* were equivalent to those observed at the negative control, the TEL6R 7.5kb probe, where Sir3p has not been found previously. Therefore, these results, at this level of sensitivity, demonstrate that *HMR-I*

does not recruit Sir proteins in the absence of *HMR-E* and are consistent with previous functional results (1, 7, 50).

These results lead to an alternative explanation that *HMR-E* “communicates” with *HMR-I* to facilitate silencing at a distance. To explore this paradoxical phenomenon further, we monitored silencing of a dual reporter system known as the boundary trap assay that was developed and used to investigate insulator proteins at *HML* (33). In this assay, the silenced locus was modified and the mating-type genes were replaced with *ADE2* and *URA3* genes. Gal4 binding sites flank the *ADE2* gene, whereas a second reporter, *URA3*, is not flanked by these sites and resides adjacent to the I silencer (Fig. 1B and C). The assay monitors the ability of a protein tethered to the Gal4p binding sites to insulate the *ADE2* gene from repression but not the neighboring *URA3* gene.

The dual reporter system was first used at *HML*. Unlike at *HMR*, at *HML* both silencers are able to independently initiate silencing (Fig. 1B). We tested the behavior of Gal4-Nup2p and Gal4-Sas2p. Nuclear pore proteins such as Nup2p were claimed to be “true barrier” proteins that can insulate the *ADE2* gene while maintaining the neighboring *URA3* gene in a silenced state (33). We also tested Gal4-Sas2p, since acetyltransferases are believed to function by a “desilencing” mechanism. We measured expression of *ADE2* by growth on medium lacking adenine, and we measured expression of *URA3* by growth on medium containing 5-fluoroorotic acid (5-FOA). Cells expressing *URA3* convert 5-FOA to a toxic metabolite and die. We used these assays because they are far more sensitive to changes in the expression levels than Northern blots. Furthermore, these assays allow us to determine the mitotic stability of these epigenetic states. Our results (Fig. 1B) are consistent with previously published data (33). Gal4-Nup2p insulated *ADE2* from repression while allowing *URA3* to be stably repressed in a small percentage of cells. On the other hand, Gal4-Sas2p derepressed both *ADE2* and *URA3*, presumably by disrupting silencing across the entire silenced domain.

We next constructed a dual reporter system at *HMR* that was similar to the system at *HML*, placing the *ADE2* gene near *HMR-E* and the *URA3* gene near *HMR-I* (Fig. 1C). The strain was transformed with Gal4-Nup2p, Gal4-Sas2p, or vector alone. The cell growth assays in Fig. 1C clearly show that Gal4-Nup2p functions again as a true barrier, producing colonies of cells in which *ADE2* was active but *URA3* was stably repressed. However, unlike the situation at *HML*, Gal4-Sas2p derepressed *ADE2* expression while permitting *URA3* repression at *HMR*. Thus, Gal4-Sas2p also functions as a “true barrier” at *HMR*. Importantly, the ability of cells to form colonies on medium lacking adenine but containing 5-FOA indicates that the “discontinuous” silenced state that is established in these cells is stably inherited for several generations, enabling these cells to form colonies. A dual reporter system containing *ADE2* and *MATA1* yielded similar results, suggesting that this effect was not reporter specific (data not shown). Furthermore, the fact that Sas2p, a bona fide histone acetyltransferase which is expected to behave as a “desilencer,” can function as a “true barrier” protein indicates that the molecular underpinnings for these definitions will need to be reconsidered.

We next determined whether the generation of the discontinuous silenced state required *HMR-I*. We deleted the *HMR-I*

silencer from the boundary trap strain at *HMR* and analyzed the ability of these strains to grow on medium lacking adenine but containing 5-FOA. Our results (Fig. 2A) showed that silencing of the *URA3* gene in the dual reporter system at *HMR* required *HMR-I*. When this silencer was removed, no colonies formed on the plates lacking adenine and containing 5-FOA.

This result demonstrates that the Sir proteins recruited at *HMR-E* can transpose across an active domain only when a silencer is present on either side of this domain. These results with the dual reporter systems are concordant with our earlier studies of single tethered barrier proteins (47). Silencing adjacent to *HMR-I* requires the *HMR-I* silencer if a barrier blocks the action of *HMR-E*.

To explore the discontinuous silencing phenomenon at a molecular level, we mapped the distribution of Sir3p and H4AcK16 across the *HMR* domain in the presence and absence of Gal4-Sas2p. We chose to analyze these two proteins since they are markers of active and inactive chromatin (46). In the absence of Gal4-Sas2p, there is no acetylation at either silencer or the *ADE2* gene (Fig. 2B). When Sas2p is recruited to sites flanking the *ADE2* gene, there is no detectable acetylation at the *HMR-E* and *HMR-I* silencers but there is a significant increase in H4K16 acetylation at the *ADE2* gene, consistent with the observation that *ADE2* is active in these cells.

On the other hand, Sir3p was present at the two silencers, in both the presence and absence of Gal4-Sas2p, but was reduced at the *ADE2* gene when Sas2p was tethered at the Gal4p binding sites flanking the *ADE2* gene. Thus, tethered Gal4-Sas2p does not block the normal function of the two silencers, and the growth phenotypes observed in Fig. 1 are indeed due to discontinuous silenced domains that initiate from *HMR-E*.

Interestingly, we consistently see increased levels of Sir3p at both the *HMR-E* and *HMR-I* silencers compared with the *ADE2* gene, in the presence or absence of Gal4-Sas2p. Our results demonstrate that *HMR-I* alone cannot recruit Sir proteins, but in the presence of *HMR-E* it is able to stably maintain elevated levels of Sir proteins. While the elevated levels of Sir3p at *HMR-E* can be explained by the fact that *HMR-E* recruits the Sir proteins and initiates silencing, the reason for the elevated levels at *HMR-I* were unexpected and not initially obvious. One possibility is that the increased levels of Sir proteins at *HMR-I* may be due to the two silencers being in close proximity to one another.

Rap1p localizes to *HMR-I*. Our data indicate that *HMR-E* functionally communicates with *HMR-I*, resulting in a discontinuous silenced domain, but they do not specify how this might occur. One possibility is that the two silencers reside in close proximity to one another.

To confirm these long-range interactions, we asked whether a DNA-bound protein at one end of the domain was in close proximity to the other end of the domain, similar to the experiments used to show long-range interactions in *Drosophila* (5). There is a single binding site for Rap1p at the *HMR-E* silencer, where the protein has been shown to bind (53). No Rap1p sites are known to exist at *HMR-I*. We used ChIP to map the presence of Rap1p at *HMR*, as well as at loci on chromosome 6R (Fig. 3A). While we did not observe significant binding of Rap1p to the telomere 6R 7.5-kb probe, the quantitative analyses showed that Rap1p was present immediately adjacent to telomere 6R and at *HMR*. At *HMR* we ob-

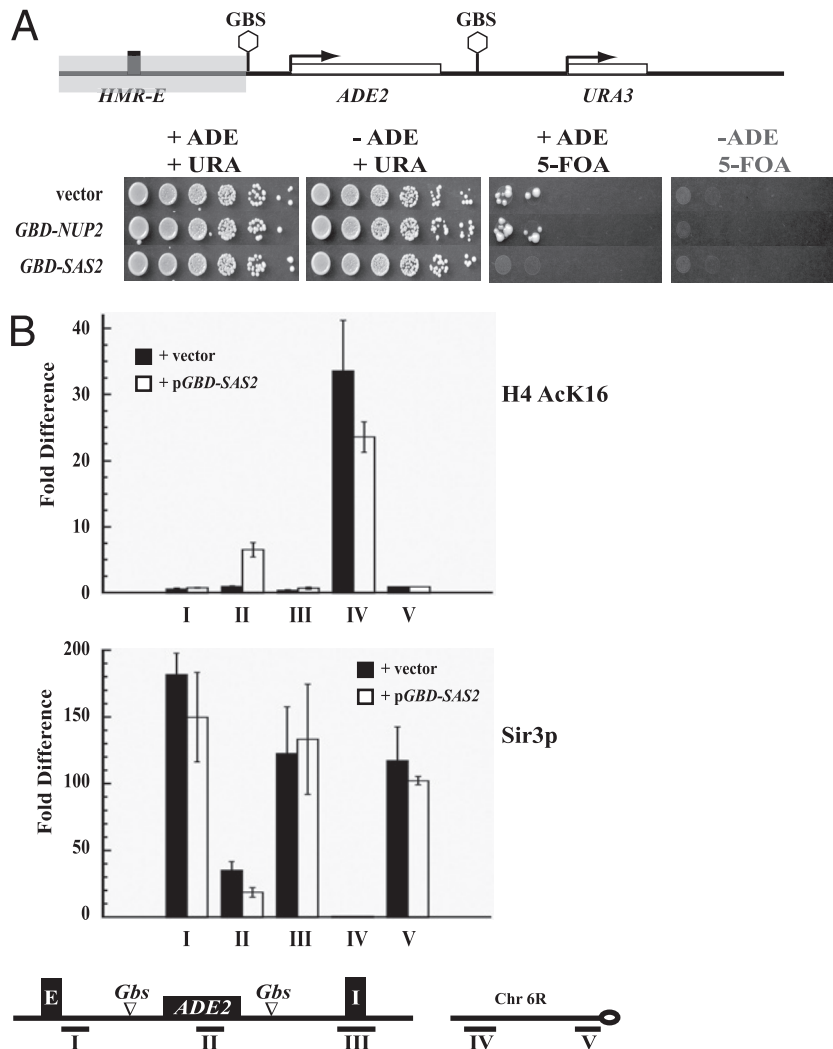


FIG. 2. Discontinuous silencing at *HMR*. (A) *HMR-I* is necessary for discontinuous silencing. Strains with a boundary trap construct at *HMR* but lacking the *HMR-I* silencer were transformed with *TRP1*-containing vector or with *TRP1* plasmids constitutively expressing Gbd-Nup2p or Gbd-Sas2p. The strains were assayed as described for Fig. 1C. (B) Mapping the distribution of acetylated histones and Sir3p in the boundary trap constructs. Strains constitutively expressing Gbd-Sas2p or vector alone were grown in HC-trp medium, selecting for the plasmids (the medium was supplemented with 90 μ g/ml adenine). ChIP with antibodies against H4K16Ac or Sir3p were performed exactly as previously described (46). The graphs depict the enrichment of the immunoprecipitated sample over the input normalized to a telomeric probe. Enrichment and standard errors were computed from at least two independent cross-linked samples and three independent immunoprecipitation experiments. Localization of the PCR probes is depicted in the schematic.

served Rap1p binding to *HMR-E*, the silenced *MATA1* gene at *HMR* and at *HMR-I*. This result was obtained with two different commercially available antibodies (data not shown), validating the presence of Rap1p at *HMR* silencers.

We next determined if loss of Sir3p affected the distribution of Rap1p. Loss of Sir3p did not lead to any decrease in the amount of Rap1p at *HMR-E*, but there was a complete loss of Rap1p from *HMR-I*. These results are consistent with the observation that *HMR-E* was in close proximity to *HMR-I*, although it is also possible that despite Rap1p being a sequence-specific DNA binding protein, it spread along the silenced chromatin through interactions with the Sir proteins.

“Enhancer” activity at *HMR*. To investigate the spatial localization of the silencers relative to each other, we decided to develop an “enhancer assay” (Fig. 3B). The assay is premised

on the assumption that when an upstream activation sequence (UAS) is brought in close spatial proximity to the promoter of a repressed gene, it will activate that gene. We placed Gal4p binding sites upstream of *HMR-E* and placed a repressed reporter gene (*URA3*) several kilobase pairs (4 kb) downstream from the Gal4p binding sites on the distal side of *HMR*, between *HMR-I* and the tRNA barrier. Transcription of the reporter was directed toward the Gal4p binding sites (Fig. 3B). At this location, the *URA3* gene was subjected to repression by Sir-mediated silent chromatin, and in the absence of the Sir proteins, *URA3* was active in both glucose and galactose (data not shown). Interestingly, stable repression of *URA3* located downstream of *HMR-I* was dependent upon the *HMR-I* silencer, because in the absence of this silencer, the reporter was no longer stably silenced (data not shown).

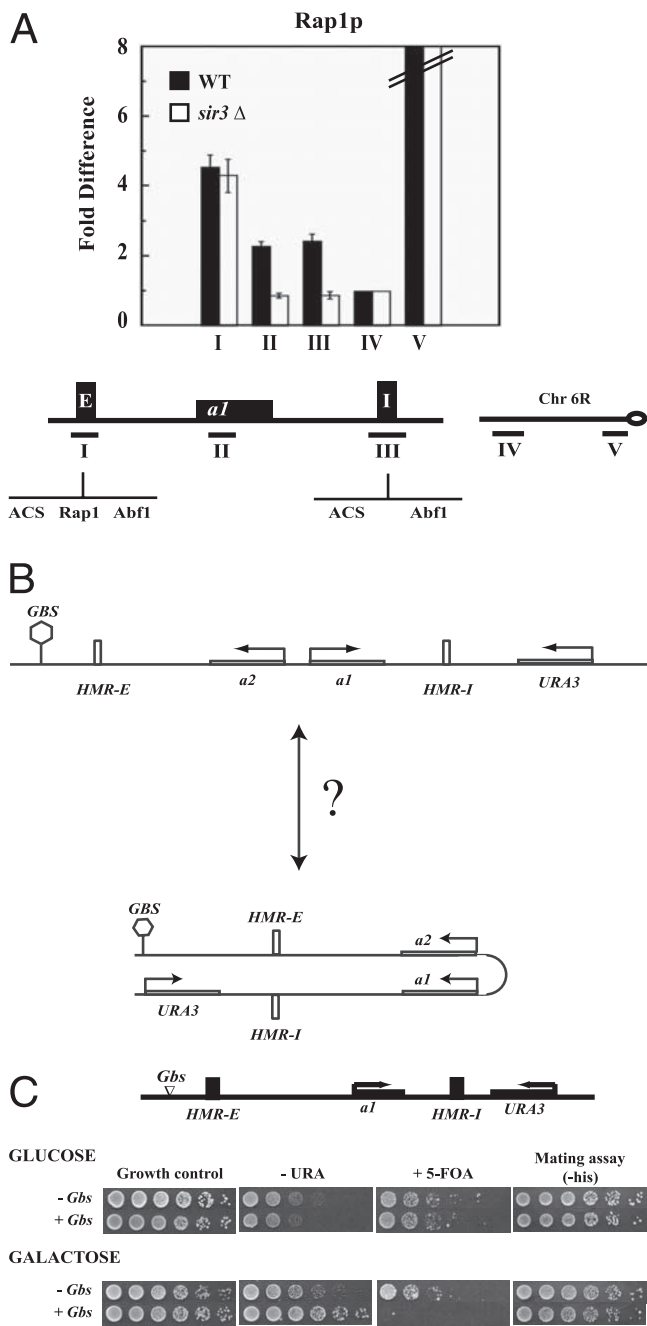


FIG. 3. Functional long-range communication at *HMR*. (A) Mapping Rap1p at *HMR*. Antibodies against the Rap1p C terminus were used to map the distribution of Rap1p across the *HMR* locus in a wild-type (WT) strain and a *sir3*Δ strain. Quantitative ChIPs were performed as described for Fig. 2. The PCR probes used are shown in the schematic diagram. Error bars indicate standard errors. (B) Schematic representation of the enhancer construct at *HMR*. The locations of Gal4p binding sites and the *URA3* gene are shown. (C) “Enhancer” activity at *HMR*. Strains with *URA3* located downstream of *HMR-I* containing no Gal4 binding sites (-*Gbs*) or four *Gbs* upstream of *HMR-E* (+*Gbs*), were grown in 5 ml YPD overnight. Cells were washed, and fivefold serial dilutions were prepared. Properly supplemented YM plates containing 2% galactose (YMG) or 2% glucose (YMD) as a carbon source were used to induce or to repress expression of Gal4p, respectively. To assay for expression of *URA3*, cells were spotted onto YMG or YMD plates lacking or containing uracil or 5-FOA and photographed. To assay for expression of *MATa1* at *HMR*, cells were spotted onto properly supplemented YMG or YMD plates with mating lawns.

We next tested whether binding of Gal4p to its sites upstream of *HMR-E* could disrupt the silencing of the *URA3* gene located downstream of *HMR-I*. When cells were grown in glucose, Gal4p was not activated and *URA3* remained silenced in strains that contained or lacked Gal4p binding sites (Fig. 3C, top). When these cells were grown in medium containing galactose, however, *URA3* was activated and cells were able to grow in medium lacking uracil and not in medium containing 5-FOA (Fig. 3C, bottom). This result was observed only in strains that contained Gal4p binding sites, indicating that Gal4 binding upstream of *HMR* mediated the galactose-dependent *URA3* induction.

One possibility is that Gal4p was disrupting silencer function. If Gal4p was disrupting silencing across the entire domain, then the *MATa1* gene located in the silenced region should also be activated. We therefore monitored expression of the *MATa1* gene located between the two silencers by performing a mating assay and selecting for diploids. The appearance of diploids (Fig. 3C) demonstrated that at this level of sensitivity, *MATa1* was silenced.

We also investigated whether Gal4p binding upstream of *HMR* was disrupting Sir3p binding at *HMR* by quantitative ChIP. If this was the case, then in strains containing Gal4p binding sites grown in galactose, one might expect to see a reduction in the levels of Sir3p at *HMR*. We mapped the levels of Sir3p by ChIP across the entire *HMR* domain in strains grown in galactose with and without Gal4p binding sites. Our analyses showed only a slight change in the levels of Sir3p at the two silencers, with no discernible change at the *MATa1* gene located between the two silencers in the presence or absence of Gal4p (data not shown). These results are consistent with our mating assays (Fig. 3C) showing that silencing did not significantly change at *HMR*.

These results demonstrate “enhancer” function of a gene across a silenced domain. They also demonstrate that a protein bound to a UAS located several kilobase pairs from the promoter of a gene could derepress that gene in yeast. This result is highly unusual, since activation over such long distances has not been observed in yeast (20). The simplest explanation for these results is that the UAS was in close spatial proximity to the promoter of the reporter gene in the nucleus, which then alleviated silencing of the reporter. However one cannot rule out other possibilities due to the inherent limitations of these assays.

Loss of the barrier does not affect long-range communications. Our results suggested that the two silencers might be in close proximity to one another; we were interested in determining the DNA elements and factors that affected this localization. In chicken cells the globin insulator helps tether the globin domain to the nucleolus and aids in the formation of chromatin loops (61, 62). In yeast, tRNA genes are dispersed throughout the genome, but in situ hybridization demonstrated that the genes are clustered adjacent to the nucleolus (30). One of the *HMR* barriers is a tRNA gene (21), and it is therefore possible that tethering of the *HMR* barriers to the nucleolus might be the mechanism by which the two silencers were brought in close proximity to one another. A prediction of this model would be that the *HMR* locus would reside adjacent to the nucleolus and deletion of the barrier would result in a concomitant loss of this localization.

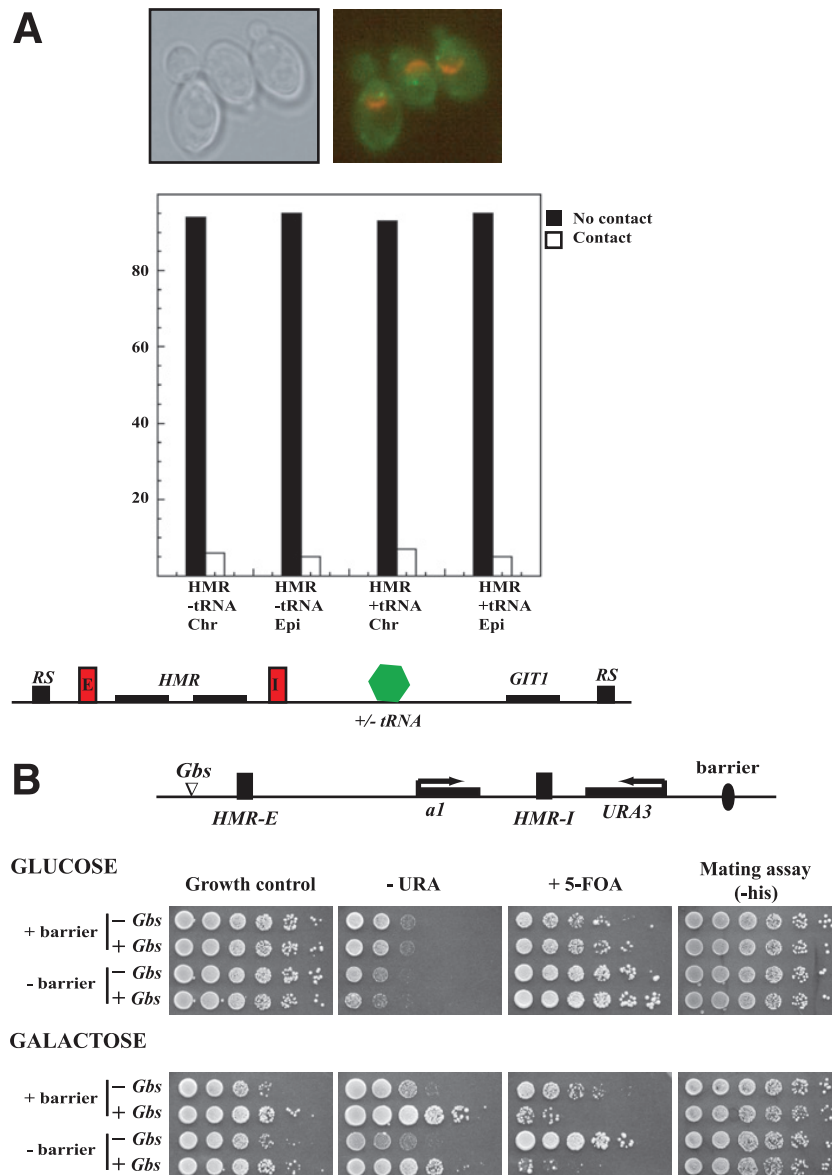


FIG. 4. Long-range communication and barrier function. (A) *HMR* does not colocalize with the nucleolus. Fluorescence analysis was performed with strains expressing Sik1-RFP and *lac*-GFP. A *lac* operator array placed approximately 4 kb from *HMR*, adjacent to the promoter of the *GITI* gene, allowed us to map the localization of the silenced domain relative to the nucleolus. The GFP and RFP signals were monitored in strains containing or lacking the tRNA barrier. Colocalization was defined as full overlap of the green and red signals within the same or adjacent image planes. Over 200 cells were examined in two independent trials. Colocalization was monitored when the *HMR* domain was present on a chromosome as well as on an episome (following recombination). A representative picture of the cells is shown above the graphs. (B) Loss of the tRNA barrier does not affect long-range activation at *HMR*. Strains containing or lacking the tRNA barrier without or with four Gal4p binding sites upstream of *HMR-E* and with *URA3* located downstream of *HMR-I* were grown overnight. Properly supplemented YMG plates containing 2% glucose or galactose as carbon source were used to induce expression of Gal4p. To assay for expression of *URA3*, cells were spotted onto YMG plates lacking or containing uracil or 5-FOA and photographed. To assay for expression of *MATA1* at *HMR*, cells were spotted onto properly supplemented YMG plates with mating lawns.

To determine whether the tRNA barrier adjacent to *HMR* associated with the nucleolus, we used a cytological approach. A *lac* operator array was incorporated adjacent to *HMR* (inserted approximately 4 kb from *HMR*) in a strain that expressed *lac*-GFP, as well as Sik1-RFP. *lac*-GFP binds the *lac* operator array to create a bright green spot of fluorescence (marking *HMR*), whereas Sik1p, a nucleolar protein, imparts red fluorescence to the perinuclear crescent-shaped nucleolus.

Stacks of fluorescent images along the Z axis using both GFP and rhodamine (red) filters were collected to determine the relative positions of *HMR* and the nucleolus. Colocalization was defined as full overlap of the green and red signals within the same or adjacent image planes. Over 200 cells were examined in at least three independent trials. The data in Fig. 4A show that in over 90% of the cases, *HMR* and the nucleolus did not contact one another. The low level of coincident colocal-

ization is similar to that found for other noninteracting chromatin landmarks (9, 12). Similar results were found when *HMR* and the adjacent barrier were liberated from the chromosome by site-specific recombination to form an extrachromosomal DNA circle. These results indicated that *HMR* and the associated boundary did not reside at the nucleolus. Furthermore, deleting the barrier did not alter the localization of *HMR* in the nucleus (Fig. 4A). It is therefore unlikely that the mechanism by which silencers are brought in close proximity is via tethering to the nucleolus.

Tethering of insulators to nuclear superstructures has been proposed to be important for insulation. In *Drosophila*, the Su(Hw) insulators cluster in the nucleus, forming insulator bodies (28), while in yeast, nuclear pore proteins localize to the silenced chromatin (10), and models suggest that tethering of insulators to the pores, forming a chromatin loop, is the mechanism by which chromatin domains are organized and maintained (32).

Therefore it was still possible that the barrier insulator elements at *HMR* were important for the observed long-range communication between the two silencers, albeit not by tethering to the nucleolus. If barrier elements were necessary for organizing chromatin domains into loops, then loss of a barrier should result in loss or diminution of long-range communications. Using the enhancer assay, we investigated the role of the *HMR* tRNA barrier in this process. We generated two strains lacking the tRNA barrier and possessing *URA3* immediately downstream of *HMR-I*. One strain contained Gal4p binding sites located upstream of *HMR-E*, while the second strain lacked these binding sites. Monitoring the expression of *URA3* in these two strains in galactose showed that loss of the tRNA barrier did not adversely affect the long-range communication between the two ends of the silenced chromatin domain (Fig. 4B).

It has been suggested that chromatin loops are formed by the attachment of barrier elements to the nuclear pore via Nup2p (32). Analyses of Nup2p mutants indicate that Nup2p is necessary for robust tRNA barrier function, but loss of Nup2p did not affect the long-range communication between the two silencers (G. Ruben and R. T. Kamakaka, unpublished data).

***HMR-E* and *HMR-I* are in close spatial proximity.** All of our analyses described thus far suggested that the two silencers were in close spatial proximity to one another. We therefore directly analyzed the spatial relationships at the native *HMR* locus in the yeast nucleus by using the 3C method, which was developed to investigate the three-dimensional relationships between DNA elements (15, 18). Cells were briefly treated with formaldehyde to cross-link DNA to proteins, followed by cleavage with a specific restriction enzyme; we digested the DNA with *Sau3A* since this enzyme generated small fragments (55). The fragments were then diluted and ligated, such that ligations were primarily between cross-linked DNA fragments. The fragments that ligated to one another were identified using PCR with specific pairs of primers. Using this method, the cross-linking frequency between two restriction fragments is expected to be roughly proportional to their proximity to one another in the nucleus.

The DNA between the *HMR-E* and *HMR-I* silencers does not contain many *Sau3A* sites. To improve the resolution of our analyses, we introduced three additional *Sau3A* sites

within this region (in the *MATa2* gene). Furthermore, since regions at *HMR* are homologous to regions at *HML α* and *MAT*, we performed this analysis with a strain in which these two loci were deleted. All of the primers that we used in this analysis were oriented in the same direction. Therefore, a PCR product can arise only after restriction fragments were digested and religated. This eliminated PCR products that might arise from incomplete digestion of the cross-linked samples.

We initially analyzed the ligations with a fixed oligonucleotide located in a *Sau3A* fragment containing the *HMR-E* silencer (primer A) with restriction fragments that encompassed the silenced domain and beyond to determine which fragments were in close proximity to the reference fragment. Our analyses showed that the *HMR-E* fragment ligated most frequently to a single *Sau3A* fragment (amplified with primer G) containing the *HMR-I* silencer (Fig. 5A).

To ensure that the PCR amplification efficiencies between different primer pairs were comparable, we digested plasmid DNA containing the *HMR* locus with *Sau3A* in the absence of cross-linking, followed by ligation under conditions that favored intermolecular ligation. PCR analyses indicated that all of the primer pairs were approximately equally efficient in amplifying the ligated products (Fig. 5A). We confirmed the equivalent PCR amplification efficiencies of the primers used by twofold serial dilutions of the un-cross-linked (intermolecular) ligation reaction prior to PCR (data not shown). These data showed that the differences in amplification observed across the *HMR* domain, in the nucleus, were due to differences in cross-linking/ligation of various fragments to the reference fragment (*HMR-E*) and not due to differences in PCR amplification. As an additional control, we purified the PCR products obtained from the cross-linked nuclear samples and sequenced them to unambiguously determine the identity of the ligated fragments (data not shown).

We next used the primer in the *Sau3A* fragment containing *HMR-I* (primer G) as the reference primer and assayed the proximity of this fragment to other fragments across *HMR*. Our results, shown in Fig. 5A, revealed that the *HMR-I* silencer-containing fragment ligated most frequently to the *HMR-E* silencer-containing fragment (primer A) as well as to a fragment downstream of *HMR-E* (primer C) that harbored the end of the *MATa2* gene. From these data we inferred that *HMR-I* was in close proximity to *HMR-E* and the 3' end of the *MATa2* gene. It is possible that *HMR-I* is in close proximity with both fragments simultaneously or exchanges rapidly between these two fragments.

The 3C method was initially used to demonstrate the proximity between various centromeres in yeast (18). We also tested this interaction with our cross-linked samples. Consistent with previously published data, we found that *EcoRI* fragments at Cen IV (primer 14) ligated only to fragments at Cen III (primer 6) and not to other chromosome III *EcoRI* fragments (primers 5 and 7) (data not shown).

Long-range interactions require silencing. Our results suggest that long-range communication between the two *HMR* silencers was not a fortuitous result of the clustering or long-range interactions between the barrier elements that flank the two silencers at *HMR*. Silencing at *HMR* utilizes the Sir proteins that interact with the chromatin to mediate silencing. We therefore investigated whether the communication between

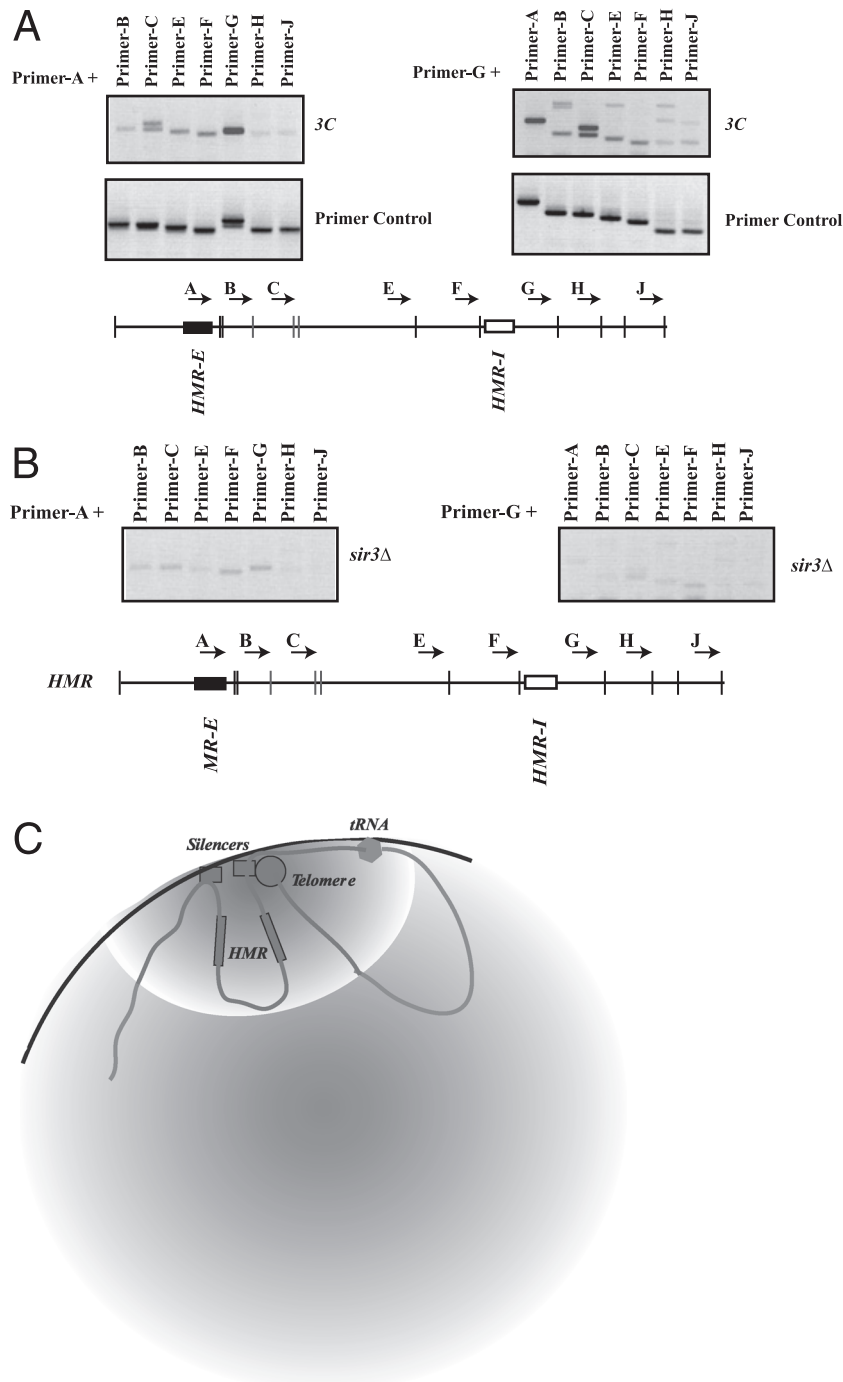


FIG. 5. Spatial organization at *HMR*. (A) 3C analysis of *HMR*. Wild-type strains (lacking *HML* and *MAT*) were cross-linked, digested with *Sau3A*, and ligated, and the ligation products were analyzed by PCR (labeled 3C). Reference primer A or G was used along with the other primers across the domain. The location and orientation of the primers are shown schematically. The primer control panel involved intermolecular ligations and PCR analyses of un-cross-linked DNA. (B) Loss of Sir3p affects long-range interactions. Strains with a deletion of the *SIR3* gene were analyzed with the 3C assay as described above. (C) Schematic representation of the *HMR* domain in the yeast nucleus.

the two silencers was a result of silencing. We determined whether the long-range interaction between the two silencers was disrupted in a *sir3Δ* mutant by 3C analyses. Our results showed that in the absence of this repressor, the extent of ligation between the *HMR-E*- and *HMR-I*-containing *Sau3A*

fragments was dramatically reduced (Fig. 5B), suggesting that these long-range interactions required the Sir proteins.

Since *HMR-E* no longer ligated to *HMR-I* in a *sir3Δ* mutant, to ensure that the cross-linking and ligation were normal in this sample, we examined the ligation between the centromeric

fragments as controls, since Sir3p is not present at yeast centromeres and Sir3p mutations have not been shown to affect centromere function. Our analysis of centromeric chromatin demonstrated that indeed CEN III remained in close proximity to CEN IV in the absence of Sir3p (data not shown).

Our results in their totality demonstrate that silencers separated over several kilobase pairs of DNA functionally and structurally interact with one another. We have begun to identify the determinants necessary for these interactions and show that the Sir proteins are necessary for this long-range communications between silencers.

DISCUSSION

The control of eukaryotic gene expression involves communication between regulatory elements that are often separated by great distances. There are now numerous examples of distal enhancers and LCRs that contact the genes they activate (reviewed in reference 60). More recent studies have even found interactions between regulatory elements and genes that reside on entirely different chromosomes (39, 54). In this report, for the first time, we show long-range interactions between the silencers that flank the *HMR* locus in yeast, and we identify the determinants required for these interactions. Interestingly, we have shown that deletion of the tRNA insulator element or the nuclear pore protein Nup2p did not affect functional long-range communication between the two silencers but that loss of Sir3p did result in diminution of these interactions, suggesting that silencing itself may be important for organizing this chromatin domain.

Silencers and the mechanisms for silencing. Numerous studies have shown that *HMR-E* is sufficient to nucleate silencing at *HMR*. *HMR-I* cooperates with *HMR-E*, but it cannot initiate silencing on its own (42, 50). Therefore, *HMR-I* is analogous to proto-silencers that have been found at telomeres and *HML* (6, 13). At telomeres, proto-silencers and proteins with barrier activity are interspersed in the subtelomeric blocks to yield domains of discontinuous silencing (24, 25, 38), and these functional assays have led to models where the proto-silencer elements might interact with one another, but direct long-range interactions at telomeric loci have not been demonstrated using the 3C technique. Long-range interactions between the proto-silencers and terminal telomeric sequences, which function as silencers, may indeed be occurring, similar to what we observe at *HMR*. The distribution of terminally bound Rap1 and Ku at telomeres is consistent with telomere loop formation (43, 56), and enhancer assays like the one we have employed in this study have also suggested looping within silent chromatin at telomeres (16). However, this is the first report to unambiguously describe long-range interactions and the formation of chromatin loops at an internal silenced locus in yeast.

Placement of the dual reporter constructs of the boundary trap assay at the silent mating-type loci created discontinuous silencing states. At *HMR*, Sir3p was found at both silencers but was consistently reduced at the insulated reporter gene in between. In agreement, the H4K16Ac mark for active chromatin was found in a reciprocal pattern. How can a discontinuous state be created when *HMR-I* does not function on its own? One possibility is that both silencers are held in close

proximity so that *HMR-I* shares the nucleation activity of the more potent *HMR-E* silencer. In this case, silent chromatin would nucleate at both silencers and spread from both until encountering synthetic (or natural) barriers. Thus, when a pair of barrier proteins is situated between *HMR-E* and *HMR-I*, a domain of active chromatin will reside between domains of silent chromatin.

An alternative possibility is that the insulated *ADE2* construct counteracts silent chromatin that has spread from a sole nucleation point at *HMR-E*. In this scenario, *ADE2* activation would occur following a cell cycle event, such as DNA replication, that compromises silencing efficiency (4). Silent chromatin would persist on both sides of the activated domain because the silencers stabilized the existing repressed state (2, 13, 14, 42, 50). However, it is hard to visualize how this mechanism lends itself to stable inheritance (which we observe in our assays). An alternative model that combines these two scenarios is possible where binding of Sir proteins to *HMR-E* facilitates interactions between *HMR-E* and *HMR-I*. Once *HMR-I* is brought in proximity to *HMR-E*, it can also nucleate silencing, which then would spread from both silencers.

Sir3p and long-range repression. The long-range communication between the two silencer fragments is dependent upon Sir3p. Sir3p is a structural repressor protein that binds the histones in nucleosomes to mediate repression. In vitro binding studies with oligonucleosomes have shown that Sir3p oligomers bind multiple chromatin fragments and "cross-links" nucleosomal arrays (27, 29, 40). It is therefore possible that Sir3p binding to chromatin cross-links the silenced domain and the resulting compaction brings distal sites together. The dependence on Sir3p is reminiscent of long-range repression in *Drosophila*, where Polycomb-mediated repression involves interactions between chromatin memory module elements and distally repressed promoters via the association of Polycomb group proteins (17, 44). We note, however, that Sir3p is not sufficient to hold *HMR* loci on sister chromatids together (12). Instead, silent chromatin recruits cohesin, which mediates pairing of the twin silent chromatin domains. Additional factors could similarly facilitate interactions between distal silent chromatin segments within the same chromatin fiber.

Regulatory elements important for long-range communication. An interaction specifically between the two silencer-containing DNA fragments raises the question of which DNA elements, if any, are required. Our results with the boundary trap system demonstrate that *HMR-I* is necessary. Preliminary data using the 3C technique also suggest that the silencer elements are necessary. ORC and Abf1p bind *HMR-I* and might be involved in mediating these long-range interactions. Rap1p might also aid long-range communication. When bound to two sites on naked DNA, the protein induces loop formation (31). Further experiments will be necessary to dissect the roles of these elements and proteins in long-range communication.

In an alternative scenario, the two silencers could be brought in close proximity by insulator elements that flank the silenced domain. It has been suggested that chromatin loops are formed through association of insulator elements with the nuclear pores (32, 33, 52). It is therefore possible that the barrier elements flanking *HMR* (21, 22) or the hypersensitive sites at this locus (45) associate with nuclear pores or active chromatin hubs and cluster in the nucleus, the consequence of which

would be to align *HMR-E* and *HMR-I* in close proximity. However, we have shown that deleting the barrier or Nup2p, which interacts with the barrier, had little effect on long-range communication between the silencers. It therefore seems unlikely that chromatin barriers play a major role in the observed interactions between *HMR-E* and *HMR-I*.

Sir spreading, long-range interactions, and silencing. While we have shown that the *HMR* silencers are in close proximity to one another, it is unclear whether this long-range communication between silencers has any functional significance at the native locus. It is possible that the two silencers are fortuitously brought in close proximity to one another simply by the compaction of chromatin. However, data show that the *HMR-I* silencer is important for stabilizing the repressed state (50). In its absence, the silent state is reduced and silencing is weakened in a population of cells. Importantly, our data with the boundary trap assay showed that *HMR-I* becomes an essential silencing element when propagation of silent chromatin from *HMR-E* is blocked. One possibility, as described above, is that an interaction between the silencers permits the robust nucleation activity of *HMR-E* to act locally via a spreading mechanism as well as distally at *HMR-I* via long-range interactions. Another possibility (the two are not mutually exclusive) is that silencers associate with one another and with telomeres to bring both ends of the silenced domain into a nuclear compartment that favors silencing (Fig. 5C). *HMR* resides at the nuclear periphery and frequently colocalizes with the highly concentrated foci of Sir proteins associated with telomeres (26, 57). Consistent with this is the demonstration that telomere 3L is in proximity to the silenced *HML* locus (37). The clustering of regulatory elements in the nucleus is a recurring theme utilized by a variety of elements (60) to robustly activate and repress genes. It is thought to increase the local concentrations of proteins and thus improve the probability that these elements will function efficiently (19).

The role of the Sir proteins in this scenario would be to stabilize the long-range interactions that arise due to tethering of the silencers. This would be consistent with *in vitro* evidence showing that the Sir proteins preferentially bind two DNA fragments (27, 29) and with our 3C data showing that the long-range interactions are lost in the absence of Sir3p. This role for the Sir proteins is also consistent with the observation that loss of silencing affects telomere-telomere interactions and formation of telomeric foci in yeast.

Besides playing a role in tethering the *HMR* domain to telomeric foci, the role of the silencers would also be to nucleate the spread of Sir proteins (41, 51). Our quantitative analysis demonstrating increased concentrations of Sir proteins at silencers with reduced levels of these proteins the further one traverses from the silencer would be consistent with this role for the silencers, though other models are equally possible.

The mechanism by which long-range silencing occurs may be more complicated than previously anticipated. Interactions between silencer elements mediated by the repressor proteins may compete with interactions between enhancer and promoters to affect the three-dimensional organization and functional status of the nucleus. Further studies should help us to understand the significance of long-range interactions in chromatin domain organization and gene regulation.

ACKNOWLEDGMENTS

We thank Genevieve Fourel, David Donze, Grant Hartzog, Michael Lichten, Orna Cohen-Fix, Masaya Oki, and Catherine Fox for comments on the manuscript; C. Fox and U. Laemmli for specific strains; and Riza Ysla for technical assistance.

This work was supported by grants from the NIH to R.T.K. (GM078068) and M.R.G. (GM51402).

REFERENCES

- Abraham, J., J. Feldman, K. A. Nasmyth, J. N. Strathern, A. J. Klar, J. R. Broach, and J. B. Hicks. 1983. Sites required for position-effect regulation of mating-type information in yeast. *Cold Spring Harbor Symp. Quant. Biol.* **47**:989–998.
- Ansari, A., and M. R. Gartenberg. 1999. Persistence of an alternate chromatin structure at silenced loci *in vitro*. *Proc. Natl. Acad. Sci. USA* **96**:343–348.
- Ansari, A., and M. Hampsey. 2005. A role for the CPF 3'-end processing machinery in RNAP II-dependent gene looping. *Genes Dev.* **19**:2969–2978.
- Aparicio, O. M., and D. E. Gottschling. 1994. Overcoming telomeric silencing: a trans-activator competes to establish gene expression in a cell cycle-dependent way. *Genes Dev.* **8**:1133–1146.
- Blanton, J., M. Gaszner, and P. Schedl. 2003. Protein:protein interactions and the pairing of boundary elements *in vivo*. *Genes Dev.* **17**:664–675.
- Boscheron, C., L. Mailet, S. Marcand, M. Tsai-Pflugfelder, S. M. Gasser, and E. Gilson. 1996. Cooperation at a distance between silencers and protosilencers at the yeast HML locus. *EMBO J.* **15**:2184–2195.
- Brand, A. H., L. Breeden, J. Abraham, R. Sternglanz, and K. Nasmyth. 1985. Characterization of a "silencer" in yeast: a DNA sequence with properties opposite to those of a transcriptional enhancer. *Cell* **41**:41–48.
- Bulger, M., and M. Groudine. 1999. Looping versus linking: toward a model for long-distance gene activation. *Genes Dev.* **13**:2465–2477.
- Bystricky, K., P. Heun, L. Gehlen, J. Langowski, and S. M. Gasser. 2004. Long-range compaction and flexibility of interphase chromatin in budding yeast analyzed by high-resolution imaging techniques. *Proc. Natl. Acad. Sci. USA* **101**:16495–16500.
- Casolari, J. M., C. R. Brown, S. Komili, J. West, H. Hieronymus, and P. A. Silver. 2004. Genome-wide localization of the nuclear transport machinery couples transcriptional status and nuclear organization. *Cell* **117**:427–439.
- Chambeyron, S., and W. A. Bickmore. 2004. Does looping and clustering in the nucleus regulate gene expression? *Curr. Opin. Cell Biol.* **16**:256–262.
- Chang, C. R., C. S. Wu, Y. Hom, and M. R. Gartenberg. 2005. Targeting of cohesin by transcriptionally silent chromatin. *Genes Dev.* **19**:3031–3042.
- Cheng, T. H., and M. R. Gartenberg. 2000. Yeast heterochromatin is a dynamic structure that requires silencers continuously. *Genes Dev.* **14**:452–463.
- Cheng, T. H., Y. C. Li, and M. R. Gartenberg. 1998. Persistence of an alternate chromatin structure at silenced loci in the absence of silencers. *Proc. Natl. Acad. Sci. USA* **95**:5521–5526.
- Cullen, K. E., M. P. Kladde, and M. A. Seyfred. 1993. Interaction between transcription regulatory regions of prokaryotic chromatin. *Science* **261**:203–206.
- de Bruin, D., Z. Zaman, R. A. Liberatore, and M. Ptashne. 2001. Telomere looping permits gene activation by a downstream UAS in yeast. *Nature* **409**:109–113.
- Dejardin, J., and G. Cavalli. 2004. Chromatin inheritance upon Zeste-mediated Brahma recruitment at a minimal cellular memory module. *EMBO J.* **23**:857–868.
- Dekker, J., K. Rippe, M. Dekker, and N. Kleckner. 2002. Capturing chromosome conformation. *Science* **295**:1306–1311.
- Dillon, N., and R. Festenstein. 2002. Unravelling heterochromatin: competition between positive and negative factors regulates accessibility. *Trends Genet.* **18**:252–258.
- Dobi, K. C., and F. Winston. 2007. Analysis of transcriptional activation at a distance in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **27**:5575–5586.
- Donze, D., C. R. Adams, J. Rine, and R. T. Kamakaka. 1999. The boundaries of the silenced HMR domain in *Saccharomyces cerevisiae*. *Genes Dev.* **13**:698–708.
- Donze, D., and R. T. Kamakaka. 2001. RNA polymerase III and RNA polymerase II promoter complexes are heterochromatin barriers in *Saccharomyces cerevisiae*. *EMBO J.* **20**:520–531.
- Drissen, R., R. J. Palstra, N. Gillemans, E. Splinter, F. Grosveld, S. Philipsen, and W. de Laat. 2004. The active spatial organization of the beta-globin locus requires the transcription factor EKLF. *Genes Dev.* **18**:2485–2490.
- Fourel, G., E. Lebrun, and E. Gilson. 2002. Protosilencers as building blocks for heterochromatin. *Bioessays* **24**:828–835.
- Fourel, G., E. Revardel, C. E. Koering, and E. Gilson. 1999. Cohabitation of insulators and silencing elements in yeast subtelomeric regions. *EMBO J.* **18**:2522–2537.
- Gartenberg, M. R., F. R. Neumann, T. Laroche, M. Blaszczyk, and S. M.

- Gasser. 2004. Sir-mediated repression can occur independently of chromosomal and subnuclear contexts. *Cell* **119**:955–967.
27. Georgel, P. T., M. A. Palacios DeBeer, G. Pietz, C. A. Fox, and J. C. Hansen. 2001. Sir3-dependent assembly of supramolecular chromatin structures in vitro. *Proc. Natl. Acad. Sci. USA* **98**:8584–8589.
 28. Gerasimova, T. I., K. Byrd, and V. G. Corces. 2000. A chromatin insulator determines the nuclear localization of DNA. *Mol. Cell* **6**:1025–1035.
 29. Ghidelli, S., D. Donze, N. Dhillon, and R. T. Kamakaka. 2001. Sir2p exists in two nucleosome-binding complexes with distinct deacetylase activities. *EMBO J.* **20**:4522–4535.
 30. Haeusler, R. A., and D. R. Engelke. 2004. Genome organization in three dimensions: thinking outside the line. *Cell Cycle* **3**:273–275.
 31. Hofmann, J. F., T. Laroche, A. H. Brand, and S. M. Gasser. 1989. RAP-1 factor is necessary for DNA loop formation in vitro at the silent mating type locus HML. *Cell* **57**:725–737.
 32. Ishii, K., G. Arib, C. Lin, G. Van Houwe, and U. K. Laemmli. 2002. Chromatin boundaries in budding yeast: the nuclear pore connection. *Cell* **109**:551–562.
 33. Ishii, K., and U. K. Laemmli. 2003. Structural and dynamic functions establish chromatin domains. *Mol. Cell* **11**:237–248.
 34. Ito, H., Y. Fukuda, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153**:163–168.
 35. Kamakaka, R. T. 1997. Silencers and locus control regions: opposite sides of the same coin. *Trends Biochem. Sci.* **22**:124–128.
 36. Laemmli, U. K., E. Kas, L. Poljak, and Y. Adachi. 1992. Scaffold-associated regions: cis-acting determinants of chromatin structural loops and functional domains. *Curr. Opin. Genet. Dev.* **2**:275–285.
 37. Lebrun, E., G. Fourel, P. A. Defosse, and E. Gilson. 2003. A methyltransferase targeting assay reveals silencer-telomere interactions in budding yeast. *Mol. Cell. Biol.* **23**:1498–1508.
 38. Lebrun, E., E. Revardel, C. Boscheron, R. Li, E. Gilson, and G. Fourel. 2001. Protosilencers in *Saccharomyces cerevisiae* subtelomeric regions. *Genetics* **158**:167–176.
 39. Ling, J. Q., T. Li, J. F. Hu, T. H. Vu, H. L. Chen, X. W. Qiu, A. M. Cherry, and A. R. Hoffman. 2006. CTCF mediates interchromosomal colocalization between Igf2/H19 and Wsb1/Nf1. *Science* **312**:269–272.
 40. Liou, G. G., J. C. Tanny, R. G. Kruger, T. Walz, and D. Moazed. 2005. Assembly of the SIR complex and its regulation by O-acetyl-ADP-ribose, a product of NAD-dependent histone deacetylation. *Cell* **121**:515–527.
 - 40a. Litt, M. D., M. Simpson, F. Recillas-Targa, M. N. Prioleau, and G. Felsenfeld. 2001. Transitions in histone acetylation reveal boundaries of three separately regulated neighboring loci. *EMBO J.* **20**:2224–2235.
 41. Luo, K., M. A. Vega-Palas, and M. Grunstein. 2002. Rap1-Sir4 binding independent of other Sir, yKu, or histone interactions initiates the assembly of telomeric heterochromatin in yeast. *Genes Dev.* **16**:1528–1539.
 42. Mahoney, D. J., R. Marquardt, G. J. Shei, A. B. Rose, and J. R. Broach. 1991. Mutations in the HML E silencer of *Saccharomyces cerevisiae* yield metastable inheritance of transcriptional repression. *Genes Dev.* **5**:605–615.
 43. Martin, S. G., T. Laroche, N. Suka, M. Grunstein, and S. M. Gasser. 1999. Relocalization of telomeric Ku and SIR proteins in response to DNA strand breaks in yeast. *Cell* **97**:621–633.
 44. Maurice, C., and R. Paro. 2002. A cellular memory module conveys epigenetic inheritance of hedgehog expression during *Drosophila* wing imaginal disc development. *Genes Dev.* **16**:2672–2683.
 45. Nasmyth, K. A. 1982. The regulation of yeast mating-type chromatin structure by SIR: an action at a distance affecting both transcription and transposition. *Cell* **30**:567–578.
 46. Oki, M., and R. T. Kamakaka. 2005. Barrier function at HMR. *Mol. Cell* **19**:707–716.
 47. Oki, M., L. Valenzuela, T. Chiba, T. Ito, and R. T. Kamakaka. 2004. Barrier proteins remodel and modify chromatin to restrict silenced domains. *Mol. Cell. Biol.* **24**:1956–1967.
 48. O'Sullivan, J. M., S. M. Tan-Wong, A. Morillon, B. Lee, J. Coles, J. Mellor, and N. J. Proudfoot. 2004. Gene loops juxtapose promoters and terminators in yeast. *Nat. Genet.* **36**:1014–1018.
 49. Patrinos, G. P., M. de Krom, E. de Boer, A. Langeveld, A. M. Imam, J. Strouboulis, W. de Laat, and F. G. Grosveld. 2004. Multiple interactions between regulatory regions are required to stabilize an active chromatin hub. *Genes Dev.* **18**:1495–1509.
 50. Rivier, D. H., J. L. Ekena, and J. Rine. 1999. HMR-I is an origin of replication and a silencer in *Saccharomyces cerevisiae*. *Genetics* **151**:521–529.
 51. Rusche, L. N., A. L. Kirchmaier, and J. Rine. 2002. Ordered nucleation and spreading of silenced chromatin in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **13**:2207–2222.
 52. Schmid, M., G. Arib, C. Laemmli, J. Nishikawa, T. Durussel, and U. K. Laemmli. 2006. Nup-PI: the nucleopore-promoter interaction of genes in yeast. *Mol. Cell* **21**:379–391.
 53. Shore, D. 1994. RAP1: a protean regulator in yeast. *Trends Genet.* **10**:408–412.
 54. Spilianakis, C. G., M. D. Lalioti, T. Town, G. R. Lee, and R. A. Flavell. 2005. Interchromosomal associations between alternatively expressed loci. *Nature* **435**:637–645.
 55. Splinter, E., F. Grosveld, and W. de Laat. 2004. 3C technology: analyzing the spatial organization of genomic loci in vivo. *Methods Enzymol.* **375**:493–507.
 56. Strahl-Bolsinger, S., A. Hecht, K. Luo, and M. Grunstein. 1997. SIR2 and SIR4 interactions differ in core and extended telomeric heterochromatin in yeast. *Genes Dev.* **11**:83–93.
 57. Taddei, A., F. Hediger, F. R. Neumann, C. Bauer, and S. M. Gasser. 2004. Separation of silencing from perinuclear anchoring functions in yeast Ku80, Sir4 and Esc1 proteins. *EMBO J.* **23**:1301–1312.
 58. Tolhuis, B., R. J. Palstra, E. Splinter, F. Grosveld, and W. de Laat. 2002. Looping and interaction between hypersensitive sites in the active beta-globin locus. *Mol. Cell* **10**:1453–1465.
 59. Vakoc, C. R., D. L. Letting, N. Gheldof, T. Sawado, M. A. Bender, M. Groudine, M. J. Weiss, J. Dekker, and G. A. Blobel. 2005. Proximity among distant regulatory elements at the beta-globin locus requires GATA-1 and FOG-1. *Mol. Cell* **17**:453–462.
 60. Valenzuela, L., and R. T. Kamakaka. 2006. Chromatin insulators. *Annu. Rev. Genet.* **40**:107–138.
 61. Yusufzai, T. M., and G. Felsenfeld. 2004. The 5'-HS4 chicken beta-globin insulator is a CTCF-dependent nuclear matrix-associated element. *Proc. Natl. Acad. Sci. USA* **101**:8620–8624.
 62. Yusufzai, T. M., H. Tagami, Y. Nakatani, and G. Felsenfeld. 2004. CTCF tethers an insulator to subnuclear sites, suggesting shared insulator mechanisms across species. *Mol. Cell* **13**:291–298.