Insulator dynamics and the setting of chromatin domains

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Summary

The early discovery of *cis*-regulatory elements able to promote transcription of genes over large distances led to the postulate that elements, termed insulators, should also exist that would limit the action of enhancers, LCRs and silencers to defined domains. Such insulators were indeed found during the past fifteen years in a wide range of organisms, from yeast to humans. Recent advances point to an important role of transcription factors in insulator activity and demonstrate that the operational observation of an insulator effect relies on a delicate balance between the ''efficiency'' of the insulator and that of the element to be counteracted. In addition, genuine insulator elements now appear less common than initially envisaged, and they are only found at loci displaying a high density of coding or regulatory information. Where this is not the case, chromatin domains of opposing properties are thought to confront each other at ''fuzzy'' boundaries. In this article, we propose models for both fixed and fuzzy boundaries that incorporate probabilistic and dynamic parameters.

Introduction

Chromatin insulators are elements that can shelter genes from the effects of silencers or enhancers (see Fig. 1). They are envisioned to perform a partitioning of genomes into independently regulated chromosomal domains and should therefore play a critical role in genome function. Whereas certain properties appear to be unique to the insulators that act in the protection against either silencers or enhancers,[1,2] there is enough similarity between both types of systems to suggest that there is a shared mechanism. Long-range effects have been shown to involve the propagation of a chromatin of specific composition and structure in the case of silencers,[3] and recent advances suggest that this is also the case for enhancers.[4–6] Progress is being made in the deciphering of the histone codes in both active and repressive chromatin domains, although more is known about the proteins that are involved in the establishment and maintenance of chromatin function in repressive, heterochromatin-related structures. In addition, recent work in the yeast *Saccharomyces cerevisiae* has helped elucidate the function of insulators that oppose the effects of endogenous silencers. The aim of this review is to present a dynamic molecular model of insulator function that has emerged from recent experiments on both heterochromatin and insulators. For a more comprehensive review describing the variety of insulators reported so far, the reader is referred to Ref. 7. Of note, the term ''heterochromatin'' is used here in its now widely accepted sense to denote any condensed and transcriptionally silent chromatin that can induce mosaic silencing of transgenes,[8] rather than the original description as the fraction of the nuclear material that remained condensed after mitosis. The ideas that will be discussed in this article derive in large part from work done in yeast. Where possible, the findings will be discussed with respect to their applicability to multicellular eukaryotes, in which much higher degrees of condensation are thought to be achieved in heterochromatin.

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Abbreviations: LCR, locus control region; UAS, upstream activating sequences; GRF, general regulatory factor.

A dynamic and probabilistic vision of heterochromatin

Silencing has long been recognized as a manifestation of the state of heterochromatinization of the affected gene(s). The initial suggestion for this arose from the study of ''position effect variegation'' (PEV) in *Drosophila*, which occurs when a euchromatic gene is placed adjacent to either centromeric heterochromatin or a telomeric domain.[9] In such circumstances, expression of the locus is said to variegate, namely it is active in some cells and silent in others. Silencing is a stable phenomenon in that it can be propagated over cell generations and even through meiosis in some instances. Recent studies in both yeast and higher eukaryotic cells now show however that, in seeming contrast to the stability of the silenced state, heterochromatin itself is highly dynamic, sustaining turnover rates of its components that, while significantly lower than euchromatin, are nevertheless very rapid, in the order of one minute for HP1.[10–12] Accordingly, heterochromatin is no longer viewed as a package of inert material, and recent studies have demonstrated that the condensed chromatin

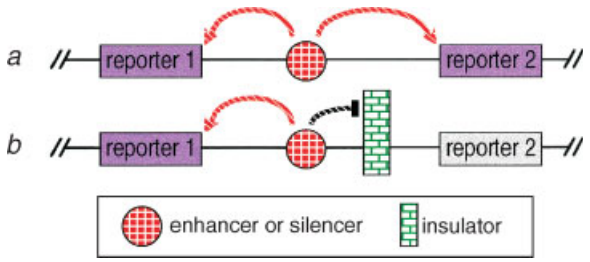

Figure 1. Insulators are chromosomal elements that can shelter genes from the effects of silencers and enhancers. Genes are usually subject to regulation by long-distance-acting elements, which either enhance (enhancers) or repress expression (silencers). **a:** Such elements can be shared, for example by two reporter genes. **b:** However, interposition of an insulator will specifically block communication between the enhancer or silencer and the downstream gene, without affecting the capacity of the enhancer or silencer to regulate the second reporter gene.

domains of higher eukaryotic cells are readily accessible to large macromolecules.[13,14] Finally, the variegating aspect associated with silencing reveals that a probabilistic aspect has to be taken into account.[15] This notion of stochasticity is now widely accepted to be responsible for the inherent variability observed in many cellular processes.[16–18]

The current vision of heterochromatin assembly and maintenance is largely derived from the mass action model initially proposed by Tartof and colleagues on purely genetic grounds.[19,20] Heterochromatin can thus be defined as a complex of DNA and a set of chromosomal proteins that comprises both (1) proteins that are thought to confer on heterochromatin its specific physical features, namely condensation and regular positioning of nucleosomes, and (2) enzymes responsible for the covalent modifications that mark it. This assembly is ruled by a self-reinforcing network of mutual interactions and is therefore expected to be strongly cooperative. The fact that assembly predominates over disassembly, thus affording stability, is also the consequence of the non-homogenous repartition of heterochromatin components in the nucleus. Indeed, heterochromatin regions tend to aggregate, creating reservoir compartments that may also play an active role in the silencing phenomenon.[20–23] The assembly is initiated at one or several initiation points (silencer/protosilencer)[3,24] and the complex may then spread in *cis* down the chromatin fiber, as initially suggested by decreasing frequencies of PEV for genes as a function of distance from heterochromatin.[9] However, examples of apparent discontinuity in the spreading of silencing rather imply that pseudo-propagation involving the looping out of unaffected domains, possibly aided by protosilencers and locally ruled by mass-action law, actually occurs.[3,20,25,26] Continuous propagation, in contrast, must be envisaged as a default mode that occurs when affected chromosomal regions are immediate neighbors. In sum, propagation of heterochromatin from a focal point of assembly can be compared to a dynamic wave signal emanating from a generator and vanishing at a distance, and possibly skipping some chromatin domains that are envisioned to loop out. Along with this dynamic view, the vocabulary describing silencing efficiency must now evolve to incorporate kinetic and probabilistic parameters.

10 years ago, variegation was proposed to arise through the competition between the transcription process and the packing of chromatin into a repressive structure.[27] It can now be better envisioned from a probabilistic point of view in the context of the inherent "breathing" of heterochromatin as a pseudo-stable equilibrium between two states of chromatin (see below).

Conversely, wherever highly efficient silencing is required, genomes appear to have evolved silencing elements that surround the locus plus sometimes internal, protosilencer elements that serve the function of a silencing relay.[3] The apparent cooperation at a distance of silencers that may be considered, to a first approximation, to operate independently, may simply involve the combination of probabilities that the affected gene(s) will be silent. For instance, silencing of the silent mating type cassette *HML* in *S. cerevisiae* is guaranteed by the *HML E* and *HML I* silencers that bracket the locus, together with a central elementary protosilencer. Silencing emanating from the telomere repeats is reinforced and stabilized by middle-repetitive subtelomeric elements at natural chromosome ends in *S. cerevisiae* and also probably in *S. pombe*.[25,28,29] The classical telomere fragmentation techniques that allow natural telomeric regions to be replaced by artificial ones through homologous recombination combined with the seeding of a new telomere, usually eliminate these subtelomeric elements, yielding an unidirectional silencing system that displays variegation[30,31] (see Fig. 3a).

Reconciliating the barrier and active models of insulation into the antisilencing principle

The current operational definition of insulators involves an important positional constraint: the insulator should be active in protecting a domain from silencing only when it is interposed between the silencer and this domain. In particular, the insulator must leave intact the capacity of the silencer to repress a gene situated in another segment than the one beyond the insulator. Hence the classical representation of the insulator as a wall against which heterochromatin propagation comes to a halt (Fig. 1). Essentially, two alternative molecular mechanisms can be envisioned (Fig. 2). (1) In the passive, barrier model, a large and stable DNA-bound complex forms a physical block to a propagating chromatin structure (Fig. 2a). Chromatin encroaching onto a stable nuclear substructure, such as nuclear membrane or an internal matrix/scaffold, may be envisaged as a variant scenario. Alternatively, insulators

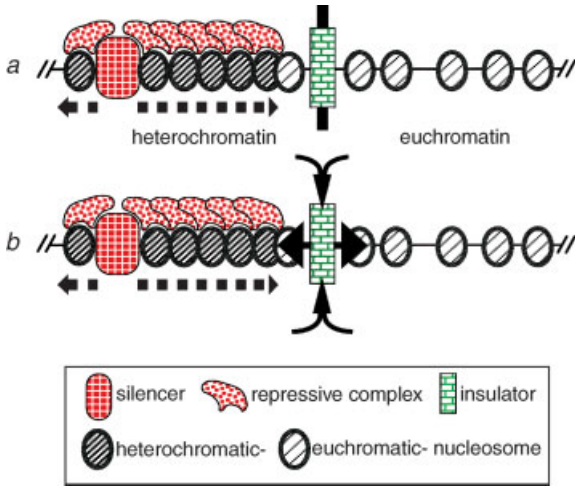

Figure 2. Models of molecular scenarios for insulator effect. Heterochromatin assembles from a silencer, can propagate up to an insulator, and is dynamically maintained on this chromatin segment (dotted arrow). Nucleosomes bear specific heterochromatic marks and are closely spaced, by contrast with euchromatin regions at which nucleosomes are more irregularly spaced and bear euchromatic mark. **a:** In the passive, road-block model, a large and stable DNA-bound complex forms a physical block to the propagating heterochromatin, or serves as a "pit of potential" that absorbs incoming topological constraints. **b:** In the active model, the insulator serves as an entry site for enzymatic activities that either covalently modify chromatin or enhance nucleosome mobility around the insulator to create a less conducive substrate for heterochromatin assembly. It should be noted that, in both models, destabilization of heterochromatin dynamic assembly at the insulator itself is predicted to make propagation up to the insulator less likely, as indicated by a euchromatic nucleosome on the "silencer side" of the insulator. It is also possible that specific nucleosome marking may occur at the insulator that is not characteristic of either the heterochromatic or the euchromatic nucleosome.

may also be seen as simple topological barriers that would arrest the propagation of topological changes associated with long-range chromatin folding or unfolding.[32,33] (2) In the active, antisilencing model (Fig. 2b), the insulator directly interferes with the mechanism of heterochromatin formation or maintenance by serving as an entry site for enzymatic activities that either covalently modify chromatin, or modulate nucleosome properties, or affect DNA topology.

Support for each of these two models was recently strengthened through the identification of factors that can recapitulate insulator activity when tethered to DNA. A variety of transcription activation domains, as well as the histone acetyltransferases Sas2, Esa1 and Gcn5, were shown to operate as autonomous insulators[34–36] and also to cooperate with a distant, natural subtelomeric insulator in defining an insulated domain.[34] Strong levels of histone acetylation

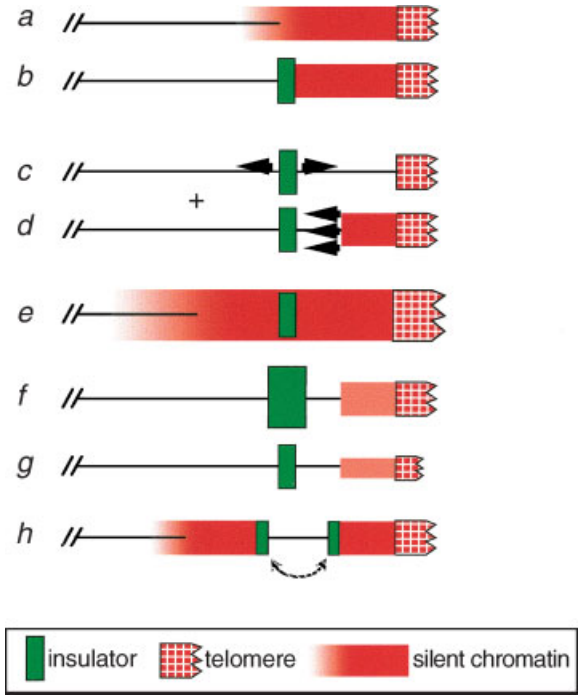

Figure 3. Quantitative changes in insulator effect may translate into seemingly qualitative changes. **a:** In *S. cerevisiae*, telomeres function as silencers from which heterochromatin emanates and propagates into subtelomeric areas. **b:** Insertion of an insulator sequence protects downtream regions against silencing, and therefore seems to act as a barrier to silent chromatin propagation. However, this barrier effect may rather be envisioned in a dynamic view as the equilibrated sum of two extreme situations: **c:** the full elimination by the insulator of any adjacent heterochromatin structure through a cascade, all-or-none destabilization effect; **d:** the continuous re-invasion of the chromatin fibre by heterochromatin emanating from the silencer. Let us now consider how the system evolves when low-, middle-, or high-strength insulators and silencers are combined, as represented by symbols of increasing size. **e:** Increasing the silencer's potency overrides the insulator effect by making (**d**) predominant over (**c**). **f:** Conversely, increasing the insulator potency, for example through the concatemerization of core sequences, seemingly repels silent chromatin by making (**c**) predominant over (**d**). **g:** A similar, apparent bi-directional anti-silencing effect can be observed with a middle-strength insulator if the silencer's potency is decreased. Two distant insulators define an insulated domain that is protected from the influence of surrounding chromatin, and they do so in a synergistic fashion. **h:** In other words, even low-strength insulators can thus cooperate efficiently in protecting from a middle-strength silencer. **i:** Finally, a single low-strength insulator is predicted to protect from a low-strength silencer.

are indeed found at natural insulators.[37,38] Insulator activity was further found to correlate with chromatin remodeling either due to direct interaction with histones or through the recruitment of chromatin remodeling/modifying machineries.[39,40]

In addition, screening *S. cerevisiae* genome for elements endowed with insulator activity was found to retrieve the upstream activating sequences (UAS) of many genes, and highlighted the action of general regulatory factors Rap1p, Abf1p and Reb1p as potent effectors of the insulation effect.[35,41,42] These findings further imply that promoters not only serve to regulate the expression of companion ORFs but also partition the genome into functionally independent domains. On the other hand, physically tethering each side of a short chromatin segment to the inner basket of the nuclear pore complex appears sufficient for its protection against neighboring silencers,[43] which rather suggests a link between insulation and the establishment of a topologically independent domain. Further support for the latter model comes from an in vitro reconstituted system that formally recapitulates enhancer–insulator interactions.[44] However, the ability of some insulators to impart a *cis*-requirement on enhancer–promoter interactions and block enhancer action in *trans* is clearly at odds with a simple topological barrier model of insulation action.[45]

It should be noted at this point, first, that the "passive" and the "active" models are less antinomic than first thought. They may even be seen as the two ends of a spectrum, in which many transcription factors partition with an unsoluble nuclear fraction and thus concomitantly play an active, enzymatic role together with a more passive role anchoring chromatin to a stable nuclear structure. Secondly, the barrier image of insulators (Fig. 3a,b) is likely simplistic, as this effect may rather be envisioned in a dynamic view as the sum of two extreme, theoretical situations: (i) the full elimination by the insulator of any heterochromatin structure through a cascade destabilization effect, as a counterpart to the strongly cooperative interactions between heterochromatin components (Fig. 3c); and (ii) the continuous re-invasion of the chromatin fiber by heterochromatin emanating from the silencer, all the more rapid or probable as the silencer is a strong one (Fig. 3d). Quantitatively modifying the relative potency of either of these two players changes the system and results in an apparent modification of the nature of the insulator effect. Thus, the protective capacity of a natural, middle-strength yeast insulator can be overridden by increasing the potency of silencing (Fig. 3e).[34,36] Conversely, insulators were observed to display bi-directional antisilencing capacity in two types of instances: (1) using potent insulators (Fig. 3f), or (2) in a favorable genetic context in which telomere position effect is known to be less efficient (Fig. 3g)[34,36,42] (G.F. and E.G., unpublished data). Strikingly similar conclusions regarding quantitative aspects of insulator function were drawn in enhancer-blocking assays in *Drosophila*, through varying the insulator anatomy and the enhancer strength[46] or the compatibility between the enhancer and the promoter of the reporter gene.[47]

Furthermore, two insulators that bracket a gene have synergistic effects in protecting that gene against the influence

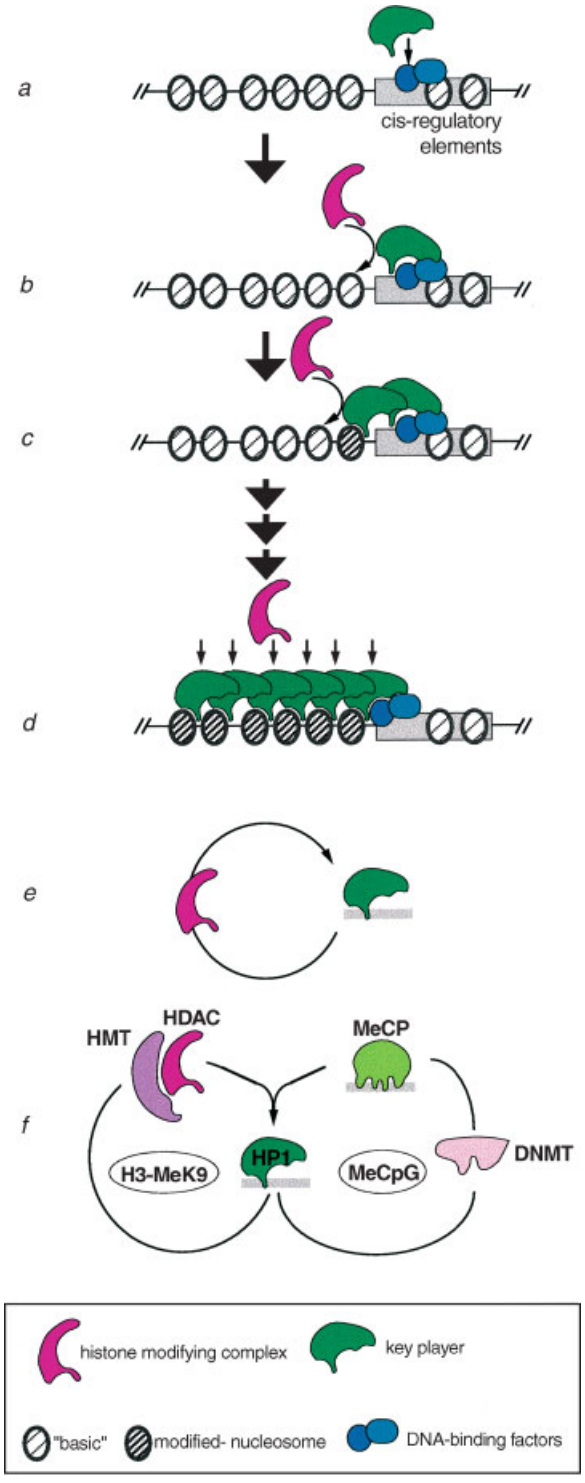

Figure 4.

of neighboring silencer/enhancer, as observed in a variety of systems (see for instance[2,25,48]) and may in addition facilitate the propagation of the silencer/enhancer effect beyond the insulated domain[34,49,50] (Fig. 3h). This synergy may rely on physical pairing, at least in some instances.[51,52] Thus, two relatively weak insulators may efficiently protect a gene when they surround it.

Altogether, the ability of an insulator to behave as a true barrier or a bi-directional anti-silencer does not seem to correlate with the underlying molecular activities but relies on a particular balance between opposing forces. A recent set of observations that were interpreted to suggest distinct mechanisms underlying barrier- and antisilencing-type behaviors[53] is actually most plausibly explained by a quantitative difference in the potency of the insulators chosen to illustrate each model.

We propose that, in essence, an insulator is an antisilencing element. Much the same conclusion was inferred for *cis*-regulatory elements such as enhancers and locus control regions (LCR) that can oppose a repressive status imposed for instance on randomly integrated transgenes in higher eukaryotic cells.[54–57] It is therefore not surprising to find the same molecular players as key effectors at the heart of both types of phenomena. In particular, dissection of insulators, enhancers, locus control regions (LCR), and promoters upstream activating sequences (UAS) all pointed to so-called ''transcription factors'' as active participants in their respective behaviors.

Insulators and fuzzy boundaries

The concept of insulator elements arose as an explanation for the existence of independently regulated domains along eukaryotic genomes, and was perhaps rooted in part in an overly simple view of genomes depicted as one-dimensional entities. On the one hand, very few elements that seem to act solely as insulators have been identified to date.[57] On the other hand, transcription activating elements are an invariant feature of active genes. Thus, elements that behave exclusively as chromatin insulators may be less common than previously thought and many insulator elements may have evolved as elements in highly specialized contexts at loci displaying a high density of coding or regulatory information, at which domain limits must be set in a very precise position. This may be the case for example at the β-globin locus, which is closely flanked by genes displaying markedly different patterns of expression as well as a heterochromatic region,[58]

Figure 4. Positive feedback loops in the establishment and maintenance of chromatin states. Euchromatin and heterochromatin organizations both critically rely on key factors that share common features: (1) they have choice between at least two modes for interacting with chromatin, being recruited either through partners bound at specific DNA motifs such as transcription factors or through the recognition of distributed markers such as histone tags (**a,c**); (2) they can in turn amplify this specific marking each proper to euchromatin or heterochromatin through the recruitment of more of the enzyme responsible for it (**b**). Several rounds of such an iterative process eventually result in the pseudo-propagation of the chromatin state defined by its components and histone markers (**d**). The self-reinforcing network of functional interactions between the key player incorporating with chromatin (grey bar) and the histone-modifying complex can be envisioned as a positive feedback loop (**e**), which sustains propagation and confer stability in a context of dynamic assembly. Of note, these targeted appositions of chromatin markers occur over a background of global modifications, in particular for acetylation and deacetylation[95–97] that can, in some instances, allow a rapid return to the initial state when targeting is removed. This model is particularly well illustrated in the yeast *S. cerevisiae*, in which the Sir3/4 proteins are recruited to silencers through interaction with elementary protosilencer binding factors such as Rap1p. These recruit the Sir2 histone deacetylase, which converts neighboring nucleosomes into substrates for Sir3/4 binding, by removing acetyl radicals from Lys16 residues in H4 N-terminal tail. Recent dissection of the mechanisms governing heterochromatin assembly in higher eukaryotes unveiled two intertwined positive feedback loops centred on H3-K9 methylation and methylation of CpG residues, respectively, which synergize in the maintenance of a stable structure (**f**). This was recently confirmed in an elegant model of regulated recruitment of HP1 to a euchromatic gene (Ref. 65 and references therein). HMT, K9 histone methyltransferases; HDAC, histone deacetylases; DNMT, DNA methyltransferases; MeCP, MeCP1 and MeCP2 complexes. In euchromatin, transcription factors at *cis*-regulatory elements such as promoters recruit chromatin remodeling complexes and histone acetyltransferases, and the transcription machinery itself carries such activities.[96] Acetylated histones are recognition sites for bromodomain-containing proteins such as Bdf1 in *S. cerevisiae*, which is postulated to recruit more of the Sas2 and Esa 1 acetyltransferases.[6] Bromodomains are also found in subunits of several chromatin-remodeling complexes and the general transcription factor TFIID, which altogether provides strong links between transcription activation and the amplification of histone acetylation and may account for the fact that widely extended areas of histone acetylation have been found at some loci. Two other pathways contribute to euchromatin identity in a partially redundant manner, and combinations of alterations in any two pathways among the three may dramatically affect transcription and lead to synthetic lethality as reported in *S. cerevisiae*.[98,99] In transcription conducive chromatin, first, conventionnal histone H2A is commonly replaced by the universally conserved histone variant Htz1 (H2A.Z in higher eukaryotes), and, second, histone H2B may be found ubiquitinated at residue K123 (the equivalent of human K120).[4] Ubiquitination of H2B results from the ubiquitin E3 ligase activity of the Bre1–Lge1 complex in combination with Rad6,[99] and, although, it affects only a minority of nucleosomes (about 5%), it acts as a master control switch inducing the widespread methylation of H3–K79 by the HMT Dot1. In *S. cerevisiae*, about 90% of all H3 is methylated on K79 and is presumably found in every nucleosome in regions that are not subject to silencing. H3–K79 methylation in turn allows further methylation of H3 at K4 by Set1-related HMTs, as found in the coding region of active genes and which is thought to facilitate transcription by protecting active coding regions from deacetylation. For a more comprehensive review of histone modifications reported so far and the different levels of positive and negative cross-talks, the reader is referred to a recent review.[4]

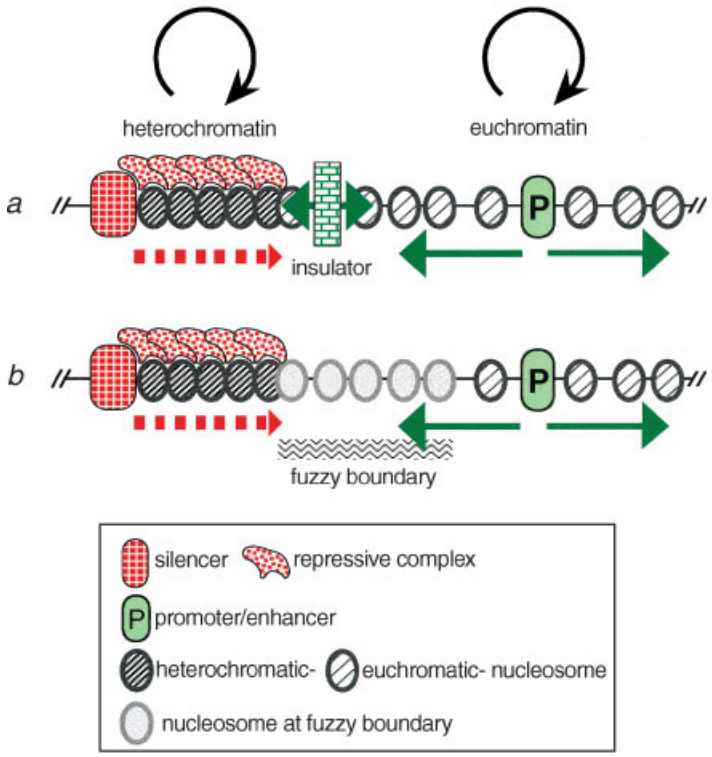

Figure 5. Insulators and fuzzy boundaries. **a:** An insulator sequence establishes the border between a heterochromatic and a euchromatic domain. These are each ruled by self-reinforcing networks of interactions (looped arrows). **b:** Whenever heterochromatin and euchromatin directly confront, an intermediary area at which either heterochromatic or euchromatic identity can be found establishes a "fuzzy boundary". Nucleosomes at fuzzy boundary therefore display either heterochromatic or euchromatic marking according to temporal and spatial conditions, and therefore the fuzzy boundary itself may be an area of relative instability. In this latter situation, the anti-silencing structure that acts to delimit the heterochromatic domain is not a bona-fide insulator but is the focus of events converging at the promoter of the euchromatic gene with long-range effect sustaining euchromatin propagation (green arrows). Conversely, the silencer can be considered to limit euchromatin propagation through promoting heterochromatin assembly (red arrow).

and between the neighboring TCRα and TCRδ gene segments.[59] However, the absence of any effect of deleting the corresponding insulators suggests that they are redundant with other regulatory elements at these loci.[60] Insulators also emerge as a recurrent feature of imprinted loci, at which they exhibit parent-of-origin differential activity and help orchestrate complex regulatory interplays.[61–63]

How then are frontiers between chromatin domains established in the absence of insulators? A competition model between two incompatible states of chromatin for the same substrates was early proposed to account for the fascinating symmetry of effects on silencing patterns observed upon dosage variation in either anti-silencing transcription factors or in heterochromatin components (reviewed in Ref. 64). Recent studies now provide compelling evidence that: (1) the establishment and maintenance of both euchromatin and heterochromatin are governed by similar general rules, involving self-reinforcing networks of interactions between specific markers, and (2) euchromatin and heterochromatin each harbors factors that antagonize the switch to the opposite chromatin state (see Fig. 4). The establishment of a particular chromatin state depends on focal points of assembly that specify the identity as either euchromatin or heterochromatin and, depending on the particular system, may not be required in the propagation/maintenance steps that follow.[10,24,65–67] Importantly, one single element may alternately function in specifying either euchromatin identity or heterochromatin identity in distinct contexts, for instance according to developmental chronology or chromosomal position.[3]

The strikingly symmetrical rules that govern heterochromatin and euchromatin formation highlight common substrates for which competition may occur as well as strictly different, distinguishing features (see Fig. 4). For instances, in *S. cerevisiae*, the bromodomain-containing Bdf1 protein can compete with the Sir2 deacetylase for binding to acetylated H4;[6] comparably, histone H3 methylated at residue K79 or the replacement of H2A by Htz1 in a nucleosome can prevent silent chromatin from forming.[68,69] These results suggest that boundaries between two types of chromatin domain may become established simply by inhibition/exclusion principles. The positioning of such a frontier, however, would be expected to be relatively unprecise and highly sensitive to changes in component dosage. We therefore propose to call this type of frontier "fuzzy boundaries", in contrast to "insulators", which specify fixed or immobile DNA elements (Fig. 5). Fuzzy boundaries may be stable enough to allow some variegation, owing to positive feedback loops operating on each side (Fig. 4). It is more likely, however, that the genuine border is permanently shifted from one position to the other within a window that actually defines the fuzzy boundary itself and, therefore, that the expression state of a neighboring gene frequently changes.

Five recent studies[6,70–73] indeed demonstrate that compromising any one of three pathways participating to euchromatin maintenance (see Fig. 4) in *S. cerevisiae* triggers the spreading of silent chromatin into neighboring gene domains, which ordinarily are not silenced in a wild-type background. In particular, because the silencing proteins are in limiting supply within the nucleus, when silencing proteins bind promiscuously and start repressing genes elsewhere in the genome, they appear to be titrated away from the silent loci where they normally reside. This effect of ''communicating vessels'' results eventually in the mild repression both of genes that are usually strongly repressed and of genes in adjacent domains. Conversely, deletion of Sir2 causes the invasion of normally silent domains by euchromatin.[70]

Altogether, it appears that the frontier between heterochromatin and euchromatin is usually set by confrontation at fuzzy boundaries, close to the ''weak domain model'' of genome organization proposed by N. Dillon and P. Sabbattini:[57] euchromatin helps to define silent domains by limiting silent chromatin propagation, and conversely silencers limit euchromatin propagation and allow decoupling in the regulation of adjacent chromosome domains.[42]

A unified vision of transcription factor function in long-range regulatory circuits

Enhancers/activators exert a complex effect that includes insulation. For instance, Sutter and colleagues[56] have shown that position effects involving silencing can be suppressed by the MTF transcription factor. Significantly, at one of the genomic sites studied, the activator was required only transiently: silencing continued to be suppressed after withdrawal of the activator. However, in the absence of the activator, the expression level promptly fell to a lower, uninduced level. This implies that the effect of MTF on silencing and transcription rate are mediated by different mechanisms, and that silencing can be suppressed by some persistent epigenetic modification. Therefore, insulation as assayed by suppression of position effects emerges as one compelling facet of transcriptional activators.

An elegant type of system was recently engineered to investigate transcription factors functions and, more specifically, to directly visualize their effect on chromatin folding. This technology entails tethering of a transcription activation domain to hundreds of tandem binding sites inserted at a controled chromosomal location. The structural changes that are observed are presumed to represent an amplification of similar structural perturbations produced over much smaller neighborhoods surrounding endogenous promoters. Thus, the targeting of BRCA1, E2F1, p53, the glucocorticoid receptor (GR) or the estrogen receptor (ER) to the same heterochromatic *lac* operator array or comparable systems results in a similar large-scale chromatin unfolding, but each has some specific features.[74–76] Chromatin unfolding is frequently accompanied by histone acetylation. In addition, the process of transcription by itself may in some cases contribute to the phenomenon. Chromatin unfolding was shown to coincide with the sequential recruitment of a number of remodeling as well as histone-modifying activities.[77] Interestingly, markedly varying kinetics of recruitment were observed for individual members of these large complexes, with the catalytic subunits of remodeling machineries recruited first. Other experimental systems have highlighted the importance of a nuclear mobility parameter, suggesting a ''dynamic organizer of chromatin'' function for transcription factors. This appealing, if as yet poorly substantiated notion, proposes that one critical function of transcription factors is to help shift a locus to a defined subnuclear location.[78]

This unifying view of transcription factors function is further supported by the amazing level of redundancy between *cis*-regulatory elements for chromatin opening at some loci, which can be so marked as to render ancillary a powerful element such as an LCR.[79] Two recent studies give definitive evidence for a looping model of long-distance promoter activation and further suggest that all the regulatory elements that functionally cooperate in the regulation of the β-globin locus actually cluster to form an ''active chromatin hub''.[80,81] In this view, the larger the number of these activating elements and the higher the affinity between them, which depends on the transcription factors bound at each and the associated cofactors, the more likely the formation of the chromatin hub. The latter then conveys engagement of the locus in a transcription-conducive state. It is conceivable that most higher eukaryotic loci have evolved many more *cis*-regulatory elements than seems necessary, in order to guarantee, in a highly redundant manner, counteraction of the potential repressive effects that may arise from the burden of silent chromatin harbored by differentiated cells.

A now widely exploited approach to evaluate the importance of a factor for the activity of a LCR-like element is based on selecting a transgenic mouse line in which this LCR coupled to a reporter gene has inserted into a strongly repressive environment such as pericentromeric chromatin. Usually the LCR gives full expression that is not subject to position-effect variegation, whereas deletion of important portions thereof results in a variegated expression pattern in transgenes located pericentromerically, which is sensitive to dosage of heterochromatin components such as HP1. Reducing the dosage of key transcription factors through a cross with a null mouse line should similarly induce silencing of the full-length LCR. Whole loci have now been dissected using such a strategy (see for instance[82]).

In view of the approximate equivalence of the different types of *cis*-regulatory elements in this model with regard to so many parameters, one may ask how it is possible that they behave differently enough in functional assays so that each of them was tagged with a specific name? Amongst these, however, it is only insulators, according to their definition, that

are expected not to have direct effects on transcription initiation and only to affect the competency of the locus with regard to transcription. In vertebrates, the highly conserved CTCF protein has been implicated at many different loci, and YY-1 was recently identified at an insulator within the murine *Peg3* gene.[63] In the yeast *S. cerevisiae*, Abf1p, Reb1p, and Rap1p emerge as potent effectors of insulation at natural subtelomeric insulators and in the UAS of a number of promoters that behave as insulators.[41,42] Strikingly, these three factors are known as the General Regulatory Factors (GRFs) and share the following characteristics. GRFs are abundant and essential. One or several binding sites for at least one of these factors are found in a very large number of *S. cerevisiae* promoters. GRFs behave as obligate synergizers: their binding motifs usually have little intrinsic regulatory activity, but instead can potentiate the effect of neighboring regulatory sites. GRFs share a common mechanism of action as the binding site for one GRF within a promoter can be exchanged with another and, even more compellingly, protein domains can be swapped among GRFs without loss of function. Finally, GRFs are highly multifunctional proteins: for instance, silencers at the mating type cassettes are built from binding sites for GRFs and, in particular, Rap1p can recruit Sir3 and Sir4 proteins through direct interaction. Remarkably, both CTCF and YY-1 display characteristic GRF features. In particular, YY-1 can nucleate PcG proteins-mediated repression when expressed in *Drosophila*[83] and was also implicated in the reiterated targeting of another repressive complex at D4Z4 repeats in human DNA sequences.[84] However, YY-1 has also been connected to gene activation through the targetting of a myriad of partners (Ref. 85 and references therein), which holds true for CTCF too.[86] Another candidate GRF with demonstrated insulating capacity is the *Drosophila* GAGA factor,[87,88] which binds to a very large number of promoters and has been further implicated in the assembly of PcG repressive complexes at polycomb response elements (PREs). What, then, is the mechanism through which GRFs operate? They act most likely as specialized ''landing platforms'' for a variety of chromatin-remodeling machineries. Such a function and other operational GRF features were recently also unveiled for two factors playing pivotal roles in the lymphoid system development, Ikaros and SATB1. The latter, therefore, emerge as prime candidates for new insulating factors.[82,89,90] A ''genome organizer'' capability is another suspected function of GRFs but it is proving more difficult to track.[42] It has nevertheless been shown for the Su(Hw) and Mod(mdg)4 factors acting at the *gypsy* insulator in *Drosophila*, which coalesce into a small number of insulator bodies at the nuclear periphery.[91] A similar observation was recently reported for CTCF which appears to tether sequences to the nucleolus.[100]

More ''classical'' transcription factors have also been reported to interact with remodeling machineries, and the major difference with GRFs may reside in the overall efficiency of the process or in the more restricted panel of interacting partners. Another key difference lies in the capacity of classical transcription factors to interact with components of the basal transcription machineries, and hence to affect transcription initiation. They may also potentially affect a variety of downstream steps (Ref. 92 and references therein). Along these lines, the major, specific effect of the β-globin LCR at its native locus was recently shown to be an enhancement of the transition from transcription initiation to elongation.[93]

Conclusions

Our current vision of genomic organization is that the prevalent mechanism of transcriptional coregulation operates via extensive chromatin domains encompassing multiple genes. Accordingly, such domains must have borders but these may be of two distinct types. First, these may be insulators, which guarantee that transition from one domain to the next occurs at a fixed position and which counteracts regulatory communication between adjacent domains. Alternatively, when gene domains are sufficiently far apart, signals may simply vanish at ''fuzzy'' boundaries. Whatever their nature, these borders as well as the chromatin that converges on them must be envisaged in a highly dynamic mode. So-called transcription factors were found to play key roles both in the organization of domains poised for activation and in the definition of their borders, as also previously highlighted.[57] They are likely to both serve a platform function, for the recruitment of chromatin remodeling machineries, and perform a role as dynamic genome organizer, together with a more passive role related to their participation in a nuclear scaffold that may contain or ''absorb'' topological constraints. General regulatory factors such as Reb1, Abf1 and Rap1 in *S. cerevisiae* and CTCF, GAGA and YY1 in higher eukaryotes appears to play universal roles in chromatin higher-order organization and have set the stage for the discovery of new players and underlying molecular mechanisms in insulation. The existence of overlapping gene domains further hints at other mechanisms such as the specificity of enhancer–promoter interactions,[57] and the potential selectivity of insulator action, as important determinants of domain organization. Finally, a challenging view proposes that domain autonomy may be an optional feature for many genes.[94]

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