Blockers and barriers to transcription: competing activities?

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In the eukaryotic cell active and inactive genes reside adjacent to one another and are modulated by numerous regulatory elements. Insulator elements prevent the misregulation of adjacent genes by restricting the effects of the regulatory elements to specific domains. Enhancer blockers prevent enhancers from inadvertently activating neighboring genes, and recent results suggest that they might function by a conserved mechanism across species. These elements appear to disrupt enhancer-promoter 'communications' by interacting with the regulatory elements and sequestering these elements into specific regions of the nucleus thus rendering them non-functional. Barrier elements insulate active genes from neighboring heterochromatin and recent results suggest that they function by specific localized recruitment of acetyltransferases that antagonize the spread of heterochromatin-associated deacetylases, thus preventing the propagation of heterochromatin.

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Abbreviations

CTCF	CCCTC binding factor
HS	hypersensitive
ICR	imprinting control region
UAS	upstream activating sequence
Xic	X-chromosome choice/imprinting center

Introduction

The eukaryotic nucleus is organized into active and inactive domains, and the modulation of genes within these domains is mediated by regulatory elements: 'enhancers', 'promoters' and 'locus control regions' are required to activate genes efficiently over significant distances, whereas 'silencers' repress the transcription of genes. Adjacent genes in a cell are frequently associated with functionally antagonistic elements, such as enhancers and silencers, and despite the close proximity of these elements they do not affect expression of the neighboring gene. Thus, junctions between the active and inactive gene domains occur commonly along chromosomes.

In this review, we focus on the sequences that insulate the effects of positive and negative regulatory elements from neighboring genes and that restrict the effects of these elements to specific domains.

Enhancer blockers

Certain boundaries between active and inactive genes can arise merely at the junction of 'open' active chromatin and 'condensed' inactive chromatin. By definition, these junctions are non-specific to sequence and fluid in their location; that is, the position of the junction is dependent on the relative concentrations of the activator and repressor proteins, and on the associated chromatin structures that spread along the DNA fiber from nucleating centers such as enhancers and silencers.

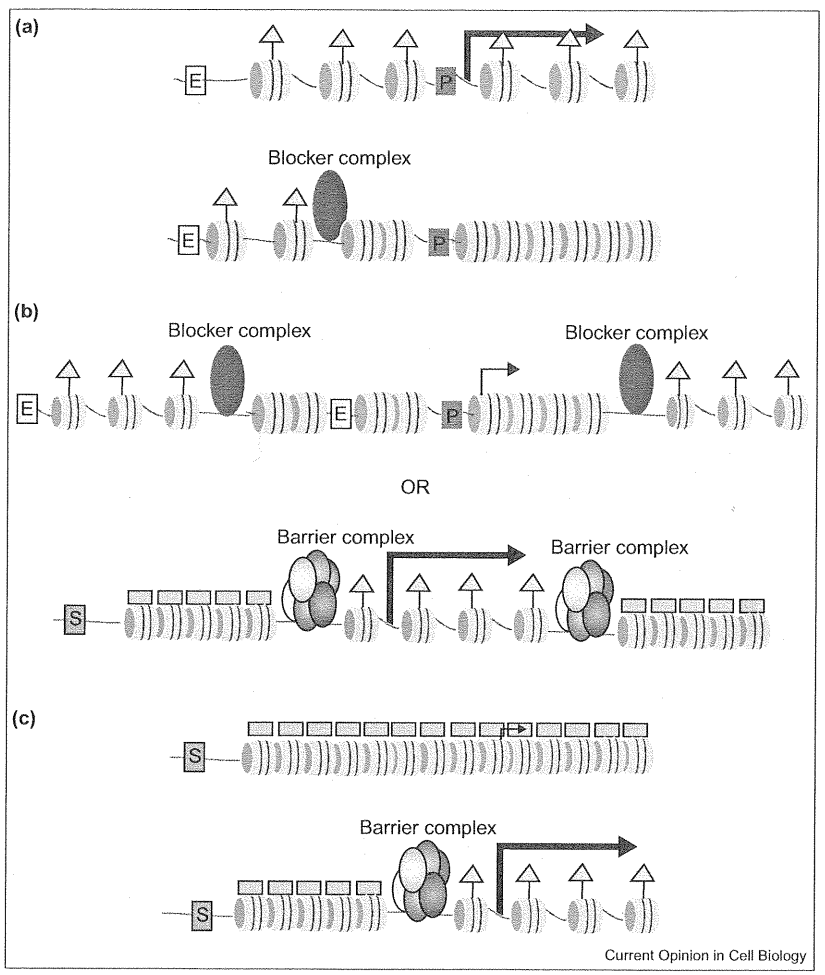
At other loci, the boundaries are mediated by specific sequence elements. These insulator elements come in different flavors with distinct functions. Some insulators can function to prevent the misregulation of adjacent genes by blocking the enhancer of one gene from activating the promoter of a neighboring gene. Furthermore, numerous eukaryotic genes have several regulatory elements, which are needed for tissue-specific and cell-specific expression. A mechanism of tissue-specific regulation, for example, could involve insulator elements that restrict the activity of the different regulatory elements to ensure correctly regulated gene expression. Finally, one can also envisage the existence of insulators that restrict the spread of active chromatin and prevent it from invading inactive regions of the genome (Figure 1).

Insulator elements were first discovered in *Drosophila* and were found to contain DNaseI-hypersensitive sites located at or near the locus boundaries of the heat-shock gene *hsp70* [1]. These elements were called special chromatin structures (*scs*/*scs'*) and were shown to protect a transgene against chromosomal position effects when they flanked the gene, presumably by blocking the action of neighboring chromosomal enhancers from influencing transcription from the transgene. Independent studies have shown that the *gypsy* transposable element can block the effect of an enhancer when located between the enhancer and a promoter [2]. Specific sequences containing binding sites for the Su(Hw) protein within the transposon were shown to mediate the enhancer-blocking effects. The *scs* and *gypsy* elements were shown subsequently to be able to both block the activity of enhancers and protect transgenes from position effects (reviewed in [3]).

Proteins with insulator functions

The proteins that mediate the insulator functions at the *scs* and *gypsy* elements have been identified. The *scs* and *scs'* elements contain binding sites for the proteins Zw-5 and BEAF-32, respectively, which are required for insulator function; in contrast, the protein that binds the *gypsy* insulator and mediates its function is the Su(Hw) protein — a zinc-finger protein that has also been shown to act as an activator and a repressor. At certain loci, this protein requires the modifier mdg4 to mediate its insulator function. Additional insulator elements have been identified in *Drosophila*, as well as in other eukaryotes, and there are numerous excellent reviews on the subject [3–6].

Figure 1



Assays used to study insulator function. **(a)** Enhancer blocking assay. A putative insulator element is inserted between an enhancer (E) and a promoter (P), and enhancer-mediated transcription of a downstream reporter gene is monitored. Efficient enhancer blocking leads to inactivation of the reporter gene. **(b)** Protection against position effects. Insulator elements flank a reporter gene that is integrated into the genome, and transcription of the reporter gene is monitored. Depending on the site of integration of the reporter construct, insulators can either block adjacent native enhancers from fortuitously activating the reporter gene or block adjacent native silencers (S) from inactivating the reporter gene. **(c)** Silencer blocking assay. A putative insulator element is inserted between a silencer and a reporter gene, and silencer-mediated repression of transcription is monitored. Efficient barrier function leads to activation of the reporter gene.

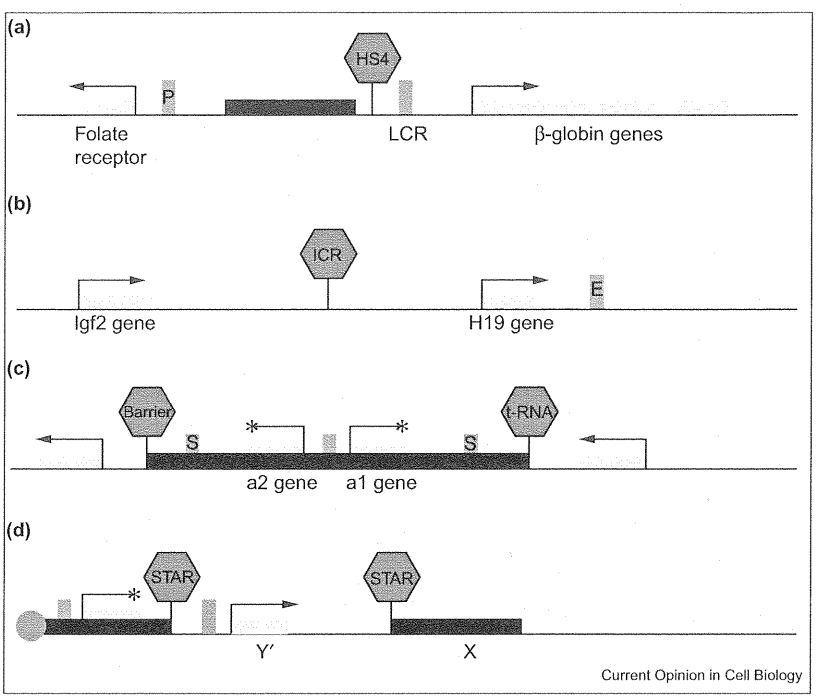
One of the best-characterized vertebrate insulator elements is the HS4 insulator of the chicken β-globin gene (Figure 2). Analysis of the chromatin structure around the active β-globin locus in chicken erythrocytes identified a 33-kb domain of open chromatin and a 16-kb stretch of heterochromatin upstream of the locus [7,8••,9••]. Further upstream of the 16-kb heterochromatin domain is a second erythrocyte-specific locus carrying the folate receptor gene, although the regulation of the folate receptor gene is distinct from that of the globin genes [10]. The constitutive DNaseI-hypersensitive site referred to as HS4 is located at the junction between the active globin domain and the heterochromatic domain.

Like the *Drosophila scs* and *gypsy* insulators, the HS4 element can protect transgenes against position effects [11] and can also function as an enhancer blocker when inserted between an enhancer and a promoter [12]. The role of the enhancer-blocking function of HS4 in the regulation of the globin locus is not clear, but it has been proposed that HS4 may be involved in blocking crosstalk between the globin locus control region enhancers and the folate receptor gene *in vivo*. An alternative possibility is that HS4 might prevent the globin LCR enhancers from disrupting the adjacent 16-kb heterochromatin domain. The conserved protein CCCTC binding factor (CTCF) mediates the enhancer-blocking activity of HS4, and mutations of the CTCF-binding sites in HS4 result in the abolition of enhancer-blocking activity [13]. Again like Su(Hw), CTCF is a transcription factor that has been identified as both an activator and a repressor of transcription (reviewed in [14]).

Recent evidence suggests that mouse CTCF is also involved in insulator function [15–18]. Imprinting of the linked *Igf2/H19* locus in mouse endodermal cells results in the expression of *H19* from the maternal allele and *Igf2* from the paternal allele (Figure 2). Transcription of these genes is regulated by a cell-type-specific enhancer located proximal to the *H19* gene. An imprinting control region (ICR) is located between the *Igf2* and *H19* genes and DNA methylation of CpG residues at the ICR are involved in regulating this

Figure 2

Genetic loci with potential insulator elements. Active genes are shown in yellow, positive regulatory elements in blue, heterochromatic domains in black, silencers in green and insulator elements in red. **(a)** The chicken β-globin locus with the HS4 insulator. **(b)** The mouse *Igf2/H19* locus with the ICR insulator. **(c)** The yeast silenced *HMR* domain with the flanking barrier elements. **(d)** The yeast native telomeres with the STAR (subtelomeric anti-silencing region) barrier elements.
E, enhancer; P, promoter; S, silencer.



imprinted locus. The CpG residues at the ICR are methylated on the paternal allele, which results in enhancer-mediated activation of the *Igf2* gene. On the maternal allele, the ICR is not methylated and consequently *Igf2* remains inactive.

The ICR element contains binding sites for the mouse homologue of CTCF, and the DNA-binding activity of CTCF is dependent on the methylation status of the ICR — CTCF cannot bind to its cognate binding site when the site is methylated. The CpG residues at the ICR are methylated on the paternal allele, which is thought to prevent CTCF from binding, thus allowing the enhancer to activate the *Igf2* gene. On the maternal allele, the CTCF-binding sites are not methylated and the consequent binding of CTCF blocks the enhancer from activating the *Igf2* gene.

Intriguingly, CTCF has also been proposed to be involved in the selection of X-chromosome inactivation in mammals [19••]. The X-chromosome choice/imprinting center (Xic) determines which X-chromosome is repressed and which chromosome is maintained in an active state. The Xic contains binding sites for CTCF, and it has been proposed that CTCF may function in the choice of X-chromosome inactivation in a manner analogous to the mechanism postulated for the *Igf2/H19* locus.

Models

Although it has been assumed that all enhancer blockers function by a similar mechanism, recent results suggest that there are differences in the properties of these elements and their mechanism of function [20]. Enhancer-blocking activity, which is mediated by insulator elements, has been shown to be dependent on position. It requires the insulator to be located between the enhancer and the promoter, which is consistent with the element acting to block propagation of a signal from the enhancer to the promoter. Insulation is not caused by inactivation of the enhancer or the promoter [21–23], which suggests that enhancer-blocking activity occurs by interfering with enhancer–promoter 'communications'.

Because enhancers can, under certain circumstances, function in *trans* [24] and because enhancer blockers can even insulate enhancers in *trans* [25], it is possible that enhancer blockers function as decoys by forming 'non-productive' enhancer–insulator complexes [3]. Whether an element is able to insulate will therefore depend on the ability of the insulator to interact with the enhancer or promoter complexes (or to block the transducing signal that emanates from these elements) in direct competition with the interactions between the enhancer and promoter. Consistent with this model are recent results from studies in *Drosophila*, which have shown that insulator function depends strongly on enhancer and promoter strength: an insulator that could not block a strong enhancer was found to block a weak enhancer easily [26,27].

But simply increasing the number of insulator elements does not increase the enhancer-blocking activity. On the contrary, duplication of the Su(Hw) insulator neutralized

the enhancer-blocking activity and paradoxically increased the activity of the enhancer [28••,29••]. These results have led to the suggestion that multiple insulator elements preferentially take part in interactions with one another rather than in enhancer–insulator or promoter–insulator interactions, thereby neutralizing their enhancer-blocking activity. Further experiments should help elucidate this interesting observation.

Enhancer blocking may also be facilitated by the sequestration of gene regulatory regions to nuclear domains that are either deficient in transcription activators or enriched in repressors, thus affecting enhancer and promoter strength and increasing the probability that enhancer–promoter communications would be disrupted. This is consistent with the observations that Su(Hw) foci are located on the nuclear periphery [30] — a region in yeast that has been shown to be predisposed to silencing genes [31].

Enhancer blocking can thus be visualized as a competition for interactions between the enhancer and the promoter on the one hand and between the enhancer or promoter and the insulator on the other hand. Blocking probably arises by the separation of the enhancer and promoter elements into separate domains in the nucleus and this is probably facilitated by interactions between these elements and the insulator elements. However, the detailed molecular mechanism by which enhancer blockers function remains to be determined.

Barriers

In addition to sequences that function to regulate the activity of enhancers, certain sequences, recently termed 'barriers', block the spread of repressed chromatin and insulate neighboring active regions of the genome from repressive effects.

The *Drosophila* Su(Hw) protein has been shown to protect partially a transgene from neighboring heterochromatic regions, thus acting as a barrier to the spread of silenced chromatin [22]. Similarly, it has been proposed that an element within the globin HS4 insulator may be involved in blocking the spread of neighboring heterochromatin. One function of HS4 may be to prevent the heterochromatic region encroaching into the active locus in chicken erythrocytes. In support of this, HS4 can confer position-independent expression to a stably transfected transgene when it flanks the reporter gene, and so can prevent the transcriptional extinction of the transgene [11,32].

Specific hypoacetylation and methylation of the histone tails has been shown to be associated with the transcriptionally repressed heterochromatin. Analysis of the histone acetylation and methylation patterns of the chicken β-globin locus in a variety of cell types at different stages of erythrocyte differentiation has shown that the region immediately surrounding the HS4 insulator has a constitutively higher level of histone acetylation, whereas the adjacent heterochromatin domain is hypoacetylated and methylated on lysine 9 of histone H3, and the active globin domain contains acetylated histones [7,8••,9••].

These results suggest that the barrier element present in HS4 may act as an entry site for histone acetyltransferase activity to protect the globin locus from the influence of the condensed heterochromatin immediately upstream of HS4. Although the CTCF protein has been shown to bind to the HS4 insulator and mediate enhancer-blocking activity, it does not seem to be involved in protecting transgenes from position effects and may not be involved in acting as a barrier to the spread of the neighboring heterochromatin.

Barrier elements from *Saccharomyces cerevisiae*

The most thoroughly characterized barrier elements are from the budding yeast *Saccharomyces cerevisiae*. Transcriptional silencing in yeast has been shown to exist at numerous loci, including the cryptic mating-type loci *HML* and *HMR*, as well as most of the telomeres, and these loci are typically associated with hypoacetylated histones and the Sir proteins (Sir2p, Sir3p and Sir4p). Silencing at these loci uses sequence-specific factors bound at silencers to initiate silencing, and the repressed chromatin domain is thought to spread from the silencers along the chromatin fiber (reviewed in [33]). But the silenced chromatin does not spread indefinitely, and has been shown to be restricted to a specific region along the chromosome [34,35].

Elements have been found at the boundary between the active and repressed domains at several loci and these elements block the spread of silencing. Other genomic elements have been identified that can functionally block the spread of silenced chromatin when placed between a silencer and a reporter gene.

Studies at the *HML* locus with the upstream activating sequence (UAS) *TEF2* have shown that this UAS acts as a barrier to the unidirectional spread of silencing [36]. Further analysis has revealed that the DNA-binding sites for Rap1p (repressor and activator protein) present in the UAS are required for this barrier effect and several Rap1-binding sites are sufficient to block the spread of silenced chromatin.

At most native yeast telomeres, a transcriptionally repressed domain is found adjacent to the telomeric repeats. Centromere-proximal to these silenced telomeric domains is the STAR (subtelomeric anti-silencing region) elements (Figure 2) [37,38]. These elements block the spread of silencing from the telomere and contain several Tbf1p- and Reb1p-binding sites that are necessary to block the spread of silencing. Furthermore, artificially recruiting these proteins also results in termination of the silenced domain, which shows that these proteins have an active role in barrier function [39•].

Analysis of silenced chromatin at the *HMR* locus has demonstrated that the domain is not restricted to the region between the two silencers but extends out in either direction from the

silencers [35]. A search for barrier elements at the *HMR* locus has led to the identification of a specific tRNA gene at the right boundary of the *HMR* domain that can block the spread of silenced chromatin. Deletion of this tRNA gene leads to a further spread of silencing (Figure 2) [40••].

Mutations in the tRNA promoter elements or the proteins that bind the promoter reduce barrier activity [40••]. Extragenic mutations in the acetyltransferase genes *SAS2* and *GCN5* also reduce tRNA barrier activity, and Gal4-fused Sas2p protein, when recruited artificially to a barrier, could block the spread of silencing [40••]. These results led to the suggestion that stable binding of the transcription factors coupled with the recruitment of chromatin modifying activities results in barrier formation.

Models

Heterochromatin seems to spread from silencer elements through the interaction of repressor proteins, such as Sir3p, Sir4, Swi6 and HP1, with nucleosomes in a process that is probably facilitated by deacetylating and methylating histone tails. A simple model of barrier function can be deduced from the characterizations of the barrier elements described above.

In this model, the ability of a complex to bind stably to chromosomal DNA in competition with the propagating heterochromatin would constitute a barrier. The ability of factors bound to the barrier to recruit chromatin-modifying activities would increase the probability that the barrier remained in place, thereby decreasing the probability of the spread of heterochromatin. According to this model, barrier activity would result from chromatin-modifying activities that create localized regions of open chromatin to block the propagation of heterochromatin. The active recruitment of acetyltransferase activity that modifies histones would function by countering the effects of deacetylating and methylating histone tails, thus preventing the binding and spread of repressor proteins.

Conclusions

So far, research indicates that elements that insulate gene domains from neighboring domains are integral to the process of gene regulation and that these elements use different mechanisms to modulate the positive and negative gene-regulatory elements. Further experimentation should help to define better the molecular mechanisms involved in the process of insulation.

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