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Review

Boundary elements and nuclear organization

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Abstract

Functional compartmentalization of eukaryotic genomes is thought to be necessary for the proper regulation of gene expression. Chromatin insulators or boundary elements have been implicated in the establishment of this compartmentalization, as they may be involved in creating independent chromatin domains. Recent advances in understanding the mechanisms of insulator function suggest a role for boundary elements in determining transcriptional identity of chromatin and in organizing chromatin into structural compartments within the nucleus. Insulators may thus be involved in setting up topological chromatin domains associated with particular transcriptional states.

Keywords: Chromatin; Transcription; Nucleus; Insulator

1. Introduction

The connection between the organization of chromatin inside the nucleus and regulation of gene expression has emerged as one of the most intriguing questions of modern biology. Chromatin has long been thought to be organized in a non-random manner inside the eukaryotic nucleus, and the factors that control such an organization and the functional consequences of it remain under intense investigation. Perhaps the best-known example of a non-random chromatin organization is its subdivision into two physically and functionally distinct compartments—euchromatin and heterochromatin (reviewed in Elgin and Grewal, 2003; Grewal and Moazed, 2003). Cytologically distinguishable since the early days of microscopy, these compartments display differential physical and molecular marks, which often translate into dramatic consequences for the control of various cellular processes, most notably, the regulation of transcriptional activity. Thus, heterochromatin is thought to represent a highly condensed state of chromatin, restrictive to active transcription and, consequently, home to elements that need to be silenced. On the other hand, euchromatin is defined as less condensed chromatin that replicates early in S phase and where most of gene expression is thought to take place during the rest of the interphase. The phenomenon of position effect variegation (PEV), originally discovered in fruit flies, is thought to reflect this kind of partitioning. PEV, which generally involves dependence of a gene's activity upon local chromatin environment, has been observed in a variety of organisms and its existence argues for the presence of repressed versus active domains of both gene expression and chromatin structure. The persistence of such separate chromatin states inside the nucleus implies the existence of regulatory elements that must be able to enforce the division and functional independence of distinct chromatin domains as well as prevent encroachment of differential transcriptional states onto each other.

The current view of global gene expression also postulates the presence of functional chromatin domains, which represent units of independently occurring transcriptional activity. Thus, superimposed on the euchromatin versus heterochromatin level of organization, eukaryotic genomes are thought to be compartmentalized into functional domains of gene expression, which need to maintain independence from surrounding units to establish a proper differentiated or developmental state (Dillon and Sabbattini, 2000). The lack of promiscuity in enhancer-driven activation of genes, given the large distances separating promoters from their regulatory elements and the physical proximity of neighboring genes, argues for the presence of such autonomous domains. Furthermore, a variety of cytological and molecular evidence

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supports the existence of structural partitioning of the genome into topological domains, which may correlate with functional domains of transcription. The delineation of the *Drosophila* polytene chromosomes into discreet bands of condensed versus decondensed chromatin and the correspondence of such bands to regions of differential transcriptional activity was one of the earliest clues (Tissieres et al., 1974; Nowak and Corces, 2000). Other early biochemical experiments, such as high salt extraction of chromatin-associated factors, resulted in visually discernable loops of nuclear DNA, attached at specific sites to the remaining nuclear matrix scaffold (Pederson, 2000; Nickerson, 2001). Such topological organization of nuclear DNA was postulated to represent structural partitioning of the genome, which may aid in the establishment of functional independence of gene expression. This notion is supported by the fact that DNA sequences that form the basis of attachment to the nuclear matrix, termed MARs (matrix attachment regions), appear to possess gene regulatory properties (Forrester et al., 1999; Fernandez et al., 2001). Recently, physical clustering of co-expressed genes was found prevalent throughout eukaryotic genomes, including yeast, *Drosophila* and humans, reinforcing the notion of transcriptional compartmentalization through physical separation (Cohen et al., 2000; Caron et al., 2001; Spellman and Rubin, 2002).

Thus, in addition to the maintenance of distinct euchromatic and heterochromatic states, cross talk between autonomous domains of gene expression must also be prevented. In either case, functional independence of a chromatin domain can be achieved in a variety of ways, including establishment of a physical block to *cis* spreading of a chromatin state, recruitment of specific activities to a limited locus, or targeting to a subnuclear compartment associated with either silencing or activation. All of these mechanisms may involve the action of specialized regulatory elements, which phenotypically would behave as chromatin domain boundaries. Such elements, termed boundary elements or insulators, have been characterized in a variety of organisms and may play an important role in the organization of independent chromatin domains inside the nucleus.

Boundary elements or insulators (used interchangeably in this review) are defined by their ability to ensure independence of gene expression by protecting genes from surrounding signals. Insulators have been characterized by two experimentally defined properties involving altered gene expression. First, when positioned between an enhancer and a promoter, an insulator is capable of disrupting enhancer–promoter interactions, without rendering the enhancer inactive (as it is still capable of activating a "non-insulated" promoter) (Geyer and Corces, 1992; Kellum and Schedl, 1992). This property of insulators has been termed enhancer blocking. Second, when flanking a transgene, insulators are able to shield the transgene from position effects, particularly from the repressive effects of heterochromatin, allowing for position-independent gene expression. The second property of boundary elements is often referred to as barrier activity, since it involves blocking the spread of one chromatin state into another (Sun and Elgin, 1999). It should be further noted that some characterized boundary elements, particularly those found in yeast, act primarily as barriers to heterochromatin. Other boundary elements possess both properties, enhancer-blocking and barrier, and in some cases, these activities have been found to be physically linked but separable (for a summary of identified insulator properties, see West et al., 2002). It is particularly interesting that boundary elements possessing both properties are able to exert two seemingly opposing effects on transcription—their barrier function prevents transcriptional repression, while the enhancer-blocking property interferes with transcriptional activation. The two defining properties of insulators originate from the experimental assays used to identify and characterize such elements, yet these properties should arise from the endogenous functions insulators play in the regulation of gene expression. Understanding the mechanisms by which insulators are able to exert enhancer-blocking or barrier effects will undoubtedly promote further understanding of their normal roles in chromatin activity.

2. Mechanisms of insulator function

Both of the defining properties of insulators can be explained by a view in which insulators function as boundaries of chromatin domains, possibly imposing a change in chromatin organization in the created compartments. The precise mechanism by which insulators are able to demarcate chromatin domains remains unknown, but several possibilities have been suggested. Based on the existing evidence for two types of functional association of boundary elements, proposed explanations of insulator action can be grouped into two broad categories—those linking insulators to transcriptional regulation and those associating insulators with structural chromatin organization. It should also be noted that, in many cases, the barrier function of boundary elements may involve mechanisms distinct from those underlying the enhancer-blocking activity. Yet, recent findings have pointed to some functional parallels between the two insulator properties, as discussed below.

The first set of models concentrates on the emerging connection between boundary elements and the transcriptional activation machinery (Fig. 1). Thus, the barrier function is explained by the ability of insulators to block the spread of repressive chromatin by recruiting histone-modifying or gene-activating factors and possibly by serving as sites of nucleation for the permissive chromatin state (Donze and Kamakaka, 2002; Kuhn and Geyer, 2003). In this view, insulators behave as active boundaries by imposing a unidirectional state of transcriptional activation on surrounding chromatin or by counteracting the incoming repressive chromatin effects (Donze and Kamakaka, 2002). The involvement of boundaries in transcriptional activation works well to explain their function as heterochromatin barriers,

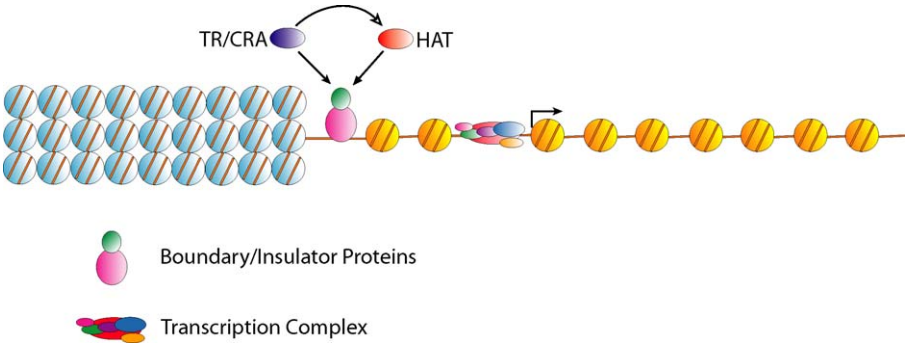

Fig. 1. Insulators as barriers to transcriptional repression. Boundary elements may block the spread of repressive heterochromatin by recruiting histone-modifying enzymes (such as HATs) or transcriptional regulators (TR) or chromatin remodeling activities (CRA) to confer a transcriptionally permissive state on a chromatin domain. Blue spheres represent nucleosomes in condensed chromatin containing histone modifications characteristic of heterochromatin. Yellow spheres represent nucleosomes in open chromatin with histone modifications characteristic of active chromatin.

and correlates with at least one model for their enhancer-blocking activity. Termed the "promoter decoy model", it suggests that insulators are able to interact with enhancer-bound factors, involved in gene activation, and in this manner, prevent the proper interaction of enhancer with its target promoter (Geyer and Clark, 2002). In the case of either the barrier function or the enhancer-blocking activity, this view of insulator function highlights the documented interaction between boundary elements and transcriptional regulators.

Other proposed models focus on the role of insulators in directing the physical organization of chromatin into separable and independent structural domains, interaction between which is somehow prevented (Labrador and Corces, 2002; West et al., 2002). Crucial to this idea is the assumption that insulators interact with each other or with a nuclear attachment substrate, converging together at fixed points and establishing the physical isolation of "looped out" chromatin fiber. In this view, insulators behave primarily as structural boundaries between autonomous domains of gene expression by possibly coalescing into nuclear "insulator bodies" (Fig. 2), though functional involvement of insulators in determining the transcriptional identity of these domains is not excluded. Thus, positioning an insulator between an enhancer and a promoter results in partitioning of the two into separate functional domains, preventing their proper communication and perhaps causing them to assume incompatible structural traits. Similarly, two insulators can create an independent domain of expression for the flanked transgene, relieving it from the effects of nearby condensed chromatin and in some way, conferring the information necessary for activation. The means by which the information between two domains cannot be exchanged are poorly understood, but recent findings suggest possible ways such as topological restrictions or functional separation due to recruitment to nuclear bodies. In fact, the views of chromatin boundary elements as transcriptional regulators or as structural organizers are not mutually exclusive. Insulators may possess the dual property of directing nuclear organization and of recruiting transcriptional regulators, as they may be part of a strategy that modern genomes have evolved to simultaneously establish both. The primary role of boundary elements may thus be structural partitioning of chromatin into higher-order physical domains associated with a particular transcriptional identity.

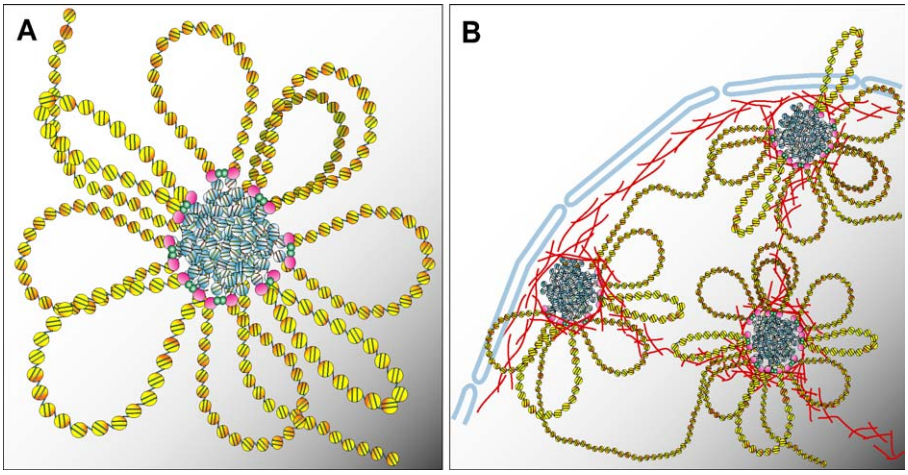

Fig. 2. Insulators as organizers of independent chromatin domains within the nucleus. The self-interaction of boundary proteins (pink and green) or their interaction with a fixed nuclear substrate (red) form insulator bodies that may participate in the structural partitioning of chromatin into independent topological domains. Functional independence of the created chromatin compartments may come from physical separation of regulatory elements located in individual domains, from association of specific bodies with activating or repressive signals within the nucleus, or from blocking the propagation of topological strain.

3. Insulators and transcriptional regulation

The ability of boundary elements to prevent repression by blocking the spread of heterochromatin has been described for a variety of identified insulator elements (for a list of such elements, see West et al., 2002), suggestive of their role in preserving the separation between heterochromatin and euchromatin. Recently, the heterochromatic repressive chromatin has been characterized by several molecular marks, primarily, the enrichment in methylation of histone H3 lysine 9 and decrease in methylation of H3 lysine 4, hypoacetylation at several lysine residues of histones H3 and H4 as well as the binding of heterochromatin protein 1 (HP1) (reviewed in Grewal and Moazed, 2003). On the other hand, the euchromatic or transcriptionally competent state is associated with hyperacetylation of H3 lysines 9 and 14 and methylation of H3 lysine 4. Hyperacetylation of histones is thus correlated with transcriptional activation and is thought to aid in the establishment of an open or "trancriptionally receptive" state. Gene activation also requires the action of general and specific transcription factors, some of which may be responsible for the recruitment of histone-modifying enzymes such as HATs (histone acetyl transferases), as well as the assembly of a ready RNA polymerase complex. Recent analysis of yeast and vertebrate boundary elements has implicated the dynamics of histone acetylation and methylation and the action of transcriptional activators (TAs) in the mechanism of barrier establishment (Donze and Kamakaka, 2001; Fourel et al., 2002; Mutskov et al., 2002; Kuhn and Geyer, 2003). These findings correlate with reports of promoters and promoter-like regulatory elements behaving as insulators (Ohtsuki and Levine, 1998; Bi and Broach, 1999), further emphasizing the role of establishment of transcriptionally competent chromatin in the action of boundary elements.

Endogenous boundary elements have been found at borders between active and non-active regions of chromatin. The *scs* and *scs'* (specialized chromatin structure) insulator elements delimiting the *hsp70* heat-shock locus in *Drosophila* were the first insulators to be characterized and corresponded to hypersensitive sites located at the presumed borders of the transcriptional domain of a heat-shock gene (Avramova and Tikhonov, 1999). In this case, the flanking chromatin boundaries may primarily function in limiting the spread of activated chromatin and preventing its effects on nearby loci. The *S. pombe* silenced mating type locus is similarly flanked by two inverted repeats with presumed boundary activity, as they delimit the transition from the heterochromatic area, enriched in methylated H3 K9 and Swi6 (homologue of HP1), into the euchromatic area, which exhibits high levels of methylated H3 K4 (Noma et al., 2001). Another well characterized *Drosophila* boundary element, the *gypsy*/Su(Hw) insulator, may similarly associate with the borders of condensed versus open chromatin. The *gypsy* insulator consists of a 350 bp sequence located in the 5′ transcribed, untranslated region of the *gypsy* retrotransposon, the active core of which includes binding sites for the Su(Hw) (suppressor of Hairy-wing) protein (Geyer and Corces, 1992). Endogenous binding sites of Su(Hw), which may function as genomic insulators, are frequently found at the borders between bands and interbands of polytene chromosomes, suggestive of their role in this type of partitioning (M. Labrador and V. Corces, unpublished observations).

The chicken *β-globin* 5′ HS4 insulator is present at the border between a globin gene expression domain and a block of repressive heterochromatin. The *β-globin* gene expression is controlled by the LCR (locus control region) regulatory element, the 5′ end of which is comprised of five hypersensitive sites (HS). The 5′ HS4 region has been found to possess both of the insulator characteristics as it protects transgenes from position effects in cell culture and from PEV in *Drosophila* (Chung et al., 1993; Walters et al., 1999) and blocks enhancer-driven activation of a promoter (Chung et al., 1997). The 5′ HS4 boundary is known to bind CTCF (CCCTC-binding factor), an evolutionarily conserved protein implicated in the activity of most described vertebrate boundary elements and thought to be necessary for the enhancer-blocking activity of HS4 (Bell et al., 1999). Recently, a detailed molecular characterization of the *β-globin* 54 kb region by ChIP (chromatin immunoprecipitation) analysis has shown that the HS4 insulator sequence maps to the transition point between the heterochromatic block, constitutively enriched in methylated H3 K9, and the active region, marked by hyperacetylation of H3 and H4, upon developmental induction of transcription (Litt et al., 2001a, 2001b; Burgess-Beusse et al., 2002). Remarkably, the HS4 locus was found to exhibit constitutively elevated levels of histone acetylation, even in cells that do not normally express the *β-globin* gene. These findings were further supported by subsequent demonstration that the HS4 insulator is required to maintain high levels of acetylated histones and expression of an integrated transgene (Mutskov et al., 2002) and correlated with previous reports that treatment of transgene-bearing cells with deacetylase inhibitors mimicked the presence of insulators (Pikaart et al., 1998). Together, the link of insulator activity to histone acetylation has led to the proposal that boundary elements may function as entry sites for HATs to counteract the spread of methylated and deacetylated heterochromatin. In this view, insulators function as chain terminators of encroaching heterochromatin by recruiting histone acetylases and acting as nucleation sites for establishment of an open chromatin state. Interestingly, the position effect protection by the HS4 insulator was not found to depend on the binding sites for CTCF, suggesting that the barrier activity of HS4 is separable from the enhancer blocking. Consistently, the CTCF-binding 3′ HS boundary element found at the 3′ end of the *globin* gene cluster is not associated with a peak of acetylation. The *globin* locus is followed by an odorant gene at its 3′ end as opposed to a block of what appears to be constitutive heterochromatin at its 5′ end, and it has been suggested that this organization reflects the differential need for enhancer-blocking versus barrier activity, respectively (West et al., 2002).

Recently, a similar genomic arrangement of a heterochromatic block followed by an active transcriptional unit, separated by a CTCF-binding insulator element, was described for the mammalian *c-myc* locus (Gombert et al., 2003). The authors have similarly employed ChIP assays to map the point of transition between H3 K9 methylation and acetylation upon induction and have identified a putative insulator region, conserved between the human and murine *c-myc* loci, which contained CTCF-binding sites. The insulator sequence was shown to possess enhancer-blocking activity in the CTCF-binding 40 bp region and a separable adjacent boundary activity, necessary for position-independent expression of a reporter transgene. Interestingly, the 160 kb region surrounding the *c-myc* expression unit is further flanked by two MAR sequences, separating it from the closest neighboring genes on either end. Insulators have been proposed to be structurally related to MARs, as discussed below, though functional boundary activity of MARs has not been clearly shown.

The involvement of histone acetylation in barrier function against heterochromatin was also demonstrated at the yeast heterochromatin-like mating type loci. The spread of the silenced *HMR* domain of the *S. cerevisiae* mating type locus is thought to be delimited by a boundary element, comprised of a *tRNAthr* gene (Donze et al., 1999). The *tRNAthr* gene can protect a nearby reporter gene from silencing and appears to block the spread of the silencing Sir2/3/4 complex into adjacent euchromatin. Mutations in the Sas2 and Gcn5 HATs disrupt the barrier function of the *tRNAthr* boundary element, and their direct tethering to the boundary region creates a potent block to the spread of silencing (Donze and Kamakaka, 2001). Additionally, the integrity of the RNA pol III promoter of the *tRNA* gene appears important for the barrier function even through active transcription by RNA pol III does not, suggesting that the boundary activity results more from recruitment of chromatin-opening activities than actual transcription.

Another example of a yeast boundary element, associated with transcriptional activation, is the UAS_{rpg} (upstream activating sequence of ribosome protein genes) element found in the promoters of the *TEF1* and *TEF2* genes (Bi and Broach, 1999). The boundary activity within the UAS_{rpg} appears restricted to the binding sites for the transcriptional regulator Rap1p, which among other roles, has been shown to be involved in targeting the NuA4 histone acetyltransferase complex. Two other TAs, Tbf1p and Reb1p, were found to bind within the STAR (sub-telomeric anti-silencers) boundary elements of the yeast heterochromatin-like telomere regions, where silenced domains are thought to exist in a discontinuous array (Fourel et al., 1999). Direct tethering of Tbf1p and Rep1p or their transcriptional activation domains similarly provided barrier function against telomeric silencing (Fourel et al., 2001). These three proteins, Rap1p, Tbf1p and Rep1p, belong to the functional category of general transcriptional factors (GRFs), which can enhance both activation and repression of transcription and appear to regulate a large number of promoters in yeast. These properties are reminiscent of the functional behavior of some metazoan insulator proteins, such as CTCF and GAGA. GAGA is an insulator-associated protein implicated in the activity of several *Drosophila* boundary elements, which appears to synergize with both the repressive polycomb group (PcG) complexes and the activating trithorax (trxG) proteins (Strutt et al., 1997; Mulholland et al., 2003). It has been proposed that GRFs and GRF-like proteins possess boundary activities and exert their regulatory effects by directing genomic loci to particular functional subcompartments in the nucleus (Fourel et al., 2002).

Curiously, multiple TAs have been found to exhibit barrier activity in yeast, as judged from the direct tethering approach to assess the expression state of a reporter gene. Factors with activating acidic or proline-rich domains act as barriers in such assays, yet as suggested by previous studies, transcription itself is not required for their boundary activity (Fourel et al., 2001). The dispensability of transcription supports the notion that, like HATs, TAs may act as barriers by creating open or active chromatin structure, which may be accomplished by recruitment of chromatin remodeling or modifying activities or targeting to transcriptional subnuclear compartments as proposed for GRFs.

As previously suggested, one of the main difficulties in envisioning such mechanisms and a problem with all existing insulator models is the inability to explain the polar effect of these sequences on enhancer–promoter interactions (West et al., 2002). This also applies, to a lesser extent, to the barrier properties, as whatever functional effect an insulator is proposed to exert on its surrounding chromatin, it has to operate exclusively in one direction. In the case of the barrier activity, it is possible that this polarity is enforced by factors other than the insulator itself, such as strong silencer elements located in heterochromatin. In this manner, a HAT or TA recruiting boundary works in both directions to open up and de-silence chromatin while repressive complexes coming in from one side override these activating effects until an insulator sequence is reached. A boundary and boundary-associated proteins may then function as either an active (chromatin remodeling or activity targeting) barrier or a passive (physical block or chain terminator) barrier, or both. The hypersensitive nature of many described insulator sequences, such as *scs/scs′*, *β-globin* 5′ HS4 and 3′ HS and *gypsy*, may reflect the specialized chromatin structure needed for breaking the continuity of repressive spreading.

The difference between these two types of barrier function of boundary elements, the desilencing versus the blocking, has been recently explored by the work of Ishii and Laemmli (2003). The authors utilized a system in yeast where two reporter genes, *ADE2* and *URA3*, were positioned between the E and I silencers of the silent *HML* mating type locus. The *ADE2* gene was flanked by UAS elements, and the expression state of both reporters was assessed in the presence of Gal4 DNA binding domain fusions with various proteins, known to be involved in some aspect of boundary activity. In

this manner, the system allowed to distinguish between boundary activities (BAs), with the *ADE2* gene being ON and *URA3* being OFF, and desilencing activities (DAs), with both genes being ON. DAs were presumed to impose open chromatin states in both directions from their binding site, while BAs should have protected reporter genes from heterochromatic silencing unidirectionally. It is perhaps expected that a tested HAT activity, Gcn5, and transcriptional regulators Reb1p and Tbf1p appeared to behave primarily as DAs, but not BAs. The *Drosophila scs*-binding BEAF protein behaved as a genuine BA in this assay, but surprisingly, CTCF and GAGA did not, being identified primarily as DAs. These results suggest that BEAF may function primarily as a blocking barrier to the spread of heterochromatin and/or that it can set up an isolated domain through possible self-interaction. Given the known additional functions of CTCF and GAGA as complex transcriptional regulators, these factors may be involved in further specifying the accessible state of chromatin of insulated domains, thus acting as bidirectional desilencers.

Although not tested in this assay, it has been previously reported that, in yeast, Su(Hw) protein can also act as a heterochromatic barrier when bound to an introduced *gypsy* sequence (Donze and Kamakaka, 2001). In *Drosophila*, Su(Hw) is known to tightly associate with an additional protein component Mod(mdg4), which is required for *gypsy* insulator activity (Gerasimova et al., 1995). Mod(mdg4) and GAGA share a conserved BTB/POZ domain, believed to be involved in homo-oligomerization, and both genetically interact with trithorax group genes. Mod(mg4) was not found to be necessary for the barrier function of Su(Hw) in yeast, yet it is possible that Mod(mdg4) may exert desilencing effects in its native system, similar to those of GAGA. This would agree with previous findings that certain mutations in Mod(mdg4) behave as enhancers of variegation, a property associated with factors involved in counteracting heterochromatic spread and establishment of open chromatin (Gerasimova et al., 1995).

In the study described above (Ishii and Laemmli, 2003), several mammalian TAs, which are thought to be inactive in yeast, were found to act as DAs, supporting the notion that the barrier function of transcriptional regulators and regulatory sequences is independent from actual transcription. Nonetheless, several studies link boundary activity with transcriptional initiation. Transcriptional domains of the *Drosophila* bithorax complex have been shown to produce discrete transcripts, while deletion of a proposed insulator element results in faulty transcript generation, suggesting that the deleted boundary is necessary to initiate transcription correctly (Drewell et al., 2002). This account is supported by recent findings demonstrating that transgenes experiencing position effect silencing produce transcripts incorrectly initiated at upstream start sites, and that the *β-globin* 5′ HS4 insulator can enforce initiation at the endogenous start site (Frazar et al., 2003). One explanation for these results, consistent with chromatin opening properties of boundary elements discussed above, is that insulators participate in the formation of transcriptionally permissive chromatin, allowing for the correct targeting and activity of the RNA Pol II complex at uncovered initiation sites. Cases of promoters acting as insulators, such as the *even-skipped* (*eve*) promoter in *Drosophila*, have been reported (Ohtsuki and Levine, 1998; Bi and Broach, 1999). In the case of *eve*, the promoter–insulator element is believed to associate with the enhancer-bound regulators to aid in the enhancer "trapping". When positioned away from the transcription initiation site, such an element would compete for the enhancer binding, preventing proper gene activation, and thus behave as an insulator. In fact, as mentioned above, the enhancer-blocking activity of insulators can be explained by the behavior of boundary elements as decoy promoters that are able to trap enhancer-associated factors. Although such methods of transcriptional regulation undoubtedly exist, it is not clear how common this type of mechanism is among enhancer-blocking elements (discussed further below).

4. Insulators and structural chromatin organization

The connection between boundary activity and transcriptional activation provides an understanding of the barrier function of insulators, but it is difficult to reconcile with the enhancer-blocking property of many insulator elements. For situations where the barrier and enhancer-blocking activities have not been found to be separable, it is problematic to envision how the same protein factors can impose a state of unidirectional activation or chromatin opening and at the same time block activation of a promoter by an enhancer. The activation of eukaryotic promoters is thought to depend upon a variety of factors, including the presence of regulatory elements such as enhancers and silencers, local chromatin organization and the location within the nucleus. Enhancer elements and general LCRs are known to be necessary for the proper levels of expression of genes in specific tissues and at specific developmental time points (Li et al., 1999). The exact mechanism by which they operate remains obscure, though several models have been put forward. They include the spread of chromatin state change from the enhancer to the promoter (tracking model) and the looping of the intervening chromatin to allow the interaction of enhancer-bound factors with the promoter-bound RNA Pol II complex (looping model) (Blackwood and Kadonaga, 1998; Bulger and Groudine, 1999). Yet, irrespective of the operational model of enhancer action, the question of how the genome enforces non-promiscuous regulation of each gene, given the abundance and relative proximity of long-range regulatory elements, remains unresolved. The explanation of the enhancer-blocking function of insulators is thus closely connected to the understanding of the mechanisms of enhancer–promoter communication.

One model of enhancer blocking, which can theoretically account for the barrier function of insulators as well, involves

the physical separation of enhancer and promoter into independent structural domains. This view implies that a non-insulated enhancer and its target promoter exist in the same physical domain, which allows for their communication. Taking into account existing models of enhancer function, this communication may involve the spreading of an activating signal from an enhancer down the chromatin fiber, in which case an insulator can block such propagation. Alternatively, it may involve the direct physical contact between an enhancer and a promoter, where a boundary element prevents such looping by establishing new structural loops. Two recent reports on the physical structure of the active *β-globin* locus provide evidence for the looping model of enhancer action, demonstrating direct physical contact between an LCR and the genes it regulates. A study by Tolhuis et al. (2002) utilized the 3C (chromosome conformation capture) technique (Dekker et al., 2002) to analyze the spatial organization of a transcribing versus non-transcribing murine *β-globin* locus. They found that the HS sites of the LCR physically contact the expressing *β-globin* genes in adult erythroid cells, while in cells that do not express *globin* genes, such as brain cells, no such association was observed. These results were confirmed by a separate report (Carter et al., 2002), which employed the RNA-TRAP technology to label chromatin in the vicinity of actively generated transcripts, where the hypersensitive sites of the murine LCR, located 40–60 kb away from the target promoters, were found to be in close proximity of the expressing *globin* genes.

These findings provide support for the existence of chromatin looping to establish enhancer-directed activation, and implicate, though indirectly, a disruption of this type of organization in enhancer blocking. Thus, boundary elements may prevent "productive" looping required for transcription, by creating loop domains that are conformationally restrictive to the enhancer–promoter contact. Insulators may mediate such structural partitioning by physical interaction with each other, resembling the reported clustering of LCR and active gene bound factors, or with a relatively fixed substrate such as the nuclear envelope/lamina or the nuclear matrix (Fig. 2). The existence and importance of physical association of insulator proteins or insulator DNA have been reported for several characterized boundary elements, while other findings have implicated attachment to nuclear substrates in boundary activity.

Investigation of the *gypsy* insulator in *Drosophila* has provided support for the model of insulators as partitioners of structural and functional chromatin domains. The Su(Hw) and Mod(mdg4) proteins, genetically identified as necessary for *gypsy* boundary activity, are found at several hundred endogenous binding sites throughout the genome, as judged form the analysis of polytene chromosomes (Gerasimova and Corces, 1998). Although insulator properties of these sites have not been established, recent support for endogenous Su(Hw)-mediated boundary activity has been gained from the identification of a genomic Su(Hw)-binding insulator in the 3′ region of the *yellow* gene (Golovnin et al., 2003; Parnell et al., 2003). In diploid nuclei, the binding sites for *gypsy* insulator proteins are seen to merge into large aggregates, associated often with the nuclear periphery, forming "insulator bodies". The genomic binding sites of *gypsy* insulator proteins are thought to follow this coalescence, acting as points of attachment for the structural domains of looped out chromatin. This is supported by findings showing that genomic loci can change both expression status and location within the nucleus, relative to the nuclear periphery and insulator bodies, upon introduction of a *gypsy* insulator (Gerasimova et al., 2000). The second component of the *gypsy* insulator, Mod(mdg4), carries a BTB/POZ oligomerization domain, which may mediate the proposed aggregation of insulator complexes. The nuclear association of insulator proteins correlates with the enhancer-blocking activity of the *gypsy* insulator, as hypomorphic mutations of Mod(mdg4) disrupt both the boundary function of *gypsy* and the nuclear organization of insulator bodies.

The *scs* and *scs′* boundary elements were similarly found to physically contact each other, supporting the loop domain model of insulator function (Blanton et al., 2003). The proteins associated with these DNA elements, BEAF and Zw5 (Hart et al., 1997; Gaszner et al., 1999), were shown to interact both in vivo and in vitro. Additionally, the Zw5 protein, which normally binds the *scs* element, is able to immunoprecipitate *scs′* sequences, suggesting the existence of physical linkage between the *scs* and *scs′* loci. Moreover, the *scs* and *scs′* DNA sequences were found in close proximity in vivo, as judged from results of the chromosome conformation capture analysis, while closer sequences located in the 15 kb region separated by *scs* and *scs′* boundary elements were not found to pair with *scs′* to the same extent. In this case, it appears that the direct physical association of *scs* and *scs′* bound proteins is involved in setting up a structural domain, which functionally may translate into restriction of the strong transcriptional activity of a heat-shock gene.

The involvement of physical pairing in boundary activity is highlighted by the ability of several insulator proteins to participate in homeotypic interactions. The BTB domain, common to Mod(mdg4) and GAGA proteins is capable of self-interaction and can be seen to form dimers and oligomers (Espinas et al., 1999; Katsani et al., 1999; Read et al., 2000; Ghosh et al., 2001). The GAGA factor has been implicated in the regulation of expression domains of the bithorax complex (Cavalli and Paro, 1998), in the enhancer-blocking activity of the *eve* promoter (Ohtsuki and Levine, 1998), and recently has been linked to a new boundary element identified in *Drosophila*. A novel insulator element, SF1, identified in the *Antennapedia* gene complex, is thought to promote independent regulation of the neighboring *Sex combs reduced* and *fushi tarazu* genes (Belozerov et al., 2003). This boundary sequence contains a highly conserved cluster of GAGA sites, which are found to be essential for its activity. GAGA has also been implicated in the activity of a well-characterized *Drosophila* bithorax complex (BX-C) insulator, *Fab-7*, which is involved in boundary formation between

developmentally important regulatory sequences (reviewed in Mihaly et al., 1998; Brown et al., 2003). The *Fab-7* element has been shown to support long-range interactions between distant genomic loci, as the endogenous *Fab-7* sequence was demonstrated to recruit transgenic *Fab-7* elements to the BX-C nuclear location (Bantignies et al., 2003). The role of GAGA in bringing together separate DNA loci in the nucleus was also demonstrated by its ability to stimulate activation of a promoter by an enhancer located in trans on a separate chromosome (Mahmoudi et al., 2002). Thus, the capacity of GAGA to self-aggregate may be tied to its involvement in both transcriptional regulation and boundary activity, as chromatin domains resulting from such interaction can help bring enhancers and promoters together or keep them physically apart, depending on other interactions.

The ability of insulators to interact with each other can also be seen in the unusual behavior of two boundary elements situated in tandem. According to Cai and Shen (2001), two closely linked Su(Hw)/*gypsy* insulators positioned between an enhancer and a promoter cancel each other out and allow efficient activation of the promoter. This property is not enhancer-specific and is thought to reflect the ability of insulators to loop out the intervening DNA and perhaps create topologically closed domains. A separate study similarly demonstrated that two copies of Su(Hw) insulators neutralize insulator activity and instead, perhaps even promote enhancer–promoter communication (Muravyova et al., 2001). This ability of *gypsy* boundary elements to cancel each other out can perhaps be partially explained by the need of insulators to be recruited to insulator bodies, where functional division of chromatin domains is established. Without this recruitment, the blocking of the enhancer may not be carried out properly by the insulator, as supported by studies that correlate insulator body formation and enhancer-blocking ability of the *gypsy* insulator (Gerasimova and Corces, 1998). When positioned too close together, two insulators may participate in "unproductive" interactions with each other and fail to be recruited to the functionally important clusters, thus allowing for the enhancer and the promoter to still exist in the same domain and communicate with each other (Mongelard and Corces, 2001). Perhaps, the lack of a transcription unit between two boundary elements prevents their proper organization or targeting, resulting in such "unproductive" pairing. Alternatively, it has been suggested that structural domains created by insulators are topologically restrictive, implying that enhancers propagate some type of topological change or require conformational freedom to activate promoters (Xin et al., 2003). One insulator is able to block this topological change, while two closely positioned insulators may pair and effectively create a topologically closed domain, which can be circumvented. Both of these explanations agree with the ability of boundary elements to partition chromatin into topologically independent domains and view the local interaction of two tandem insulators as representative of their normal pairing abilities.

Interestingly, the insulator neutralization property appears to be specific to the *gypsy* insulators, as pairs of other characterized boundary elements, such as SF1 and *scs/scs′*, do not exhibit a reduction in enhancer blocking (Kuhn et al., 2003; Majumder and Cai, 2003). The behavior of *scs* and *scs′* in the enhancer-blocking assay is surprising as the two elements appear to physically pair in vivo (Blanton et al., 2003), similarly to the *gypsy* insulators. These results indeed support the notion of different mechanisms involved in the action of different boundary elements, suggesting that self-interaction is not important for all enhancer-blocking elements. Alternatively, the physical pairing of some insulators may result in different functional consequences than the pairing of others. It is possible that the association between *scs* and *scs′* results in a topological domain that cannot be bypassed by an enhancer. This block could be due to a distinct conformational character or other functional specification adopted by the *scs–scs′* domain, as opposed to a *gypsy*/Su(Hw) domain. For other boundary elements, an interaction of insulator proteins with other chromatin-associated factors may take precedence over any existing self-interaction (Majumder and Cai, 2003), resulting in preservation of the enhancer-blocking ability in a paired scenario.

Support for the role of insulators in the organization of structural domains also comes from studies on prokaryotic enhancer blocking. In a recent report, an in vitro insulator-like element was constructed to analyze the mechanism of enhancer blocking by boundary elements, using DNA-binding *lac* repressor to create topologically isolated loops on a circular plasmid (Bondarenko et al., 2003). The *lac* repressor is known to form tetramers, with each tetramer able to bind two separate *lacO* (*lac* Operator) DNA sites and thus, positioning an enhancer between two *lacO* sequences should effectively place it in a closed off loop. This study reinforced the idea that enhancers, positioned inside topologically restricted domains, cannot activate promoters located outside of such domains. Furthermore, formation of an inhibitory loop can be negated by the presence of an extra insulator-like element, which allows enhancer and promoter to exist in the same topological domain, thus regaining gene activation. These results, consistent with the demonstrated ability of Su(Hw) insulators to cancel each other out when positioned in tandem, support topological constraints imposed by insulators as one of the primary mechanisms of enhancer blocking.

The establishment of topological loop domains can come from pairing or clustering of boundary proteins, or, alternatively, it can be enforced by an interaction of an insulator with a fixed substrate. The nuclear membrane and lamina are possible candidates for providing a fixed interaction surface to insulator proteins, as is the nuclear matrix (though the biochemical definition and composition of the nuclear matrix remains debated, see Pederson, 2000). Since the nucleus lacks other membrane-bound organelles, nuclear subcompartments, defined primarily by high local levels of certain proteins or genomic loci, may play a similar structural role in the activity of boundary proteins.

It is possible that both the physical pairing of proteins and binding to a fixed nuclear substrate play a role in the activity

of the *gypsy* insulator, directing the formation of structural and possibly functionally important chromatin loops. The protein components of the *gypsy* insulator have recently been shown to be components of the nuclear matrix, as they remain bound to DNA in intact insulator bodies, following high salt extraction (Byrd and Corces, 2003). This biochemical treatment of cells, in combination with fluorescence in situ hybridization, allowed for actual visualization of extended DNA loops, emanating from the protein-rich scaffold where Su(Hw) and Mod(mdg4) are seen to reside. The *gypsy* insulator is seen to affect the position of its surrounding DNA relative to the nuclear matrix, suggesting that the insulator may serve as an attachment point of DNA to the matrix and a basis of the presumed looping domains. In correlation with similar experiments carried out in vivo (Gerasimova et al., 2000), this organization is dependent on the integrity of the insulator proteins and of the nuclear matrix. This study additionally suggests that *gypsy* insulator sequences may in fact be biochemically or functionally similar to MARs.

MAR sequences can exhibit insulator properties of position effect protection. One such MAR element has been identified at the 5′ boundary of the chicken *lysozyme* gene, although the matrix-bound region appears separable from, yet in close proximity to the sequences with assayable insulator properties (Phi-Van and Stratling, 1996). Additionally, a recent study on the human *apolipoprotein B* locus has identified a CTCF-binding enhancer-blocking element in close vicinity (~2 kb) of a MAR element (Antes et al., 2001). And although vertebrate MAR sequences appear to be separable from any associated boundary activity (Phi-Van and Stratling, 1996), their physical linkage to regions with insulator properties seems intriguing and suggestive of an organizational basis. MARs may play a role in the structural organization and more or less permanent attachment of chromatin to a solid substrate, while insulators may participate in functional partitioning of the genome by the same attachment mechanism.

Recently, tethering of insulator loci to a subnuclear compartment was implicated in the activity of the CTCF insulator protein (Yusufzai et al., 2004). According to these findings, the nucleolar protein nucleophosmin/B23 was shown to form a complex with CTCF and to be present at the chicken *β-globin* CTCF-binding insulators. Furthermore, the CTCF-binding HS4 insulator array was shown to localize to the nucleolus in a CTCF-dependent manner, suggesting that nucleophosmin may recruit CTCF-associated insulators to the nucleolar periphery for chromatin domain establishment. The authors hypothesize that CTCF may utilize the nucleolar surface as an attachment point for boundary loci, generating structural loop domains in the process.

Correlation between boundary activity and components of the nuclear scaffold has also been observed in yeast, where nuclear pore components have been implicated in the establishment of boundaries against silencing (Ishii et al., 2002). A screen utilizing a reporter gene system similar to the one described earlier (Ishii and Laemmli, 2003), led to identification of proteins involved in nuclear-cytoplasmic transport, such as Cse1p, Mex67p and Los1p, and nuclear pole complex component Nup2p. Fusions of these proteins to the DNA binding domain of Gal4 protected silencing of an *ADE2* gene flanked by UAS binding sites, while the adjacent unflanked *URA3* gene remained silenced by the nearby mating type locus. The authors further demonstrated that physical targeting of the silent *HML* locus to the nuclear pore resulted in the block to the spread of repression. And although no endogenous insulator elements in yeast or other organisms have been found to function by attachment to the nuclear pore, these results support the necessity of nuclear substrate tethering in boundary activity.

5. Coming together: possible roles of insulators in nuclear organization and functional identity of chromatin

Although evidence exists to support establishment of topologically independent domains through physical association of insulators or their attachment to a fixed substrate, this view does not necessarily explain why an enhancer can no longer activate a promoter when the two are located in separate structural domains. If, as recently suggested (Carter et al., 2002; Tolhuis et al., 2002), the looping model of enhancer action is the main mode of promoter communication, then physical partitioning by insulators may not readily provide a reason for the disruption of contact. One possibility is that the physical separation imposed by boundary elements is functionally “severe”, preventing any direct association between an enhancer and a promoter. This theoretically can be accomplished by recruitment to insulator bodies, which may organize chromatin in more complex ways than uniformly looped out structural domains. In fact, it is possible that insulator clustering results in some chromatin domains being exposed or recruited to more active areas in the nucleus, while other loops may become condensed or “hidden” or targeted to silent regions (Labrador and Corces, 2002).

Recently, functionally important clustering of regulatory elements to produce high levels of transcription has been proposed, based in part on findings that the remotely positioned LCR of the mouse *β-globin* genes contacts several actively transcribing promoters (De Laat and Grosveld, 2003). This “active chromatin hub” is thought to bring regulatory elements from remote genomic loci together, into a high local concentration of the transcription machinery, looping out intervening gene regions in the process, which somehow results in silencing of the looped out genes. It has been previously suggested that formation of insulator bodies would also result in the high local concentration of promoters, which may be a way to cluster and functionally determine active chromatin sites (West et al., 2002). And although it appears that different boundary elements may employ varying mechanisms of action, this may provide some connection between involvement of insulators in topological

domain establishment and their functional association with transcriptional regulators. Organization of transcriptionally permissive clusters could require interaction with factors, involved in local chromatin opening and transcriptional activation, such as HATs and GRFs. The "insulator neutralization" property of two tandem insulators in part suggests that the presence of a promoter between two boundary activities prevents this neutralization or "unproductive" behavior. Perhaps, the presence of activated transcription in the domain created by two insulators provides a signal to be organized in a particular manner.

It is also possible that boundary elements may aggregate in a fashion functionally opposed to that of the active chromatin hub—the regions to be moved away from the sites of active transcription may cluster in ways similar to those that must be actively transcribed. Clustering of silenced genes and their associated silencing proteins has been previously suggested for another type of nuclear bodies—the PcG bodies (Buchenau et al., 1998; Bantignies et al., 2003). PcG proteins are involved in maintenance and propagation of transcriptional repression for multiple genes during development (reviewed in Orlando, 2003). It has been proposed that polycomb group complexes may aggregate to form repressive nuclear domains, clustering distant PcG-associated DNA loci in order to maintain their silenced state (Saurin et al., 1998). Intriguingly, functional association between PcG-mediated silencing and boundary elements has been described for two *Drosophila* insulators. The Fab-7 regulatory element is known to contain both the insulator sequence and a PcG-binding PRE (PcG response element), which is responsible for the developmentally patterned repression of a BX-C gene *Abdominal-B*. Mutations in *PcG* genes appeared to disrupt the previously described long-range interaction between distant Fab-7 loci, implicating PcG bodies in the organization of higher-order chromatin structure (Bantignies et al., 2003). Integrity of PcG proteins also appears to be important for the activity and nuclear organization of the *gypsy* insulator (Gerasimova and Corces, 1998), suggesting that some insulator functions may involve nuclear clustering of silenced loci.

Generally, these findings also raise the question of whether all insulator complexes or clusters are "created equal" (Fig. 2), since functional variation may exist between them. Some may be more equivalent to active chromatin hubs in that they expose high levels of promoters or regulatory elements, while others may package the chromatin fiber away from the reach of the transcriptional machinery. These ideas will be tested when novel protein factors associated with characterized boundary elements are discovered, and some indication exists that additional proteins must be essential for the activity of known insulators. For instance, a minimal sequence of 454 bp from the 3′ region of *yellow* was found to possess insulator activity in an enhancer-blocking assay, but a smaller 125 bp fragment, containing the two Su(Hw) binding sites, did not confer full insulator activity in the enhancer-blocking assays. The integrity of Su(Hw) sites was still necessary for the enhancer-blocking activity of the larger fragment, but these findings suggest the presence of binding sites for additional proteins in the endogenous insulator region, which, together with Su(Hw) protein, confer full insulator activity. Additional factors may be needed to further specify the functional identity of chromatin or regulate its organization by regulation of known insulator proteins. A few regulatory mechanisms, resulting in differential binding of CTCF and/or its associated insulator function, have been reported, including DNA methylation at the mouse *H19/Igf2* locus and the thyroid hormone-regulated enhancer blocking at the chicken *lysozyme* locus (Bell and Felsenfeld, 2000; Kanduri et al., 2000; Lutz et al., 2003).

The control of *H19* and *Igf2* gene expression in mammals is regulated by the imprinting control region (ICR), which contains a CTCF-binding insulator. The differential methylation of insulator sequences in the ICR of the paternal versus the maternal chromosomes directs binding of CTCF, which in turn regulates monoallelic expression of *H19* and *Igf2*. Thus, in the paternal chromosome, the CTCF-binding sites within the ICR are thought to be methylated, which abrogates CTCF binding and its associated insulator activity, allowing for *Igf2* to be activated by the downstream enhancers. The same ICR sites remain unmethylated on the maternal chromosome, resulting in the block of *Igf2* expression by the functional CTCF insulator and activation of the *H19* gene instead (Bell and Felsenfeld, 2000; Kanduri et al., 2000). In this case, insulator activity appears to be regulated through the differential binding of an insulator-associated protein. Interestingly, the loss of enhancer-blocking function of CTCF has not been correlated with its binding at the chicken *lysozyme* locus. The regulatory region of the *lysozyme* locus includes closely spaced CTCF-binding sites and thyroid hormone response elements (TRE), which mediate the binding of the thyroid hormone receptor (TR). Presence of the thyroid hormone (T3) has been recently demonstrated to abolish enhancer blocking by CTCF, without disrupting the binding of CTCF to the regulatory element (Lutz et al., 2003). These findings suggest an interesting possibility that the activity of insulator proteins can be regulated in a manner independent of their direct binding to DNA. In the case of CTCF, disruption of the reported interaction with nucleolar proteins may interfere with its enhancer-blocking ability, but not its DNA binding. In general, it seems that insulator factors may be modulated through interaction with novel partners or perhaps through covalent modifications, to redefine the functional identity of the chromatin domains they are associated with.

Finally, another potential scenario for an insulator-driven disruption of enhancer–promoter communication is that the enhancer-generated signal is stopped by the physical divide created by boundary elements. Recently, topological change, such as negative supercoiling density or conformational change in the DNA, has been proposed as a possible enhancer-directed signal and a determining factor for organization of chromatin domains (Xin et al., 2003). One appealing aspect of this explanation is that it can theoretically unite

the tracking and the looping models of enhancer action—the enhancer-generated supercoiling can be propagated down the chromatin fiber and simultaneously, result in a more global looping of DNA. Increasing negative supercoiling has been shown to boost enhancer action in bacteria, most likely through physical shortening of the distance between the enhancer and the promoter or through promoting DNA looping to increase likelihood of contact (Liu et al., 2001). It is possible that boundary elements may regulate topological conformation by creating compartments, into which supercoiling strain cannot enter due to restrictions of chromatin's structural freedom. This implication of conformational DNA strain in insulator function may be extended to explain the barrier activity as well. In yeast, heterochromatin is believed to contain higher levels of topological strain, and positioning a reporter gene in the silent regions increases its supercoiling density, relative to the active location (Bi and Broach, 1997). Therefore, insulators may recruit factors, which remodel chromatin and topological state of the opened domain, creating lower levels of strain to assist potential transcription.

In conclusion, insulators are emerging as elements potentially involved into two regulatory activities. On the one hand, they may participate in the recruitment of chromatin-modifying and transcriptional regulatory activities to an associated locus, specifying the functional identity of adjacent chromatin. Additionally, evidence exists to support the role of boundary elements in structural organization of chromatin within the nucleus. These two roles may be integrated, as insulators may delimit higher-order chromatin domains associated with a particular transcriptional state. Recently developed techniques of in vivo chromosomal interactions analysis, such as the 3C approach, should aid in revealing the roles of insulators in structural domain organization. Using such methods, the proposed ability of boundary elements to cluster distant genomic loci for regulatory purposes can be further investigated. The association between insulators and functional identity of chromatin domains will be better understood with the characterization of novel factors involved. Potentially, as boundary elements appear to be involved in both activation and repression of transcription, additional regulatory factors, which can modify the activity of insulator proteins, may be uncovered.

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