

Insulators: exploiting transcriptional and epigenetic mechanisms

Miklos Gaszner and Gary Felsenfeld

Abstract | Insulators are DNA sequence elements that prevent inappropriate interactions between adjacent chromatin domains. One type of insulator establishes domains that separate enhancers and promoters to block their interaction, whereas a second type creates a barrier against the spread of heterochromatin. Recent studies have provided important advances in our understanding of the modes of action of both types of insulator. These new insights also suggest that the mechanisms of action of both enhancer blockers and barriers might not be unique to these types of element, but instead are adaptations of other gene-regulatory mechanisms.

Enhancer

A *cis*-acting regulatory sequence that markedly increases expression of a neighbouring gene. Enhancers are typically capable of operating over considerable distances (sometimes ~50 kb) upstream or downstream of the gene, and in either orientation.

Silencer

A *cis*-acting regulatory sequence that decreases expression of a neighbouring gene.

Upstream activating sequence

A *cis*-acting regulatory sequence in yeast that is distinct from the promoter and increases expression of a neighbouring gene.

National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892-0540, USA. Correspondence to G.F.

e-mail: garyf@intra.niddk.nih.gov
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The organization of eukaryotic genomes necessarily results in the proximity of domains with distinct functions. A domain containing genes that are transcriptionally active in a particular cell type might lie close to another domain containing genes that are not active. Elsewhere, an active gene might be surrounded by constitutively silenced chromatin structures. To an extent, the identity of these domains is maintained by classical transcriptional regulatory elements, such as enhancers, silencers and upstream activating sequences (UASs). In other cases, however, specific DNA sequences and their associated binding proteins have a role in establishing or maintaining discrete inter-domain boundaries. Such sequence elements have been given the name insulators.

In several cases endogenous insulator sites have been shown to have important functions in the regulation of gene expression. Much of our knowledge about insulator function, however, comes from experiments with transgene constructs. Most transgenes in *Drosophila* are affected by endogenous enhancers and silencers, a result of the combined effects of a largely euchromatic genome and the method of transgene delivery¹. By contrast, most transgenes in vertebrates are subject to chromatin-mediated silencing, a reflection of the high heterochromatin content of the genome. The biochemical activities that underlie the protection from these two types of regulatory interference are different, and the elements that are involved have distinct names: those that protect from activation by enhancers are known as enhancer-blocking insulators, whereas those that protect against heterochromatin-mediated silencing are known as barrier insulators². Some compound insulators possess both enhancer-blocking and barrier activities, and

enhancer-blocking insulators also protect against certain types of transcriptional repressor.

Here, we discuss both enhancer-blocking and barrier insulators. Many recent studies have been devoted to the identification and characterization of insulators. Among the reasons for this intense activity is the recent shift in emphasis from transcriptional control of gene expression by nearby regulatory elements to a recognition of the role of long-range interactions and three-dimensional organization of chromatin within the nucleus. Insulation has also emerged as a major mechanism for epigenetic control of gene expression, especially at imprinted loci. Studies of insulators have led to new models of their modes of action. These studies also suggest that both kinds of insulator element exploit the function of other regulatory elements within the nucleus. This leads us to suggest that the established distinctions between various regulatory elements in eukaryotes might be too constraining because all such elements — enhancers, silencers, promoters and insulators — make use of a shared set of strategies to regulate transcription, with a considerable overlap of function.

Enhancer-blocking elements

Enhancers are typically located some distance upstream or downstream of the genes that they regulate, and serve to raise the level of basal transcription from that gene. Enhancer-blocking insulators are defined by their ability to interfere with enhancer–promoter interactions when placed between the two, but not from a flanking position (FIG. 1). Enhancer-blocking insulators were initially thought to be rare, but recent results, especially in vertebrates, show that enhancer blocking has a crucial role in a wide range of regulatory systems.

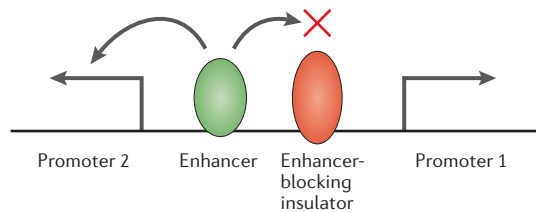


Figure 1 | Testing for enhancer-blocking insulator function. An enhancer-blocking insulator is expected to interfere with enhancer–promoter communication in a position-dependent manner. An enhancer-blocking insulator will block transcriptional activation only when it lies between a promoter and an enhancer (as in the case of promoter 1); in other situations (such as for promoter 2), activation is not blocked. A transcriptional repressor, by contrast, would reduce the level of transcription from both promoters when placed in the same position. In the example shown, the test construct contains both the experimental enhancer–promoter-1 and control enhancer–promoter-2 pairs; in other cases they can reside in separate transgenes.

Loop domains: a common theme in enhancer blocking. Although different DNA binding sequences and their associated proteins are involved in enhancer blocking in vertebrates and invertebrates, it seems that similar mechanisms of enhancer-blocking function have arisen in both: in each case, enhancer-blocking elements can interact with each other or tether the chromatin fibre to structural elements within the nucleus and so establish chromatin loop domains, in which the enhancer and promoter are separated.

Much of the early work that defined the properties of enhancer-blocking insulators was carried out in *Drosophila* using the *gypsy* retrotransposon, which has become a key experimental paradigm for these insulators. Insertion of this element *in vivo* within a cluster of enhancers at the *yellow* locus was found to effectively block the action of all enhancers that are distal to the insertion, without affecting any of the enhancers that are more proximal to the promoter³. Subsequent work has shown that the DNA sequence that is essential for the enhancer-blocking effect of *gypsy* contains a cluster of 12 binding sites for the protein Suppressor of Hairy wing (**Su(Hw)**). This protein in turn binds two others (the POZ-domain proteins **CP190** and **modifier of mdg4**)⁴, and this complex also interacts with the ubiquitin ligase Topoisomerase-I-interacting protein (**Topors**), which is bound to the nuclear lamina⁵. As a consequence of some or all of these interactions, *gypsy* elements that are located at distant chromosomal positions come together to form clusters — insulator bodies — that are localized at the nuclear periphery^{6,7} (FIG. 2). It has been proposed that the distinct domains that are created by this mechanism separate an enhancer and promoter when the *gypsy* site lies between them, and somehow interfere with enhancer–promoter interaction. Importantly, localization of clusters to the nuclear periphery is not essential to enhancer-blocking activity^{8,9}. This is consistent with the idea that Su(Hw)-mediated loop-domain formation is the crucial event; the location of the insulator bodies might be less important.

The *scs/scs'* paired elements, which flank the endogenous *Hsp70* (heat-shock protein 70) locus at cytological position 87A7, are another well-studied insulator system from *Drosophila* that provides support for the importance of loop domains^{10,11}. The proteins **Zw5** (Zeste-white 5, also known as Deformed wings)¹² and the A and B isoforms of **BEAF32** (boundary-element-associated factor of 32 kD)¹³ bind to *scs* and *scs'* respectively. The two BEAF32 isoforms form heterocomplexes at *scs'* *in vitro*¹⁴, and an interaction between BEAF32 and Zw5 has been shown to stabilize loop-domain formation at opposite ends of the 87A7 *hsp70* locus *in vivo*¹⁵. Chromatin loop formation that is mediated by *scs/scs'*, similar to that observed with Su(Hw), is presumably responsible for the enhancer-blocking activity of these elements.

Are similar structures generated by enhancer-blocking insulators in vertebrates? The first vertebrate enhancer-blocking insulator to be identified was **cHS4**, a complex element that combines enhancer-blocking and barrier activity and lies at the 5' end of the chicken β -globin locus^{16,17} (FIG. 3). The powerful enhancer-blocking activity of this element is associated with a strong binding site for **CTCF**, a ubiquitously expressed vertebrate zinc-finger protein^{18,19}. Since this initial discovery, there has been great interest in identifying other binding sites for CTCF and understanding the basis of its function.

Fortunately, one of the first searches for other CTCF binding sites indicated a role for CTCF enhancer-blocking activity in a known regulatory system, the imprinted *Igf2* (insulin-like growth factor 2)–**H19** locus. In embryonic tissues *Igf2* is expressed only from the paternal allele, under the control of downstream endodermal enhancers, and expression is correlated with paternal-allele-specific CpG methylation over the imprinted control region (ICR) that lies between the enhancers and the *Igf2* promoter. In both mouse and human genomes the ICR contains multiple CTCF-binding sites that function as an insulator, and paternal-specific methylation of these sites abolishes both CTCF binding and insulator activity, which allows imprinted *Igf2* expression (REFS 20–22). These results established a role for enhancer-blocking insulators in the regulation of endogenous loci, which has now been demonstrated at many other sites^{23–26}.

What is the molecular basis of CTCF insulator activity? The same model that is proposed for Su(Hw) in *Drosophila* seems likely to apply: CTCF molecules can interact with each other to form clusters and therefore generate closed loop domains²⁷. It has been proposed that CTCF can also tether the chromatin fibre to the nucleolar surface through interactions with the nucleolar protein **nucleophosmin** (also known as B23). This would create 'open' loop domains in which CTCF sites contact the nucleolar surface but not each other. Such a model is supported by the finding that multicopy, stably integrated **cHS4** transgenes are localized to the nucleolar surface in a CTCF-dependent manner. It should be emphasized that the essential property of this model of enhancer-blocking insulators is the ability to position the enhancer and promoter in separate domains.

Enhancer-blocking insulator

A *cis*-acting regulatory sequence that blocks the action of an enhancer on a promoter when placed between the two, but not otherwise.

Barrier insulator

A *cis*-acting regulatory sequence that prevents the extension of a heterochromatic region into a euchromatic region when placed at the junction between the two.

Imprinted loci

Genes that are expressed from only one of the two parental copies, the choice being dependent on the sex of the parent from which the copy was derived.

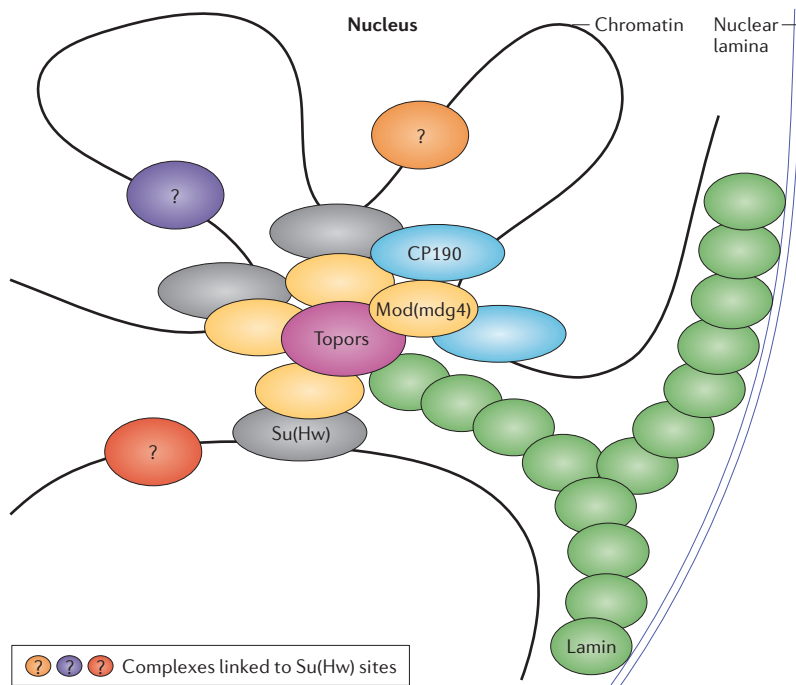


Figure 2 | Insulator-body formation and enhancer blocking are closely correlated in *Drosophila melanogaster*. Insulator bodies are formed by the association of individual Suppressor of Hairy wing (Su(Hw))-containing nucleoprotein complexes that are located at distant chromosomal binding sites. As a consequence of the formation of these bodies, the chromatin fibre that separates individual complexes is sequestered into a loop. Insulator bodies are preferentially located at the nuclear periphery because of their binding of nuclear lamin. When considering a mechanistic role for insulator bodies, the following aspects need to be considered: the topological consequences of loop formation, which might limit contact between an enhancer and promoter; preferential localization at the nuclear periphery, which could in some cases help to stabilize loop formation; and the nucleoprotein complex at the base of the loop, which is not restricted to Su(Hw) and associated factors, but also includes activities that are recruited by sequence elements near the chromosomal Su(Hw)-binding sites. Not all insulator bodies are the same; some protein components that are depicted in the figure are associated only with a fraction of them. It is not known whether this heterogeneity has any functional consequences. CP190, centrosomal protein 190 kD; Mod(mdg4), modifier of mdg4; Topors, topoisomerase-I-interacting protein.

How do insulator-induced domains prevent enhancer action? Understanding how enhancer-blocking insulators work requires knowledge of how enhancers work, and *vice versa*. In the past it has been difficult to explain the position dependence of enhancer-blocking insulators: unlike silencers, they exert their effect only when they lie between the promoter and enhancer. The insulator-mediated loop-domain model has led to two possible explanations of the source of this position dependence, each based on a specific model of enhancer action (FIG. 4). First, enhancers might function by directly interacting with their designated promoters (the direct-contact model). In this case, enhancer-blocking insulators might have a steric effect that prevents enhancers from contacting other promoters, either by favouring intra-loop enhancer–promoter interactions or preventing inter-loop contacts. Alternatively, there could be an activating signal that travels processively from enhancer

Nucleosome
The basic structural subunit of chromatin, which consists of roughly 200 base pairs of DNA and an octamer of histone proteins.

to promoter (the tracking model of enhancer action). This signal could be, for example, a helicase complex that modifies histones or alters nucleosome structure, or it could be RNA polymerase itself, launched from the enhancer. This signal could be blocked by an enhancer-blocking insulator as it tries to traverse the nucleoprotein structure at the base of the loop that the insulator generates. An extensive body of literature is concerned with the mechanism of action of UASs and enhancers^{28,29}. Evidence exists for both direct-contact and tracking models of enhancer action, which need not be mutually exclusive.

Although it is fairly simple to imagine how a tracking signal might be stopped by an insulator complex, it is more difficult to visualize how chromatin loop formation can influence direct contact between enhancer and promoter. A potential mechanism has been described in studies of the direct contact between the *Escherichia coli* *glnAp2* promoter (a downstream promoter in the gene that encodes glutamine synthetase, *glnA*) and an enhancer that is dependent on the nitrogen-regulation protein *NtrC* (also known as *GlnG*), which were separated by 2.5 kb on a closed circular supercoiled template^{30,31}. Expression was inhibited when the template was relaxed, or when the lac repressor, *LacI*, bound at sites that are present on either side of the elements, dimerized to place the enhancer and promoter in separate loops. The data support a model in which enhancer action depends on direct contact between the enhancer and promoter that is achieved by a slithering mechanism in which the interwound supercoil explores various conformations until contact is made. Relaxation or fixation of the DNA structure by the *LacI* interactions is postulated to prevent this contact. A related mechanism has been suggested in eukaryotes from a study that used an SV40 minichromosome carrying an enhancer and enhancer-blocking elements³².

Enhancer blocking and chromatin hubs. These traditional views of enhancer–promoter communication by direct contact, which are focused on the regulation of single genes, might need to be modified in light of recently proposed models for the regulation of eukaryotic gene expression^{33,34}. Studies that were initially carried out at human and mouse β -globin loci showed that promoters, gene-proximal enhancers and far-upstream activators — which can be separated by many kilobases — tend to be co-localized within the nucleus in so-called chromatin hubs. The genes that are controlled by these elements are transcribed when the hubs make contact with RNA polymerase II molecules, which are distributed as multimolecular aggregates^{34,35} within the nucleus that form ‘factories’ for transcription. In recent studies, interactions that are even more distant have been detected at these factories, both within and between chromosomes^{34,36,37}.

How do active promoters find their way to these hubs? Although they might do this simply by a process of random encounter over large distances (direct contact), West and Fraser³⁸ have suggested that distant

enhancers and promoters might be brought together by action of the RNA polymerase in the course of transcribing the intergenic regions that lie between the enhancer and the promoter. Intergenic transcripts, which were first described some years ago^{39,40}, are well documented; in this new view they are by-products of a mechanism that enables contact between promoters and even very distant enhancers.

As the polymerase is fixed in the factory it would pull the promoter towards it as it transcribes, in contrast to simpler tracking models in which the polymerase moves freely along the DNA from enhancer to promoter. In either case the presence of an enhancer-blocking insulator could interfere with tracking or with the architecture of the chromatin hub in a variant of the direct-contact model.

The model that is described above is only one example of the more general class of tracking mechanism that can be envisioned. A variant that has been discussed at length elsewhere⁴¹ proposes a role for the *Drosophila* protein **Chip** in stabilizing the binding of certain homeodomain proteins to distributed sites in the region between promoter and enhancer, creating clusters that bring enhancers and promoters closer together^{42,43}. It was suggested that Su(Hw) sites would interfere with this process. As has been pointed out⁴¹, the clustering process must be processive to account for the enhancer-blocking insulator properties of Su(Hw). The best direct evidence for tracking comes from a study of CTCF. This insulator protein can block the advance of RNA polymerase II, as has been shown using stably replicated minichromosomes that carry the *chS4* insulator: the polymerase accumulates upstream of and within the insulator⁴⁴.

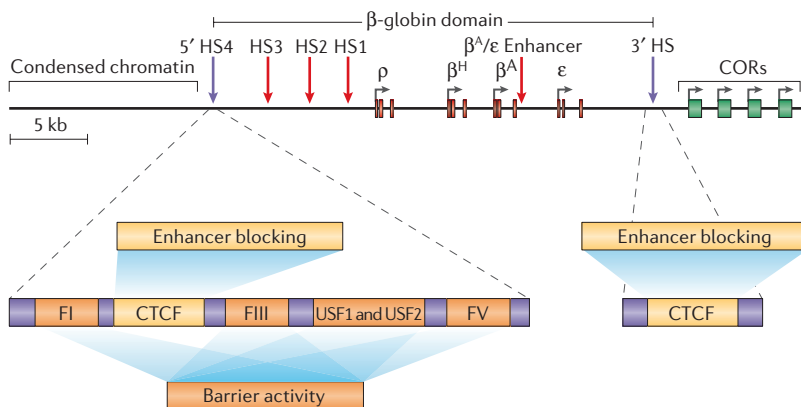


Figure 3 | The chicken β -globin locus. In chicken (*Gallus gallus*), the 5' HS4 and 3' HS insulator elements define the limits of a chromatin domain that encompasses the developmentally regulated β -globin gene cluster and its locus-control region (LCR), which is comprised of the HS1–3 and $\beta^{A/\epsilon}$ enhancers. This domain is flanked by a region of condensed chromatin and a cluster of chicken olfactory receptor genes (CORs) at its 5' and 3' ends, respectively. The HS4 element possesses both enhancer-blocking and barrier activity, presumably to prevent the LCR from inappropriately activating genes outside the domain and at the same time protecting the globin cluster against silencing that emanates from the flanking condensed-chromatin region. Enhancer blocking is mediated by CTCF, whereas barrier activity results from the combined effect of USF1 and USF2 and the as yet uncharacterized FI-, FIII- and FV-binding proteins. 3' HS binds CTCF and functions only as an enhancer-blocking insulator.

Challenges to simple models of enhancer-blocking action.

It is not known whether hubs exist in *Drosophila*, but the more general tracking and direct-contact models that are discussed above still apply. Both of these possibilities, however, seem too simplistic because neither one can explain all of the properties of the *gypsy* element. For example, the tracking model must confront the fact that *gypsy* elements that are inserted into an intron of *yellow* effectively block activation by an enhancer that is located further downstream, but do not interfere with the action of enhancers that are upstream of the promoter (FIG. 5a). If the anchoring complex always blocked a tracking polymerase, it is hard to see why it would not block formation of the *yellow* transcript at those developmental stages in which the upstream enhancers were active. One possibility is that polymerases that originate from enhancers are more easily blocked than ones that are initiated at promoters; in support of this, it has been shown that the insulator strength of a *gypsy* element is inversely proportional to the strength of an upstream enhancer⁴⁵.

A second difficulty for both tracking and direct-contact models is raised by the striking observation that when two *gypsy* elements are introduced tandemly, the enhancer-blocking activity is nullified^{46,47} (FIG. 5b). It has been suggested that the adjacent clusters of Su(Hw)-binding sites interact with each other to form a micro-loop, therefore preventing them from interacting with more distant sites to form a large loop domain. It is not clear, however, why such a micro-loop would not form an effective block against a tracking polymerase. Nor is it clear, in terms of the direct-contact model, why such a micro-loop would not join a cluster of other *gypsy* sites to maintain the domain organization. The situation is further complicated by the findings that the insertion of a third *gypsy* element between the enhancer and promoter restores enhancer-blocking activity in some cases but not others^{48,49}. At least in the case of *gypsy*, other structures or components must be involved in establishing an active insulator site.

Could the putative mechanisms of enhancer-blocking insulator action that are described above also result in interference with certain types of repressor activity, as well as with activities that stimulate gene expression? The models that have been suggested could account for the functions of those repressors in which a distal silencer has to make physical contact with the promoter in order (for example) to deliver a histone deacetylase. In vertebrates, however, the preponderance of silencing activities arise differently — from the expansion of heterochromatin into surrounding areas. This type of silencing is not affected by enhancer-blocking insulators, but is prevented by barrier insulators.

CTCF — a context-dependent enhancer-blocking insulator?

Given that CTCF often binds to regions of the genome that are adjacent to binding sites for other regulatory factors, it would not be surprising if its function were context dependent. Like many regulatory proteins, CTCF also is the target of modifications that can affect its properties. For example, it can be poly(ADP-ribosyl)ated,

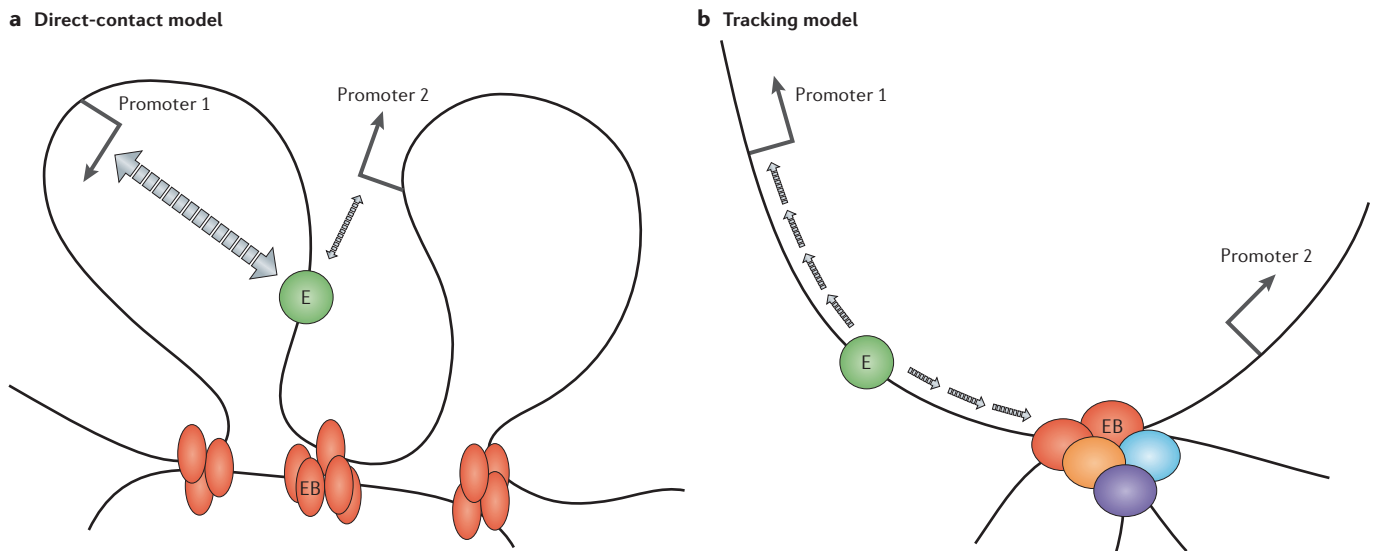


Figure 4 | Models for enhancer-blocking activity. **a** | One set of models focuses on the formation of topologically closed looped chromatin domains. They posit that enhancer-blocking elements and the enhancer-blocking (EB) proteins that bind them prevent enhancer–promoter communication by partitioning the promoter and the enhancer that it is to be shielded from into separate looped domains (here, promoter 2 is shielded from the enhancer (E), whereas promoter 1 is not). An assumption of these models is that the frequency of intra-loop enhancer–promoter interactions is higher than that of inter-loop interactions; this can be achieved by the existence of a mechanism that either facilitates intra-loop interactions or inhibits inter-loop interactions. The dashed double arrows mark enhancer–promoter interactions. Arrow thickness denotes the probability of the interaction **b** | A second group of models postulates that enhancer blocking stems from the targeting of a specific nucleoprotein complex (for example, an insulator body) to the region of the chromatin fibre that separates the enhancer and promoter (again, promoter 2 is shielded from the enhancer, whereas promoter 1 is not). Inherent to tracking models is the idea that transcriptional activation involves the processive transfer of a signal from the enhancer to the promoter. According to these models, specific interactions between the activation signal and the enhancer-blocking complex disrupt the transfer. The models can accommodate both a fixed chromatin template combined with a mobile signal (for example, RNA polymerase tracking along chromatin) and a mobile template with a fixed signal (for example, specific regions of chromatin moving into RNA polymerase II transcription factories).

and inhibition of this modification impairs its ability to function as an insulator⁵⁰. Importantly, CTCF can also function as a classical transcription factor, although this behaviour appears to be restricted to certain sites (see, for example, REF. 51). Because CTCF is an 11-zinc-finger protein for which different binding sites in DNA engage different subsets of fingers⁵², with the potential of exposing different regulatory surfaces on CTCF, its stimulatory effect on transcription could be unrelated to its enhancer-blocking function. On the other hand it is quite possible that under some circumstances the ability of CTCF to form or enter chromosomal clusters could allow it to bring promoters close to active transcription hubs. This raises the possibility that enhancer-blocking activity is only one manifestation of more general mechanisms that bring genes and regulatory elements together at multi-component sites within the nucleus.

Barrier elements

Barrier insulators protect against position-effect variation (PEV), which is the stochastic, meta-stable and heritable silencing of a euchromatic gene through the spread of heterochromatin formation (FIG. 6). Barrier elements have been isolated from several organisms (for a partial list, see REF. 41), and recent studies have led to a much more detailed understanding of their molecular

activities that sheds light on the cellular mechanisms that are used to maintain the epigenetic characteristics of chromatin domains.

Heterochromatin and euchromatin. Barrier activity can only be discussed in the context of heterochromatin and euchromatin. Biochemically, heterochromatin is the more condensed form of chromatin, as demonstrated by its reduced sensitivity to nuclease digestion, which is a reflection of the positioning of nucleosomes at regular, short intervals. Characteristic histone modifications that are seen in heterochromatic regions are high levels of methylation at the histone H3 Lys9 (H3K9) and Lys27 (H3K27) residues, combined with a lack of acetylation marks; heterochromatin is also marked by the presence of heterochromatin protein 1 (HP1). Heterochromatic DNA in vertebrates, as well as in plants, also shows extensive CpG methylation.

Recent advances have provided insight into the biochemistry of initiation and maintenance of heterochromatic structures (REF. 53). At the centre of both processes is a self-perpetuating cycle of reactions: methylation of H3K9 leads to the HP1-mediated recruitment of additional histone methyltransferase (HMT) activity. Two pathways for targeting heterochromatin formation have been described. The first targets H3K9 methylation

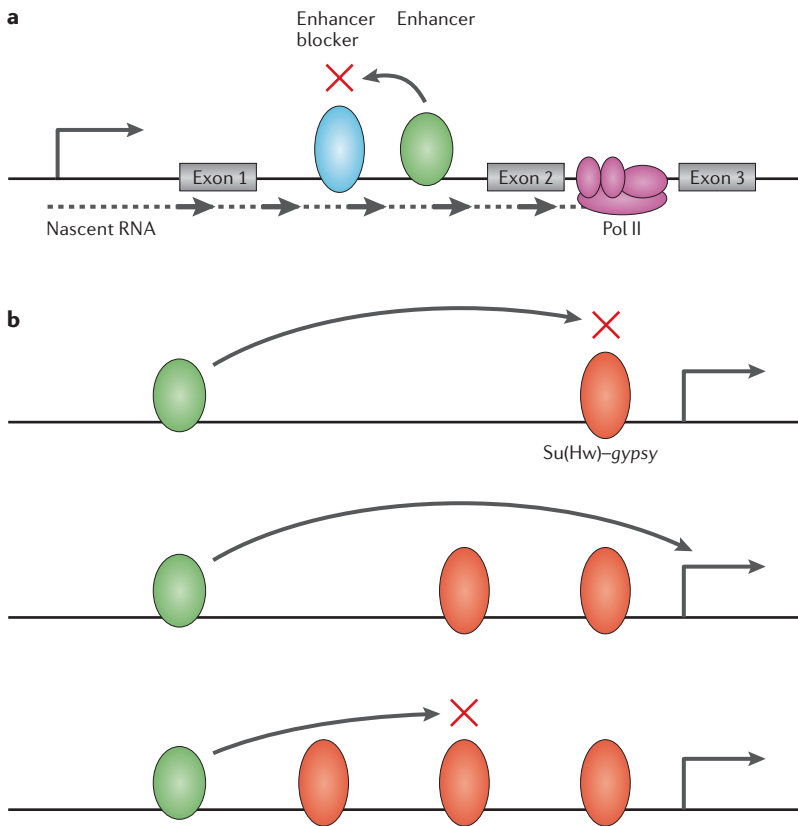


Figure 5 | Problems for simple models of enhancer blocking. Some aspects of Suppressor of Hairy wing (Su(Hw)) and *gypsy* activity are not explained by current enhancer-blocking models. **a** | An intronic *gypsy* enhancer-blocking insulator blocks enhancers that are located in the 3' direction from communicating with the promoter. It does not, however, prevent the passage of the transcribing RNA polymerase II (Pol II) complex in the forward direction. If the enhancer-blocking insulator is blocking a processive signal that originates at the downstream enhancer, why does it not also block the polymerase? **b** | Although a single copy of a Su(Hw)-*gypsy* insulator blocks enhancer-promoter communication (top), two copies inserted between the enhancer and the promoter cancel enhancer blocking (middle)^{46,47}. The proposed explanation is that the elements participate in a non-productive, local micro-loop formation instead of targeting the region to an insulator body. The validity of this explanation was challenged by the finding that insertion of a third copy of a Su(Hw)-*gypsy* element restores enhancer blocking (bottom)⁴⁸. A more recent report suggests that the non-productive self-interaction of three Su(Hw)-*gypsy* elements is influenced by their relative spacing, as well as the presence of transcriptional control elements in the space that separates them⁴⁹. It seems that the models for enhancer blocking will need further refinement to accommodate all the new data. The behaviour described above might be specific to the Su(Hw) elements: by contrast, two copies of the *scs* element inserted between an enhancer and a promoter do not abolish its enhancer-blocking activity⁴⁸.

Nuclease-hypersensitive site
Chromosomal region that is highly accessible to cleavage by deoxyribonuclease I. Such sites are associated with open chromatin conformations and transcriptional activity.

to repetitive sequence elements using small RNA molecules that are complementary to the target⁵⁴. The second relies on sequence-specific DNA-binding proteins to deliver HMT activity to specific genomic locations⁵⁵. The same biochemical cycle has been invoked to explain the spreading of heterochromatin: H3K9 methylation of nucleosomes near the initiation site leads to an extension of the HP1-bound domain (FIG. 7a).

This pathway of heterochromatin formation is conserved in most organisms. A notable exception is *S. cerevisiae*, which uses a biochemically different but conceptually similar pathway for chromatin-mediated

silencing⁵⁶. Yeast heterochromatin consists of deacetylated nucleosomes that are spaced at short, regular intervals. It is restricted to the *HML* and *HMR* silent mating loci, telomeres and rDNA repeats, and PEV is experienced by transgenes that are inserted within or near silenced chromatin regions. Silenced chromatin formation at *HML* and *HMR* is initiated by the E and I silencers, at which the binding of sequence-specific factors recruits the Sir2 histone deacetylase. Deacetylation of histone tails by Sir2 promotes the binding of the Sir3-Sir4 complex to the nucleosome, which subsequently recruits additional Sir2 molecules, stabilizing yeast heterochromatin and allowing it to spread.

Euchromatin comprises the transcriptionally active portions of the genome in which DNA is more accessible to nucleases and nucleosomes are irregularly spaced. A characteristic feature of euchromatin is the presence of nuclease-hypersensitive sites that mark the presence of sequence-specific DNA-binding proteins. Nucleosomes within euchromatin carry a combinatorial pattern of many post-translational modifications, which include high levels of acetylation and methylation of H3K4 and H3K79. Euchromatin formation is aided by processes that are associated with the activation of transcription. These include various histone modifications and nucleosome remodelling, as well as deposition of histone variants. Unlike heterochromatin, euchromatin probably does not spread through a linear polymerization-like process but instead occurs through destabilization of heterochromatic structures.

Barrier activity: breaking the nucleosome chain. Given the array of genetic tools available, it is not surprising that the most detailed characterization of barrier activity has been carried out in yeast cells. Most of these experiments have used constructs that are based on the simple architecture of a silencer (an E or I element) that is separated from the reporter transgene by a putative barrier, and screens have been carried out to identify sequence elements⁵⁷ and enzymes that are associated with barrier activity⁵⁸. Screens for enzymes have made use of a GAL4 DNA-binding-domain fusion library and four GAL4-binding sites in the barrier position. These experiments linked barrier activity to the localized disruption of the polymerization-like reaction cycle at the heart of heterochromatin spreading. Barriers function as chain terminators by modifying the nucleosomal substrate of this processive reaction (FIG. 7b). The most extreme modification of the template is nucleosome removal; various nucleosome-excluding sequence elements were shown to disrupt the spread of chromatin-mediated silencing⁵⁹. Other forms of modification are achieved through the targeted recruitment of histone acetyltransferase (HAT) and ATP-dependent nucleosome-remodelling complexes⁵⁸. Whether barrier activity is only associated with a specific subset of these enzymes remains to be seen. As discussed below, both nucleosome exclusion and the recruitment of histone- or nucleosome-modifying complexes have important roles at endogenous yeast barrier elements.

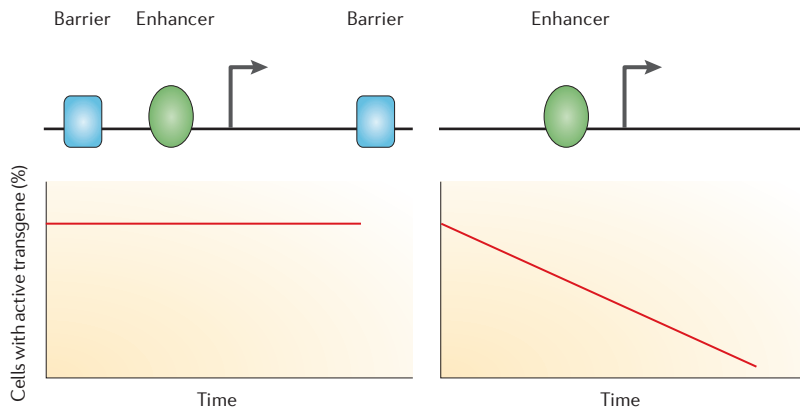


Figure 6 | Testing for barrier function. A barrier insulator protects a transgene against heterochromatin-mediated silencing in long-term tissue culture assays. The graphs show transgene activity over time in assays of this type. The barrier must flank both 5' and 3' ends of the construct as a randomly integrated transgene might experience heterochromatic encroachment from either direction. Yeast experimental constructs are an exception to this rule. They contain a single barrier that is positioned between the reporter gene and the known source of chromatin-mediated silencing activity.

Studies of cHS4, the complex vertebrate insulator that we discussed above, support the idea that a similar molecular mechanism has an important role in barrier activity in higher eukaryotes, although different enzymes are involved. cHS4 lies at the extreme 5' end of the chicken β -globin locus^{16,60}, and has both enhancer-blocking¹⁸ and barrier activities¹⁷. Whereas enhancer blocking by cHS4 is mediated by the CTCF protein, its barrier activity is independent of this protein⁶¹. The chromosomal position of cHS4 is marked in all cell types by a nuclease-hypersensitive site and a peak of euchromatin-specific histone modifications (histone acetylation and H3K4 methylation)^{62,63}. The peak of histone modifications at cHS4 is due to recruitment of HATs and HMTs by the upstream transcription factor 1 (USF1) and USF2 sequence-specific DNA-binding proteins⁶⁴. cHS4 mutations that disrupt the binding of USF1 and USF2 not only eliminate the recruitment of HATs and HMTs, but also abolish barrier activity. These findings led to the proposal that cHS4-mediated acetylation and H3K4 methylation of nucleosomes renders them resistant to H3K9 methylation and HP1 binding and therefore stops the spread of heterochromatin formation. Similar mechanisms could be based on propagation of other heterochromatin-associated histone modifications and their co-factors. It is not yet known whether all the histone modifications that are seen at cHS4 contribute equally to the barrier activity.

Can barriers function through subnuclear targeting? The idea that higher-order chromatin structures, especially chromatin loops, can limit the spread of heterochromatin has been discussed for some time. Interest in this topic was reinvigorated by two recent reports that link barrier activity and the ability to anchor the chromatin fibre, either through binding to the nuclear pore⁶⁵ or homotypic protein-protein interactions⁶⁶. These reports indicate that anchoring limits heterochromatin spreading through a molecular mechanism that is

Nuclear pore complex
A large multiprotein complex that forms a channel in the nuclear envelope of eukaryotic cells. It joins the inner and outer nuclear membranes and allows transport of proteins and nucleic acids to and from the nucleus.

different from the one described in the previous section, although no specific mechanism has been proposed. The data, however, are consistent with the possibility that the weak barrier activities of the elements that were investigated in these studies result from a combination of nucleosome exclusion and targeting of the reporter to a subnuclear compartment⁶⁷. For example, targeting to the nuclear pore complex would provide an environment that is unfavourable to chromatin-mediated silencing because of the known high concentration of transcriptional activators that favour euchromatin formation.

Can enhancer-blocking proteins also function as barriers by tethering a locus to a subnuclear compartment that is unfavourable to heterochromatin formation or spreading? Su(Hw) has been reported to partially protect transgenes from heterochromatin-mediated silencing in *D. melanogaster*⁶⁸. The molecular mechanism of this activity is unknown; however, it is tempting to speculate that the demonstrated ability of Su(Hw) to target the chromatin fibre to insulator bodies is involved. In vertebrates, there are no reports so far of CTCF directly protecting a locus against heterochromatin-mediated silencing. In fact, CTCF fails to function as a barrier in a chicken cell-based transgene assay⁶¹. cHS4, and therefore CTCF, also fails to protect a transgene that is located on the inactive mouse X chromosome from being silenced⁶⁹. There is much continued interest in this topic, however, given the recent identification of novel CTCF-binding sites at or close to transition points between silenced and active chromatin structures. One study describes such sites at the 5' end of genes that escape silencing on the inactive X chromosome⁷⁰. Another, discussed above, shows that CTCF can selectively block elongation of a transcript that is initiated at an enhancer element⁷¹. Neither report, however, provides evidence that CTCF directly limits the spread of heterochromatin, although this might ultimately prove to be true.

tRNA genes can function as barriers. Studies that show that tRNA genes can function as a barrier further challenge the concept that barrier insulation is a property of a distinct class of unique regulatory element. Sir3, a marker for silenced chromatin, is localized to a ~4 kb region that encompasses the silent α genes and the flanking E and I silencers at the *S. cerevisiae* HMR locus⁷². Transition between silenced and active chromatin takes place over a ~1 kb region at the centromere-distal end of HMR⁷³. Using a transgene assay, barrier activity within this fragment was mapped to a unique tRNA^{Thr} gene, with a minor contribution from flanking sequences⁷⁴. Deletion of conserved promoter elements abolished barrier activity, and mutations in the trans-acting factors TFIIB and TFIIC (which are basal transcription factors for RNA polymerase III) had the same effect. The tRNA^{Thr} barrier was also sensitive to mutations in a subset of genes that encode HATs. The available data support the hypothesis that high-level transcription of the tRNA promoter is necessary for its boundary activity. A possible explanation for this requirement comes from the observation that high transcription levels lead to the formation of a nucleosome-free gap at the promoter.

A recent study analysed the effects of specific mutations on the *in situ* activity of the *tRNA^{Thr}* barrier at *HMR⁷⁵*. Single mutations that affected a HAT or the *tRNA^{Thr}* promoter alone did not result in increased spreading of Sir3 protein beyond the wild-type boundary. Disruption of the barrier required a combination of two mutations: one in the *tRNA^{Thr}* promoter to eliminate the nucleosome-free gap and a second one in either the *ada2*, *eaf3* or *sas2* genes that encode histone acetyltransferases. There is no report of these HATs being specifically recruited to tRNA promoters, so it is more

likely that they contribute to barrier activity through increasing global acetylation levels. Reduced global acetylation levels in mutant strains increase the stability of silenced chromatin structures by lowering the Sir2 deacetylase activity that is required to maintain the acetylation-free status of a nucleosome.

tRNA genes also serve as barriers in other organisms. High-resolution analysis of chromatin architecture in *Schizosaccharomyces pombe* showed that the transition points between active and silent chromatin regions at the centromeres co-localize with a cluster of *tRNA* genes^{76,77}. A recent study demonstrates that an active *tRNA* gene can function as a barrier to heterochromatin spreading in *S. pombe*, although the mechanism of action is not yet known⁷⁸. Interestingly, deletion of the *tRNA* barrier not only disrupts wild-type chromatin organization at the centromere but — presumably as a consequence of this effect on chromatin — also leads to abnormal centromere function. The ability of barrier insulators to determine heterochromatic boundaries can therefore affect large-scale chromatin organization as well as local transcriptional activity. RNA polymerase III promoters (which transcribe tRNA) can also function as barriers in vertebrates, in which they have been implicated in the barrier activity of Alu elements that flank the human gene that encodes *keratin 18*⁷⁹.

A recent study has shown that clusters of Box B binding sites for TFIIC that flank the silent mating locus of *S. pombe* can function as barriers in the absence of additional Pol-III-promoter-associated factors. This report provides further insight into the potential molecular mechanisms of barriers⁸⁰. Genome-wide chromatin immunoprecipitation analysis revealed the existence of a number of TFIIC-bound Box B clusters (chromosome-organizing clamp or COC sites) that are not associated with RNA polymerase III. Microscopic examination of *S. pombe* nuclei showed that distant COC sites come together to form a limited number of clusters at the nuclear periphery. How does the formation of this structure, which has a striking similarity to Su(Hw)-mediated insulator bodies, facilitate barrier activity? The answer might come from the observation that a large fraction of COC sites are located at the 5' ends of highly active, divergently transcribed gene pairs. Perhaps the targeting of COC sites that flank the silenced *MAT* locus (which controls mating type) to a subnuclear region with high transcriptional activity prevents further spreading of heterochromatin-associated structures. Further experiments will be required to determine whether TFIIC, when it functions alone, acts through the same mechanisms as intact *tRNA* gene barriers.

Transition without a fixed barrier. Studies of the fourth chromosome in *Drosophila* revealed an organization that does not seem to depend on fixed barrier elements to separate euchromatic and heterochromatic regions. Many fourth-chromosome-linked lines were generated using a transgene construct that was specially designed to report on the chromatin status at its site of insertion⁸¹. Analysis of the lines showed that the gene-rich fraction of the fourth chromosome has interspersed

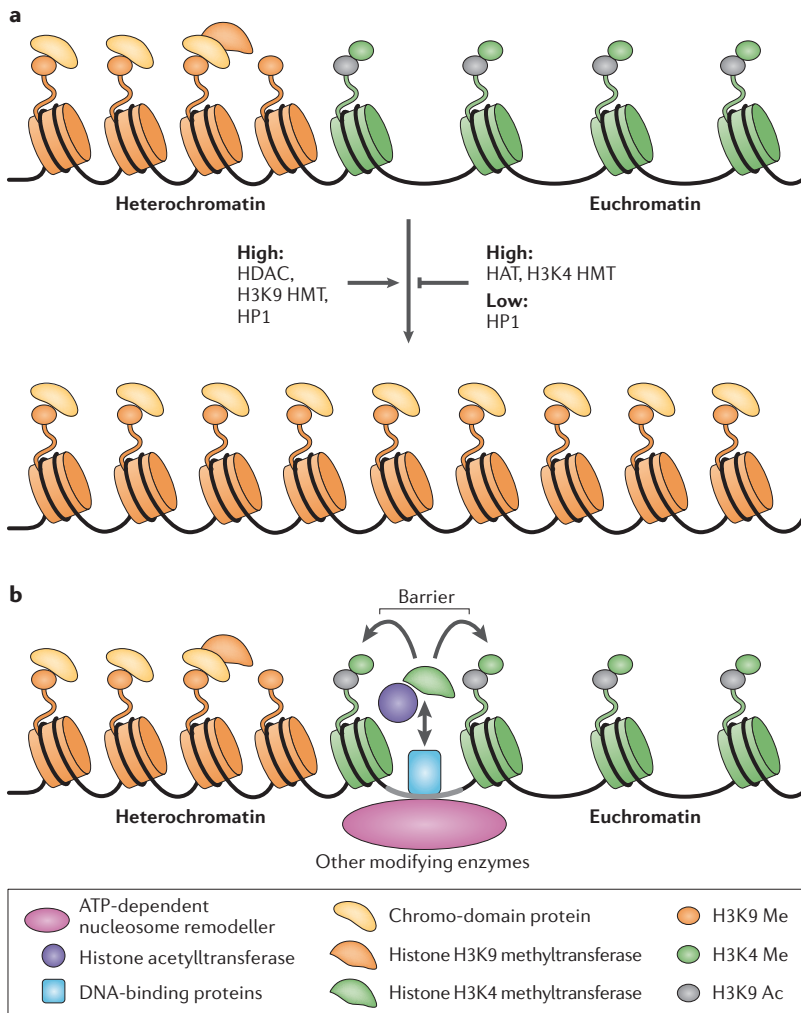


Figure 7 | A local balance of activities determines the extent of heterochromatin propagation. a | A predominance of heterochromatin-promoting enzymatic activities (for example, histone deacetylases (HDACs) and histone methyltransferases (HMTs) that promote methylation at lysine 9 of histone 3 (H3K9) and H3K27) combined with high levels of heterochromatin-specific structural proteins (for example, heterochromatin protein 1 (HP1)) leads to the propagation of heterochromatin. High local levels of enzymatic activities that are linked to euchromatin or a depletion of heterochromatin components, on the other hand, prevent further spreading into euchromatic regions. **b** | Barrier insulators change the local balance by recruiting (directly or indirectly) euchromatin-promoting enzymatic activities (histone acetyltransferases (HATs), H3K4 and H4R3 HMTs and ATP-dependent chromatin-remodelling enzymes). They might also work through tethering the chromatin fibre to a subnuclear compartment, the protein composition of which is unfavourable to heterochromatin formation, or by disrupting heterochromatin propagation by displacing nucleosomes (not shown). Ac, acetyl group; Me, methyl group.

heterochromatic and euchromatic regions, with some of the genes located in the heterochromatic parts⁸². A similar arrangement is likely to be present in the pericentric region of other chromosomes. When considering the implications of this result one needs to keep in mind that in the specific tissues in which the endogenous genes are active the chromatin organization might be different from that of the eye, which is the target tissue of the reporter transgene.

Results from genetic manipulation of individual transgenes lead to the conclusion that, on the fourth chromosome, the boundary between heterochromatin and euchromatin is not regulated by fixed barriers⁸³. Instead, it is determined by the local balance between the strength of activities that promote either heterochromatin or euchromatin formation (FIG. 8). As these activities show slight cell-to-cell variation, so does the position of the boundary, which leads to the variegated expression of a transgene that has been inserted into the region of transition. The proposed initiation site of heterochromatin formation in this case is the *1630* subclass of repetitive elements; transgenes that are inserted within ~10 kb of a *1630* element are subject to PEV. Spreading of heterochromatin beyond the ~10 kb limit must be curtailed by activities that are associated with euchromatin formation. These heterochromatin-limiting 'positive' signals are likely to be a combination of the global activity and targeted recruitment of histone- and/or nucleosome-modifying enzymes. Presumably none of the individual local-recruitment elements is strong enough to be dominant over heterochromatin spreading in a way that would allow it to behave as a fixed barrier.

The picture that has emerged from various closely related fields of chromatin research shows that the epigenetic state of a given genomic region is determined by the local balance between opposing activities that promote heterochromatin and euchromatin formation⁸⁴. These activities include both those that affect global components (such as HP1 protein levels) and targeted modifications (for example, HATs or HMTs that are recruited by USF1 or USF2 to cHS4), and can function either to stabilize or destabilize euchromatin or heterochromatin. Tight regulation of the chromatin state seems to be limited to genomic regions in which the presence of one form is clearly desirable. Examples include not only the formation of euchromatin at active genes and their regulatory regions but also the maintenance of heterochromatin at pericentromeric regions and repressed genes (for example, silenced copies of mating-type genes at the *HML* and *HMR* loci). A sharp transition between heterochromatin and euchromatin is observed only in those cases in which two tightly regulated regions are juxtaposed (for example, the chicken β -globin locus). In these situations the transition point is determined by the position of a barrier that functions as a dominant recruitment site for euchromatin-promoting activities. In other cases the transition seems gradual when examined with experimental methods, such as chromatin immunoprecipitation, that reflect the average properties of a cell population (for example, the *S. cerevisiae* *HML*

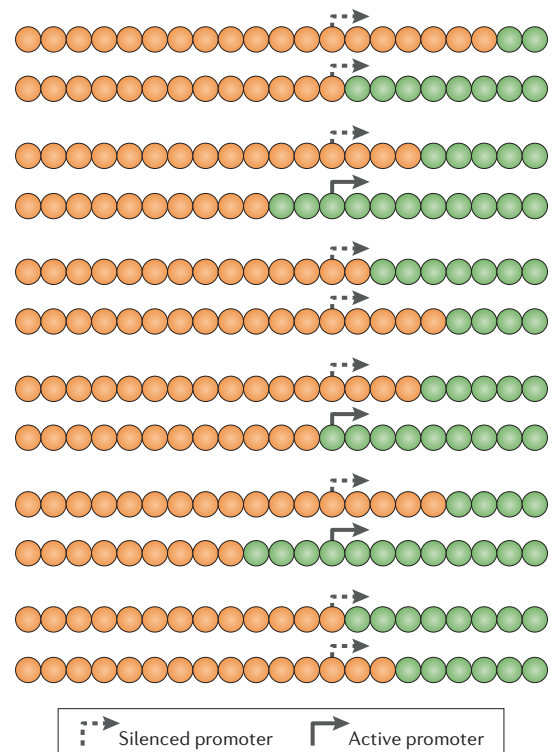


Figure 8 | Unblocked spread of heterochromatin leads to position-effect variegation. Chromatin states are shown schematically for the same chromosomal region in six genetically identical diploid cells from the same tissue. In the absence of a dominant barrier, the exact transition point between heterochromatin (orange) and euchromatin (green) is determined by a chromosome-specific balance of activities. Just as this balance shows chromosome-to-chromosome variation, so does the point of transition. When analysed with chromatin immunoprecipitation, the region of transition shows a gradual change from heterochromatin to euchromatin. The bimodal nature of the transition can only be revealed by methods that report on the status of individual cells; a gene that is located in the region of transition will have a variegated phenotype.

locus)⁷². The apparent gradual transition is probably a consequence of cell-to-cell variation in the extent of heterochromatin spreading. Even in these cases weak euchromatin-recruitment sites have a role; they are not strong enough, however, to serve as a defined barrier in all cells.

Rethinking the definition of insulators

As we have described, recent experiments strongly support the existence of a connection between enhancer-blocking insulation and the organization of chromatin structure within the nucleus. Despite this, none of the plausible models that arise from these observations is entirely satisfactory in explaining how enhancer-blocking insulation works, perhaps because more than one mechanism might be involved, both among and within organisms. By contrast, the mechanisms for barrier-insulator function seem to share the more obvious common

Locus-control region

(LCR). Originally defined as a *cis*-acting sequence element that confers tissue-specific, copy-number-dependent expression on a transgene. Molecular dissection of some LCRs showed them to be composite structures that are comprised of transcriptional activators and insulator elements.

theme of maintaining histone modifications at the boundary that are associated with ‘active’ chromatin. For both types of insulator, these mechanisms seem to reflect not a unique, highly specialized apparatus that is devoted to insulation, but rather a modification or extension of existing regulatory elements.

This blurring of the divisions between the mechanisms of insulators and other regulatory elements is quite clear in the case of barrier insulators, which recruit a wide variety of histone-modifying factors (HATs and HMTs) of a kind that are also found at enhancers. Strong enhancers might to a greater or lesser extent also confer protection against position effects, presumably through maintenance of positive histone modifications. Barrier insulators differ from enhancers in that barrier elements lack the ability to work as activators in transient transfection experiments. We note, however, that locus-control regions (LCRs), which can confer strong position-independent expression on transgenes, consist of multiple regulatory regions, some of which might function in transient assays, and others only when stably integrated into the genome. We suggest that the latter elements might have barrier-insulator properties. Whether or not this is the case, it is clear that barrier insulators and enhancers share many of the same factors and modes of action.

A similar argument can be made about the relationship of enhancer-blocking insulators to other types of transcriptional regulator and chromatin architectural element: as we noted above, the loop architecture that is connected with this kind of insulator action might be one specialized application of a more general set of regulatory mechanisms that assist in bringing together distant regulatory elements and genes (for example, in chromatin hubs), and stabilizing inter-chromosomal interactions. Proteins such as CTCF would be well suited to a role in these mechanisms, which would be quite different from their modes of action at enhancer-blocking insulators.

It is clear that insulators of both kinds share the same bag of tricks with other regulatory elements — enhancers, silencers and LCRs — which they combine in various ingenious ways to acquire specific properties. Although it is still useful to maintain the different categories of regulatory element, it should be kept in mind that there might be considerable overlap in their functions *in vivo*. As with the other elements, we should not let our classification schemes for insulators obscure our understanding of their potential versatility in controlling chromatin organization within the nucleus.

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Competing interests statement

The authors declare no competing financial interests.

DATABASES

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