Chromosomal boundaries in S. cerevisiae
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Chromatin boundary elements or insulators in metazoans delimit distinct chromosomal domains of gene expression. Recently, DNA sequences with properties similar to boundary elements were also discovered in Saccharomyces cerevisiae. These sequences block the spread of transcriptionally silent chromatin, the yeast equivalent of metazoan heterochromatin, and are referred to as ‘heterochromatin barriers’. These barriers share no sequence homology but all consist of multiple binding sites for various regulatory proteins. Current data suggest that barriers may function in yeast by recruiting a protein complex that precludes nucleosome assembly and thereby disrupts a contiguous array of nucleosomes required for the spread of silent chromatin.

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Abbreviations
CTCF CCCTC-binding factor
HML homothallic mating locus, left
HMR homothallic mating locus, right
STAR sub-telomeric anti-silencing region
UAS upstream activating sequence

Introduction
The eukaryotic genome is organized into discrete functional domains in which gene expression is either repressed or facilitated. Domains in which genes are repressed are usually defined as heterochromatin, while domains permissive for gene expression are defined as euchromatin. Heterochromatin is a more compact chromatin structure than euchromatin and contains protein complexes that propagate serially along the chromatin fiber to silence genes embedded in the structure. Euchromatin and heterochromatin domains are interspersed along the chromosome and the boundaries that demarcate them have been a focus of extensive investigations. Studies in Drosophila and vertebrate revealed that at least some domains are precisely delimited by boundary or insulator elements that preclude enhancers or chromatin outside the domain from influencing promoters inside the domain. These elements each contain binding sites for specific DNA-binding proteins but the molecular mechanism underlying boundary activity is not resolved.

Telomeres and the HML and HMR loci of S. cerevisiae represent defined chromosomal domains in which genes are transcriptionally silenced as a result of the formation of a heterochromatin-like structure across these regions. These heterochromatin domains are established and maintained through combined actions of cis-acting and transacting factors. The cis-acting elements include silencers flanking each HM locus and the C1–3A telomeric repeats. The trans-acting proteins include silencer/telomere-binding proteins, histones and the Sir2, Sir3 and Sir4 silencer proteins. The current model for silencing proposes that silencer/telomere-binding proteins recruit a complex of Sir2/Sir3/Sir4, which then propagates sequentially along the neighboring nucleosomes. What prevents silent chromatin from extending indefinitely? Studies in the past two years have revealed that certain sequences surrounding the HMR locus have the ability to block the spread of silencing from HMR ([1••]) (Figure 1). The UAS of TEF2 and STAR (sub-telomeric anti-silencing region) sequences can also block the spread of silent chromatin [2••,3••]. These yeast boundary elements were referred to as heterochromatin ‘barriers’. Finally, domains can be constrained not only by barriers to heterochromatin spreading but also by elements that establish heterochromatin formation in only one direction. This latter boundary element has as yet been described only in yeast.

TEF2-UAS: multiple Rap1-binding sites can function as a heterochromatin barrier

The yeast heterochromatin barriers were all discovered by using a silencer-blocking assay similar to the enhancer-blocking assay used in testing insulators in higher cells. This assay tests the ability of a sequence to counteract the silencing effect of a silencer/telomere on a reporter gene when it is positioned between them but not when it is reporter-distal to the silencer. We have found [2••] that the yeast TEF2 gene has silencer-blocking activity and TEF2–UAS alone is both necessary and sufficient for the silencer-blocking activity of TEF2 [2••]. TEF2–UAS can also insulate genes from the repressive effect of heterochromatin. TEF2–UAS functions as a physical barrier to the spread of silent chromatin in a DNA topology-based assay [2••,4]. TEF2–UAS consists of three variants of the consensus sequence for the DNA-binding protein Rap1. Mutations in these Rap1 sites that prevent Rap1 from binding abolish the barrier activity of TEF2–UAS, indicating that Rap1 is directly involved in the barrier activity (X Bi, unpublished data).

Boundary elements from Drosophila and chicken mediate localized alterations of chromatin structure that accompany insulator function [5]. Using the DNA topology assay for chromatin structure, we found that TEF2–UAS induces a relaxation in local DNA with a linking number change (ΔLk) of two, indicative of an alteration in chromatin structure (X Bi, JR Broach, unpublished data). Since wrapping of DNA on a nucleosomal core particle...
introduces an average ΔLk of −1, losing two nucleosomes would cause local DNA to be less negatively supercoiled (with a ΔLk of +2). Therefore, the observed linking number change may reflect the loss of two nucleosomes. Mutations in 	extit{TRF2–UAS} that abolish its barrier activity also prevent it from altering local chromatin structure (X Bi, unpublished data). Consistently, Rap1 is known effectively to interfere with positioning of a nucleosome containing its binding site [6••].

**STARs: multiple Tbf1 and/or Reb1-binding sites as a heterochromatin barrier**

Fourel et al. [3••] have found sequences within the subtelomeric repetitive sequences X and Y that can block the silencing effect of a telomere on a reporter gene. These elements (STARs) can also counteract silencer-driven repression. Bracketing a reporter gene by two STARs renders the reporter gene impervious to surrounding silencing elements. Each STAR contains multiple Tbf1 and/or Reb1-binding sites, which are required for the barrier activity. In addition, although a single site for Tbf1 or Reb1 exhibits no boundary activity, three synthetic tandem sites have silencer-blocking activity. Targeting Gal4 BD–Tbf1 and Gal4 BD–Reb1 fusion proteins also recapitulates barrier activity (E Gilson, personal communication).

The function of Tbf1 is not known but its DNA-binding domain is related to that of various telomere proteins, including TRF1 and TRF2 from mammals and Taiz1p from fission yeast [7]. Reb1 is a weak transcription activator whose binding sites are found in a variety of yeast promoters for RNA polymerases I and II, and is also required for termination of RNA polymerase I [8–10].

**HMR tRNAThr; a tRNA gene as a heterochromatin barrier**

Donze et al. [1••] examined the boundaries of the 	extit{HMR} locus and found that they are located outside of the 	extit{HMR} sequence bracketed by the E and I silencers. They further showed that a tRNA gene (HMR tRNAThr) plus some flanking sequences in the right-hand (centromere distal) boundary sequence are necessary and sufficient to support barrier activity (RT Kamakaka, personal communication). Unlike mRNA genes, tRNA genes contain promoter elements (boxA and boxB) within the transcribed sequences. BoxA and boxB are binding sites for transcription factors TFIIC and TFIIB. Mutations in these elements and those in TFIIC and TFIIB as well as in RNA polymerase III all impair barrier activity of the tRNA gene (RT Kamakaka, personal communication). Therefore, the transcriptional potential of the tRNA gene is critical for its barrier activity. Although 	extit{HMR tRNAThr} is transcribed, whether transcription per se is required for barrier activity is not known. Not all tRNA genes have barrier activity. It seems that the distance between boxA and boxB in a tRNA gene, as well as its flanking sequence affects its barrier activity (RT Kamakaka, personal communication).

In their study of the 	extit{HMR} boundaries, Donze et al. [1••] examined the effects of mutation of a large number of genes on boundary activity and found that most, including those in 	extit{RAP1, TEC1, CAC1, SPT3, SPT4} and 	extit{SPT8}, had no effect. Mutation of 	extit{SMC1}, which encodes one of the components of yeast cohesin, however, abolished activity of the right-hand boundary. More recently, Laloraya et al. [11] showed that cohesin-binding sites coincide, to the level of resolution of chromatin immunoprecipitation, with the left and right 	extit{HMR} boundaries. These data together present a strong case for a role of cohesin in boundary function. As cohesin is bound to chromosomes only between S phase and M phase, however, either cohesin is required only for establishment (rather than maintenance) of this heterochromatin boundary or else heterochromatin is not capable of spreading once it has been deposited on newly formed chromatin.

**Nucleosome exclusion and chromatin opening: a common mechanism for barrier elements?**

The diversity of the yeast barrier elements apparently discourages any unifying hypothesis for the mechanism for barrier activity, but they may have a common property: nucleosome exclusion and chromatin opening. Both Rap1 and Reb1 have been shown to disrupt chromatin formation [6••,12]. Interestingly, earlier work showed that a tRNA gene could prevent nucleosome positioning in its vicinity and this effect requires the tRNA gene to be active [13]. These observations prompted us to propose the ‘nucleosome gap model’ for heterochromatin barriers [2••]. In this model, a barrier excludes the formation of a nucleosome(s) creating a nucleosome-free gap in a regular array of nucleosomes (Figure 2). This gap prevents the spread of the Sir2/Sir3/Sir4 complex as the complex most likely propagates along chromatin by sequential interaction with adjacent nucleosomes. In essence, this model invokes a physical block to the spread of silent chromatin. The large RNA polymerase III complex (~1.5 mDa) may be responsible for the block created by 	extit{HMR}.
tRNA\(^{Thr}\) whereas multiple Rap1 or Reb1/Tbf1 proteins are required for the block in \(TEF2\)–UAS or STARs. This model does not exclude the notion that creation of the physical block may also require possible chromatin remodeling functions that may be recruited by barrier-binding proteins.

**Functional similarities between yeast barriers and metazoan insulators**

Similar to yeast barrier elements, boundary/insulator elements in metazoans are also multi-component nucleoprotein complexes. The boundary elements \(osc\) and \(osc'\) flanking the *Drosophila hsp70* genes contain binding sites for the ZW5, the BEAF-32A and the BEAF-32B proteins, respectively [14–16]. The *Drosophila gypsy* insulator is composed of 12 binding sites for the zinc-finger protein su(Hw) [17] and the mod(mdg4) protein which interacts with su(Hw). The chicken \(\beta\)-globin insulator contains the binding site for CTCF, an 11-zinc finger DNA binding protein that is highly conserved and ubiquitous in vertebrates [18••]. Recently, CTCF was also shown to bind to a boundary element at the mammalian *H19*Igf2 locus in a DNA methylation-dependent fashion [19*,20*].

Many of the protein components of the insulators are most likely involved in other genomic functions. BEAF-32A and su(Hw) each binds to many sites in the *Drosophila* genome and may have general structural and functional roles throughout the genome. In the absence of mod(mdg4), the *gypsy* insulator, bound by su(Hw) alone, becomes a silencer that mediates bi-directional heterochromatin formation. Interestingly, su(Hw) can also function as a transcriptional activator [21]. Like su(Hw), CTCF is also implicated in both transcriptional silencing and activation (see references cited in [18••]). Yeast barrier-binding proteins can also perform these diverse functions. This is best exemplified by Rap1, which binds to many sites throughout the genome and acts as a global regulator of transcriptional activation, transcriptional repression, telomere length, circular plasmid segregation, as well as meiotic recombination in a context-dependent and co-factor dependent manner [22]. For instance, Rap1 bound to UASs of glycolytic genes interact with Grcl to exert efficient transcriptional activation [23]. As a transcriptional repressor, Rap1 binds to silencers and recruits Sir3p/Sir4p to silencers through direct interactions [24]. Similarly, Rap1 interacts with Rif1 and other telomere specific proteins in executing its role in regulating telomere length [25,26]. It is not known if other Rap1-interacting factors are also required for the barrier activity at *TEF2–UAS*. The STAR-binding Tbf1 also

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**Figure 2**

Model for heterochromatin barrier function. (a) Transcriptionally silenced chromatin initiates from an organizing center 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 hypo-acetylated nucleosomes (left panel). (b) Binding of barrier proteins to a DNA-binding site, such as multiple Rap1 molecules to UAS, excludes formation of several nucleosomes across the region and this nucleosomal ‘hole’ in the chromatin blocks the migration of the \(SIR\)-complex (right panel). B, Abf1; R, Rap1; O, ORC; Sirs, complex of Sir2, Sir3 and Sir4.
binds to many sites throughout the genome [27]. Sequences of tRNA genes have also been shown to have multiple functions in the genome. tRNA genes strongly inhibit transcription from adjacent polymerase II promoters [28]. tRNA genes have also been shown to act as DNA replication pause sites [29]. In both cases, an active tRNA gene is required. The fact that barrier sequences have different functions indicates that barriers may be part of the global regulation of genome structure and function.

One intriguing finding about chromatin boundary/insulator elements from metazoans is their functional conservation among species, such that chicken elements work in human cells [30] and Drosophila elements work in Xenopus [31]. This conservation may also extend to yeast. A tRNA gene is required. The fact that barrier sequences have different functions indicates that barriers may be part of the global regulation of genome structure and function.

Sequence has a 10-fold higher boundary activity than TEF2-UAS alone. As no sequence in this fusion other than TEF2-UAS possesses independent boundary activity [29] (X Bi, unpublished data), we propose that transcription from the TEF2 promoter can further hinder silent chromatin from spreading. Consistently, the barrier activity of HMR tRNAThr is correlated with silencing-resistant transcription of this tRNA gene. Both the initiation and the elongation processes of transcription involve chromatin modification and remodeling resulting in an open-chromatin configuration. Therefore, the putative role of transcription in barrier activity can also be appreciated in the context of the nucleosome gap model described earlier.

**Directional silencing defines the boundary of HML**

The studies described above indicate that specific sequences on either side of HMR are able to block the spread of silencing emanating from HMR. That is, heterochromatin at HMR remains confined to the locus by the action of a class of heterochromatin barrier elements. A very different system operates at HML. At this locus, heterochromatin remains confined to the region not by boundary elements but rather by highly directional initiation of silencing.

By using URA3 as a reporter element for repression, we have shown [35] that repression at HML is uniformly high between the leftward and rightward silencers (E and I, respectively) but decreases sharply beyond the I silencer. The region of repression at HML correlates with the domain of histone hypoacetylation. Despite the sharp definition of the boundaries of HML, no sequence capable of blocking the spread of heterochromatin resides in the sequences flanking HML. In addition, deletion of the region spanning I did not increase the extent of repression of reporter genes inserted outside the locus. Thus, deleting the junction between active and inactive chromatin did not extend repression into the neighboring region. Inverting the orientation of I increases silencing outside of HML while weakening silencing within it. These observations prompted the conclusion that the HML I silencer establishes a boundary between active and inactive chromatin at HML but does so by organizing inactive chromatin in only one direction.

To account for the pattern of repression at HML, we assume that the E and I silencers promote formation of heterochromatin that propagates inward toward the mating type genes (Figure 3). We further assume that the repressive effect imposed by each silencer diminishes with increasing distance from the silencer, similar to the pattern of telomeric silencing [34,36,37]. Finally, we assume that the repressive effects of the two silencers are additive. Shi and Broach [38] previously showed that I is capable of promoting repression of genes at MAT only when inserted next to MAT and pointing toward the MAT genes — that is, with I oriented so that the MAT genes are to the same side.
of I as the \( \alpha \) genes are at HML. Insertion of I at this same site next to MAT but in the opposite orientation failed to elicit repression of the MAT genes. Thus, the previous demonstration of the orientation dependence of I-mediated repression is consistent with the current hypothesis that silencing spreads outward from I at HML only in the direction of the \( \alpha \) genes. Our model represents a novel mechanism for delimiting the boundaries of a eukaryotic chromosome domain.

Are HML and HMR domain organizations mechanisms completely different? HMR E also exhibits directional initiation of silencing [38] and the level of repression of a reporter gene inserted between HMR E and the left hand boundary element is significantly less than that of a gene inserted between HMR E and HMR I [11**]. Thus, directional initiation of repression may prove to be a common mechanism for confining heterochromatin to a distinct domain.

Conclusions

Results discussed in this review suggest that any sequence that can recruit proteins that exclude nucleosomes can function as a heterochromatin barrier in yeast. This conclusion reinforces the notion that heterochromatin spreads in a sequential fashion along the chromosome and distinguishes silencer organizer centers from enhancers, which appear capable of exerting effect across intervening sequences. Thus, whereas metazoan insulator elements initially appeared to possess both heterochromatin-barrier activity as well as enhancer-blocking activity, the results in yeast suggest that these two activities should have distinct mechanistic bases. In fact, more recent analysis indicates that barrier activity can be separated from enhancer-blocking activity in the chicken \( \beta \)-globin insulator. Confirmation of the proposed mechanism of heterochromatin-barrier activity awaits in vivo silencing assays. In addition, the potential role of cohesins in barrier activity needs to be extended to additional examples in order to assess whether cohesins are an integral component of barriers. If they are, then the model proposed in this review will require further refinement. Finally, the notion that boundaries can be formed by directional initiation of heterochromatin should be explored in other organisms.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:**

• of special interest
•• of outstanding interest


   The authors analyze the domain structure of the silent locus HMR and find that specific sequences constitute the left and right boundaries of HMR. Deletion of these sequences leads to the spread of silent chromatin. The right boundary contains a TFI1 LTR and a tRNA gene. Kamakaka and co-workers have since shown that the tRNA gene plus some flanking sequences is the essential part of the right boundary element (RT Kamakaka, personal communication). Mutational analysis implicates the SMC proteins, which are structural components of the chromatin, in boundary activity.


   In this report, the upstream activating sequences (UASs) of three irsosomal protein genes (RPGs), TEF1 and TEF2 from S. cerevisiae, as well as the TEF gene from Ashbya gossypii, are shown to have silencer-blocking activity. These UASs each contain multiple Rap1p-binding sites. Deletion analysis shows that the Rap1p-binding sites of TEF2-UAS are necessary and sufficient for silencer-blocking activity. A pair of these elements can insulate a reporter gene from being repressed at the HML locus. Moreover, TEF2-UAS is shown to physically block the propagation of silent chromatin in a DNA-topology-based assay. TEF2-UAS together with two other silencer-blocking elements are the first yeast sequences ever discovered to have insulator-like properties. A nucleosome-exclusion model is proposed for silencer-blocking activity of TEF2-UAS. This model may also be applicable to the other yeast barrier elements.


   In this report, the telomere-proximal portion of the subtelomeric repetitive sequences X and Y are shown to have the ability to block the silencing effect of a telomere on a reporter gene. These elements, named STARS, also had silencer-blocking activity. STARS contain multiple binding sites for Tbf1 and Reb1 and the silencer-blocking activity was recapitulated by synthetic sequences consisting of multiple Tbf1 and Reb1 binding sites.


   The authors showed that whereas the transcriptional activator Gal4 is effective in perturbing chromatin structure via a nucleosomal binding site in yeast, Gcn4 is not. Correspondingly, Gcn4 requires assistance from an accessory protein, Rap1, for activation of the HIS4 promoter, whereas Gal4 does not. Rap1 is extremely effective at interfering with positioning of a nucleosome containing its binding site, consistent with a role in opening chromatin at the HIS4 promoter. Furthermore, they obtained evidence indicating that cooperative protein–protein interactions are not involved in transcriptional facilitation by Rap1 and concluded that an important role of Rap1 is to assist activator binding by opening chromatin.


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Chromosomes and expression mechanisms


The authors identified a 42 bp fragment of the chicken β-globin insulator that is both necessary and sufficient for enhancer-blocking activity. They showed that this sequence is the binding site for CTCF, an 11 zinc finger protein highly conserved in vertebrates. CTCF has been implicated in both transcriptional silencing and activation. CTCF sites were also found in other vertebrate enhancer-blocking elements.


See annotation [20].


These two papers, [19*20*], demonstrated that CTCF, which preferentially binds to unmethylated DNA sites mediates the enhancer-blocking function of the boundary element located at the H19/Igf2 locus. These studies demonstrated that imprinted genes like H19 and Igf2 can be regulated by boundary elements.


34. Renaud H, Aparicio OM, Zierath PD, Billington BL, Chabiani SK, Gottschling DE: Silent domains are assembled continuously from the telomere and are defined by promoter distance and strength, and by SIR3 dosage. Genes Dev 1993, 7:1133-1145.


In studying the domain organization of the silent HML I locus in yeast, we document that repression across HML I is sharply delimited by the silencers but that neither the silencers nor the surrounding sequences exhibit barrier activity. Rather we explain the domain organization by postulating directional organization of heterochromatin by the silencers at this locus.

