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CHROMATIN INSULATORS AND BOUNDARIES: Effects on Transcription and Nuclear Organization

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■ **Abstract** Chromatin boundaries and insulators are transcriptional regulatory elements that modulate interactions between enhancers and promoters and protect genes from silencing effects by the adjacent chromatin. Originally discovered in *Drosophila*, insulators have now been found in a variety of organisms, ranging from yeast to humans. They have been found interspersed with regulatory sequences in complex genes and at the boundaries between active and inactive chromatin. Insulators might modulate transcription by organizing the chromatin fiber within the nucleus through the establishment of higher-order domains of chromatin structure.

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INTRODUCTION

Insulators or chromatin boundaries are DNA sequences defined operationally by two characteristics: They interfere with enhancer-promoter interactions when present between them, and they buffer transgenes from chromosomal position effects (diagrammed in Figures 1 and 2) (30). These two properties must be manifestations of the normal role these sequences play in the control of gene expression. The former property suggests insulators might be one more regulatory sequence, in the same class as enhancers and promoters, at the service of genes to ensure their proper temporal and spatial transcription. The latter attribute suggests that

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insulators might play a role in the organization of the chromatin fiber into functional domains, such that genes present in one domain are not affected by regulatory sequences present in a different one.

A function for insulators in the organization of the chromatin within the eukaryotic nucleus would fill a long-standing void in our understanding of nuclear biology. Results from cytological and molecular studies have long suggested the existence of a structural organization of the DNA within the nucleus. For example, the reproducible banding pattern of insect polytene chromosomes is suggestive of an underlying structural organization, perhaps imposed by the DNA sequence on the higher-order organization of chromatin. This specific structural layout might have a functional significance based on the correlation between transcriptional activation and decondensation of particular polytene bands (72). Similarly, the finding of active genes in the loops of lampbrush chromosomes was taken early as an indication of a direct relationship between activation of gene expression and location within a specific structural chromosomal domain (10). More recently, biochemical studies have identified DNA sequences possibly involved in the structural organization of the DNA within the nucleus. When histones and other chromosomal proteins are extracted from nuclei of interphase cells, loops of DNA containing negative unrestrained supercoils can be observed. The bases of these loops are attached to a matrix or scaffold through sequences termed MARs (matrix attachment regions) or SARs (scaffold attachment regions) (49). MARs or SARs are A/T-rich DNA sequences, often containing topoisomerase II cleavage sites, that mediate the anchoring of the chromatin fiber to the chromosome scaffold or nuclear matrix and that might delimit the boundaries of discrete and topologically independent higher-order domains. Although some of these sequences play a role in the expression of particular genes, the question of whether they are merely structural components or whether they play a functional role is still unanswered. At least some insulator elements seem to have properties that bridge those of MARs/SARs and of standard transcriptional regulatory elements, opening the possibility that the function of both types of sequences is related. Here we examine in detail the structural and functional properties of known insulators in a variety of organisms. We then review models that attempt to bring together all the characteristics of insulators and offer suggestions on their possible role in the cell.

SPECIFIC EXAMPLES OF INSULATOR ELEMENTS

The two defining properties of insulators, i.e., their ability to interfere with promoter-enhancer interactions and their capacity to buffer transgenes from silencing effects of the adjacent chromatin, have been used as experimental assays for their identification and characterization. As interest in these sequences has grown in the past few years, they have been characterized in a variety of organisms, ranging from yeast to humans (4, 15). Rather than reviewing all known insulators, we

concentrate on those that have been studied in more detail and whose analysis might offer insights into the function of these sequences.

Insulator Elements in *Drosophila*

A variety of chromatin boundaries or insulator elements have been described in *Drosophila*, including the *Mcp*, *Fab-6*, *Fab-7*, and *Fab-8* elements present in the bithorax complex (1, 36, 53, 77), the scs and scs′ elements flanking the 87A7 heat shock gene locus (47, 48), the *gypsy* insulator present in the *gypsy* retrotransposon (34, 42), and an insulator present in the *even-skipped* promoter that contains a binding site for the GAGA protein (60). These different insulators have some common characteristics that might suggest shared mechanisms of action, while at the same time they display idiosyncratic properties suggestive of particular roles in chromatin organization and regulation of gene expression.

THE SCS AND SCS′ ELEMENTS OF THE HSP70 HEAT SHOCK LOCUS The first experimental evidence of a specific DNA sequence having insulator activity was obtained with the identification of the scs (specialized chromatin structure) and scs′ elements of *Drosophila*. These sequences were identified at the borders of the 87A7 heat shock puff, suggesting that they might demarcate the extent of chromatin that decondenses after induction of transcription by temperature elevation (73). The scs and scs′ sequences contain two strong nuclease-hypersensitive sites surrounding a nuclease-resistant core, which is flanked by additional weaker nuclease cleavage sites present at intervals corresponding to the length of a nucleosome. A similar pattern of strong hypersensitive sites at the location of the proposed boundary elements is observed at the sites of the chicken β-globin 5′ boundary (14) and the insulator present in the *gypsy* retrovirus (11), and suggests that this chromatin organization might play a role in boundary function. The scs and scs' insulators differ in their DNA structure and require different proteins to mediate their function. In scs, sequences associated with DNase I hypersensitive sites are essential for complete blocking activity of enhancer function, whereas the central nuclease-resistant A/T-rich region is dispensable for this effect. Deletion of sequences associated with some hypersensitive sites leads to a reduction in enhancer blocking, whereas multimerization of subfragments with partial activity restores full boundary function (74). Further insights into the specific sequences required for boundary function have come from the identification of SBP (scs binding protein) as the product of the *zeste-white* 5 (*zw5*) gene (27). SBP binds to a 24-bp sequence of scs in vitro, and multiple copies of this sequence have insulator activity as determined by their ability to block enhancer-promoter interactions in vivo. Mutations in the sequence that disrupt SBP binding also disrupt insulator function. In addition, mutations in the *zw5* gene decrease the enhancer-blocking activity of these sequences. The ZW5 protein contains Zn finger motifs and is essential for cell viability. Null mutations in the gene are recessive lethal, but hypomorphic alleles display a variety of pleiotropic effects on wing, bristle, and

eye development consistent with a role for this protein in chromatin organization (27).

Sequences responsible for the boundary function of the scs' element have also been characterized in detail. A series of CGATA repeats that interact with the BEAF-32 proteins are responsible for the insulator activity of the scs' sequences (16, 75). Mutations in this sequence that interfere with binding of the BEAF-32 protein also abolish insulator activity, whereas multimers containing several copies of the sequence display boundary function. The latter results are similar to those obtained with the *Drosophila* scs sequences and the *gypsy* insulator, and suggest that the effect of boundary elements on transcription might require the binding of a critical number of proteins that somehow cause chromatin alterations as a consequence of their interaction with DNA.

Two related 32-kDa proteins termed BEAF-32A and BEAF-32B (for boundary element associated factor of 32 kDa) have been purified from nuclei of a *Drosophila* cell line and found to interact with scs' sequences (40, 75). These proteins bind with high affinity to a site containing three copies of the CGATA motif that flanks the two hypersensitive regions in the scs' sequence. The DNA binding activity resides in the amino-terminal region, which is different in the two proteins; the carboxy terminus is shared and it is involved in heterocomplex formation. The sequence containing BEAF-32 binding sites acts as a typical boundary element and blocks the activity of both heat shock and ecdysone responsive enhancers in stably transfected cells (75). Immunolocalization of BEAF-32 using antibodies shows the presence of this protein in specific subnuclear regions and its exclusion from the nucleolus. BEAF-32 is present in the interband regions that separate the highly reproducible and characteristic polytene bands of *Drosophila* third instar larval chromosomes. Interbands contain lower amounts of DNA than bands, and are presumed to be regions of partial unfolding of the 30-nm chromatin fiber. As expected, BEAF-32 is present at the scs'-containing border of the 87A7 chromomere, and is also found at one of the edges of many developmental puffs typically seen in polytene chromosomes at this stage of larval development (75). This observation suggests that BEAF-32 might have general structural and functional roles in defining many boundary elements throughout the *Drosophila* genome.

Recent results suggest that the ZW5 and BEAF-32 proteins can interact with each other in vitro, supporting the possibility that the scs and scs' insulators influence transcription by creating higher-order domains of chromatin organization (see below) (7). A second protein capable of interacting with endogenous scs' insulators has been recently identified (39). This protein is the transcription factor DREF; it binds to a sequence overlapping that recognized by BEAF, suggesting that the two proteins might compete for DNA binding in vivo. DREF participates in the regulation of genes encoding proteins required for DNA replication and cell proliferation. Displacement of BEAF by binding of DREF might occur during the time of rapid proliferation, and competition between the two proteins for binding to insulator sites would open the possibility for regulation of boundary function.

INSULATOR ELEMENTS OF THE BITHORAX COMPLEX The *Ultrabithorax* (*Ubx*), *Abdominal-A* (*Abd-A*) and *Abdominal-B* (*Abd-B*) genes of the bithorax complex are expressed in a parasegmental-specific pattern by a complex set of regulatory sequences arranged over 300 kb of DNA in a linear fashion, corresponding to the order of expression along the anterior-posterior axis. These parasegment-specific regulatory sequences appear to be separated by boundaries initially identified owing to the dominant gain-of-function phenotypes observed in "boundary deletion mutants" that result in the fusion of two adjacent parasegment-specific regulatory elements into one single functional unit (54). The best studied of these boundaries is the *Fab-7* element located between the *iab-6* and *iab-7* regulatory sequences that control expression of the *Abd-B* gene in parasegments PS11 and PS12 (36, 53, 77). Deletion of the boundary in the chromosomal DNA results in cross-talk between the *iab-6* and *iab-7* regulatory regions, causing homeotic phenotypes in the adult fly. These results indicate that the *Fab-7* region contains an insulator element that is involved in the normal regulation of the *Abd-B* gene. The location of the insulator has been narrowed down to a 1.2-kb DNA that contains one weak and two strong DNase I hypersensitive sites (36, 53).

In the bithorax complex, the role of the insulators that separate different parasegment-specific regulatory sequences is to avoid interactions between these sequences and to maintain proper segmental expression of the genes. This organization nevertheless poses the problem of how these regulatory elements can overcome the effect of the insulators to activate transcription of the *Abd-B* gene when appropriate. A solution to this problem might lie in a recently described sequence named the PTS (promoter-targeting sequence). This sequence, found within the *Fab-8* element, which also contains an insulator, allows distal enhancers to overcome the blocking effects of the *Fab-8* insulator (1, 78).

AN INSULATOR ELEMENT IN THE GYPSY RETROVIRUS Another insulator element found in *Drosophila* is present in the *gypsy* retrovirus. This insulator is 350 bp in length and is located in the 5′ transcribed, untranslated region of *gypsy*, upstream from the start of the *gag* open reading frame (28). Insertion of *gypsy* into noncoding regions of genes causes a tissue-specific mutant phenotype due to the inability of specific enhancers to interact with the promoter (34, 42, 44). The *gypsy* insulator does not inactivate the adjacent enhancer as this can still activate transcription of a gene located on the other side (8, 69). The *gypsy* insulator can also buffer the expression of a transgene from position effects from adjacent sequences in the genome (66), and it protects the replication origin of the *Drosophila* chorion genes from similar position effects (52). This insulator contains 12 copies of a 26-bp sequence that is the binding site for the Zn finger Su(Hw) protein. The strength of the insulator depends on the number of copies of the 26-bp basic motif: One copy causes a very small effect on enhancer activation of transcription, whereas additional copies result in a stronger effect, with an apparent linear relationship between number of copies and enhancer blocking (68, 70). As in other boundary elements, the *gypsy* insulator also contains a series of three strong DNase I hypersensitive

sites indicative of a special chromatin organization (11). This *Drosophila* insulator has recently been shown to function in *S. cerevisiae* (19).

The *gypsy* insulator is perhaps the best-studied system with respect to the characterization of protein components that interact with insulator DNA. One of these components, the Suppressor of Hairy-wing [Su(Hw)] protein, contains 12 zinc fingers involved in DNA binding and an α-helical region homologous to the second helix-coiled coil region of basic HLH-zip proteins that is absolutely required for insulator function (38). This domain mediates interactions between Su(Hw) and a second component of the *gypsy* insulator, Modifier of mdg4 [Mod(mdg4)], which contains a BTB domain (20, 32). The BTB domain is required for dimerization of the Mod(mdg4) protein and these dimers can then interact with the leucine zipper region of Su(Hw) through the carboxy-terminal region of Mod(mdg4) (35).

The Chicken β-Globin Locus and Other Vertebrate Boundary Elements

The first insulator element discovered in vertebrates is located at the 5′ end of the chicken β-globin locus and was initially characterized through its ability to interfere with activation of transcription of a reporter gene by the LCR (Locus Control Region) (14). Like the *Drosophila* insulators described above, the chicken β-globin element contains a strong DNase I hypersensitive site (64). In the genome, this element marks a boundary between the open, DNase I-sensitive and acetylated chromatin of the β-globin locus and the more condensed, DNase I-resistant and hypoacetylated chromatin located outside of the locus (41). The insulator activity was originally mapped to a 250-bp DNA fragment (13), and subsequent experiments identified a single binding site for the protein CTCF that was sufficient to confer enhancer-blocking activity (3). The CTCF protein contains 11 zinc fingers and has been previously reported to act as a repressor or activator of transcription (25). A second boundary or insulator element, also marked by hypersensitivity to DNase I, is present at the 3′ end of the chicken β-globin gene, and this element also contains CTCF binding sites (67). The fact that the β-globin locus is flanked by insulator elements supports a role for these sequences in the establishment of an open functional domain that allows the transcriptional activation of the globin genes (62).

The chicken 5′ β-globin boundary element has also been tested for its ability to protect against position effects (24, 43, 61). A reporter gene expressing a cell surface marker was introduced by stable transformation into a pre-erythroid chicken cell line under conditions in which expression of the reporter was variable from line to line. When two copies of the complete 1.2-kb β-globin boundary element surround the reporter gene, expression is quite uniform among different transformed lines. This behavior is similar to the protection against heterochromatic position effects by insulators observed in *Drosophila*. It has previously been shown that these types of position effects are associated with loss of histone acetylation (12), and the presence of the chicken β-globin insulator protects against deacetylation of histones H3 and H4 (61). This suggests that the boundary elements either promote

acetylation of the protected region or prevent the action of histone deacetylases. Interestingly, the boundary properties of the β-globin insulator are not associated with sequences that bind CTCF, and other DNA sequence elements within the boundary are required instead (63).

Binding sites for CTCF similar to those present in the chicken β-globin insulator have recently been found to be responsible for the parent-of-origin–specific expression of the *Igf2* and *H19* genes in mice. The presence of an insulator between these two genes had been proposed earlier as an explanation for the inability of enhancers present 3′ to the *H19* gene to activate expression of the *Igf2* maternally transmitted allele. Methylation of DNA sequences located between the two genes, where the putative insulator resides, in the paternally transmitted allele would lead to inactivation of the insulator and activation of *Igf2* in the paternal chromosome (51). The region targeted for methylation has now been shown to contain a series of CTCF binding sites that possess strong insulating activity (2, 37, 45, 46, 71). Mutations in these sites that prevent binding of CTCF abolish the enhancer-blocking activity. More importantly, methylation of these sites abolishes CTCF binding and insulator activity. Therefore, the imprinting phenomenon at the *H19/Igf2* locus closely correlates with the activity of the CTCF insulator, and this activity can be modulated by methylation. The ability to control the activity of an insulator by methylation opens the possibility that other mechanisms might exist in the cell to control the function of these sequences at different times of the cell cycle or during cell differentiation.

Several other elements with enhancer-blocking activity have been identified in vertebrates. Two different human MARs from the apolipoprotein B and alpha1-antitrypsin loci can work as insulators in *Drosophila* by insulating a transgene from position effects. Both elements reduced variability in transgene expression without enhancing levels of the *white* reporter gene expression (59). An insulator has also been described in the human T-cell receptor α/δ locus; this sequence, designated BEAD-1, prevents a δ-specific enhancer from acting on the α genes early in T cell development (76). A binding site for CTCF has been detected within BEAD-1, and deletion of this site abolishes enhancer-blocking effects (3). Similarly, a site within the *Xenopus* ribosomal RNA gene repeat that has limited enhancer-blocking activity when assayed in *Xenopus* oocytes (23) has been identified as a CTCF binding site (3).

Yeast Boundary Elements

Yeast insulator elements have been found at the telomeres and the mating-type loci, where they appear to separate active from silenced chromatin. Genes inserted at yeast telomeric regions or the *HM* mating-type loci are subject to silencing in a manner similar to position effect variegation in *Drosophila*. Surprisingly, the yeast *TEF1* and *TEF2* genes, when present at the *HM* loci, are resistant to this silencing. This resistance can be attributed to the presence of the upstream activation site for ribosomal protein genes (UASrpg) (5). This sequence behaves as a boundary or insulator element, since it blocks the spread of the repressive chromatin structure

associated with *HM* silencing when interposed between the *HML* α genes and the E silencer. The insulator activity has been mapped to a 149-bp fragment containing three tandemly repeated binding sites for the Rap1 protein (5).

Insulator elements are normally present flanking the *HM* loci, where they delimit the region subject to silencing by the *HMR* locus (18). Deletion of these elements causes spreading of the silenced chromatin. In addition, when these elements were inserted between a silencer and a promoter, they blocked the repressive effect of the silencer on the promoter. These elements contain a *Ty1* LTR, although the presence of LTR sequences is not sufficient to confer full insulator activity, and additional sequences from a tRNA gene are required for full boundary function. The function of this insulator requires Smc proteins, which constitute structural components required for chromosome condensation, as mutations in the *SMC1* and *SMC3* genes, but not *RAP1*, affect its activity. The structure of this insulator has been characterized in detail recently (19), and the insulator activity has been mapped to transcriptional regulatory sequences of the tRNA gene, where they normally play an important role in the regulation of the expression of the adjacent *GIT1* gene. Mutations in promoter elements of the tRNA gene, or in genes that affect the assembly of the RNA polymerase III transcription complex, affect insulator function. These results suggest that the transcriptional potential of the tRNA gene is essential for its insulator activity. Interestingly, mutations in genes encoding histone acetyltransferases, such as *GCN5* and *SAS2*, reduce insulator activity, whereas tethering Gal4-Sas2 or Gal4-Gcn5 fusion proteins to specific sites results in the formation of a robust insulator (19). These results have important implications for understanding the mechanisms of insulator function.

Additional sequences with the functional hallmarks of insulators have been found at the yeast telomeres. These sequences, called STARS (for subtelomeric anti-silencing regions), can buffer against silencing effects of both telomeric and HML sequences. In addition, when placed flanking a reporter gene, STARs can buffer its expression from surrounding silencing elements. STARS contain binding sites for Tbf1p and Reb1p, and the insulator activity can be reproduced by fragments containing multiple copies of the binding sites for these proteins (26).

MECHANISMS OF INSULATOR FUNCTION

Insulator elements are defined by their ability to interfere with enhancer-promoter interactions and to buffer transgenes against chromosomal position effects. Given these broad standards, it would not be surprising if a variety of sequences with very different roles in normal nuclear function can still fulfill the operational requirements required to be considered a boundary or insulator. For example, the boundaries characterized in yeast play a role in halting the spread of a silenced chromatin, whereas some insulators identified in *Drosophila* and vertebrates might be involved in the establishment of functional domains of gene expression. Genes in higher eukaryotes have proved to be more complex than genes in lower eukaryotes,

and this complexity requires that sequences responsible for transcriptional regulation be flexible in the way they operate. Enhancers have thus been designed to control transcription in a distance- and orientation-independent manner and, although this property allows an enhancer great flexibility in where it is positioned with respect to the gene it controls, it also entails the possibility of promiscuous interactions with neighboring genes. Insulators might keep intergenic interactions from taking place by forming boundaries that establish functional domains of gene expression. Some of this function might already be included in the promoter itself; in fact, it was found early on that enhancers could not efficiently transcribe a gene when a second gene was interposed in between (17). It might then be unwise to try to unify all the observed phenomena pertaining to insulators into a single coherent model that explains their role in transcription and, possibly, nuclear organization. This conclusion is also supported by findings suggesting that, at least in some insulators, the ability to interfere with enhancer-promoter interactions can be separated from that of buffering from position effects (63).

Two different types of models have been proposed to explain insulator function; these two models reflect the two types of activity found in insulators and perhaps also reflect the possibility that two different types of sequences are being classified as insulators when, in fact, they play different roles in nuclear function. The two models differ more in the conceptual implications for the normal role of insulators than in the actual mechanisms of how they work. The "promoter decoy" model proposes that insulators act as barriers against a signal that is propagated on the DNA from the enhancer to the promoter (33). According to this model, insulators can imitate the promoter, perhaps by interacting with some or all protein components of the transcription complex, and trick the enhancer into interacting with the insulator instead of the promoter. Although there is no evidence suggesting that this is the case for many insulators, it could certainly be true for yeast insulators found to contain promoter elements. This model seems incompatible with models of enhancer action that do not require tracking of a signal from the enhancer to the promoter. For example, experiments in *Xenopus* oocytes have shown that an enhancer can activate a promoter when the two are on separate but interlinked closed circular plasmids (22). Also difficult to explain with this type of model is the finding that surrounding the enhancer or the promoter in interlocked plasmids with insulators is sufficient to block enhancer action (23). One could argue that the insulator could also trap an enhancer as it loops out the intervening sequences to interact with the promoter. Nevertheless, this argument is not supported by the fact that the strength of at least some insulators is not affected by their position relative to the enhancer and the promoter (44).

An alternative view suggests that insulators exert their effects on transcription through changes in higher-order chromatin structure. This model is supported by the observation that insulators are usually associated with strong DNase I hypersensitive sites and tend to separate chromatin domains with different degrees of condensation (62, 73). A role of at least the *gypsy* insulator in chromatin organization is also supported by the properties of one of its protein components, Mod(mdg4).

The *mod*(*mdg4*) gene is involved in two different phenomena related to changes in chromatin structure; mutations in this gene act as classical *enhancers of position effect variegation* [*E*(*var*)] and have the properties characteristic of *trithorax-Group* (*trx-G*) genes (20, 32). Additional evidence supporting this type of model comes from analysis of the subnuclear distribution of *gypsy* insulator proteins. Results from immunofluorescence experiments, using antibodies against Su(Hw) and Mod(mdg4), indicate that these proteins are present at hundreds of sites in polytene chromosomes from salivary glands (31). Given the large number of sites and their regular distribution along the chromosome arms, one would expect to observe a diffuse homogeneous scattering of insulator sites in the nuclei of interphase diploid cells. Surprisingly, this is not the case; instead, *gypsy* insulator proteins accumulate at a small number of nuclear locations. This has led to the suggestion that each of the locations where Su(Hw) and Mod(mdg4) proteins accumulate in the nucleus is made up of several individual sites that come together, perhaps through interactions among protein components of the insulator. Interactions among individual insulator sites would thus lead to a specific arrangement of the chromatin fiber within the nucleus (Figure 3). This role for the *gypsy* insulator in nuclear organization is supported by the finding that mutations in the *su*(*Hw*) gene result in an increase in the frequency at which double-strand breaks are repaired, suggesting that the genome-wide homology search of broken DNA ends for homologous template sequences is affected when the *gypsy* insulator is not functional (50).

Interestingly, the locations where individual insulator sites appear to aggregate in the nucleus are not random; approximately 75% of them are present immediately adjacent to the nuclear lamina (29). This finding suggests that the formation of *gypsy* insulator aggregates may require a substrate for attachment, and that physical attachment might play a role in the mechanism by which this insulator affects enhancer-promoter interactions. The nuclear lamina itself might serve as a substrate for attachment, perhaps through interactions between lamin and protein components of the insulator. The preferential aggregation of insulator sites at the nuclear periphery and the possibility that this targeting might take place through interactions with the nuclear lamina led to the idea that the *gypsy* insulator might be equivalent to MARs/SARs (31). This hypothesis is directly supported by the finding of MAR activity within the DNA sequences containing the *gypsy* insulator (58). This attachment might impose a topological or physical constraint on the DNA that interferes with the transmission of a signal from an enhancer located in one domain to a promoter located in an adjacent one. According to this model, the primary role of the insulator is to organize the chromatin fiber within the nucleus, and its effect on enhancer-promoter interactions is only a secondary consequence of this organization. An important question arising from these results is whether the organization imposed by the *gypsy* insulator is static, and has a mostly structural role, or whether the organization is dynamic and has direct functional significance. In the latter case, modulation of insulator activity could mediate global changes in nuclear organization and gene expression.

A series of recently published experiments underscore the complexity of the mechanisms involved in insulator function. When a direct tandem repeat of insulators was used instead of a single copy, not only was the insulating effect not reinforced, it was indeed abolished and the enhancer was able to activate transcription (9, 57). Control experiments involving other enhancers demonstrated that the loss of insulator activity is independent of the enhancer studied. Also, the distance between insulators did not affect the results. It is difficult to reconcile these observations with a transcriptional insulator model. For example, a reasonable prediction from the decoy model would be a reinforcement of the trapping of the enhancer by a dual insulator configuration. Similarly, if insulators were entry points for chromatin-modifying enzymatic complexes, the doubling of the insulator should lead to a significant increase in its efficiency. The results seem to support models that suggest a role for insulators in establishing higher-order chromatin domains. If an enhancer-promoter pair has to reside within the same domain to be able to interact, two tandemly repeated insulators may have a tendency to preferentially interact with each other to the exclusion of other insulators because of their physical proximity, thus canceling each other (55).

OTHER FACTORS INVOLVED IN INSULATOR FUNCTION

The study of the properties of chromatin boundaries or insulators should lead to a better understanding of the mechanisms by which enhancers activate transcription in eukaryotes and of the role of complex levels of chromatin organization in the control of gene expression. Transcriptional activation in eukaryotic organisms involves changes in chromatin structure that are probably a prerequisite for the ensuing interactions of enhancer-bound transcription factors with the transcription complex present at the promoter. These changes in chromatin structure might involve alterations of higher-order levels of organization as well as changes in nucleosome structure/organization in the primary chromatin fiber involving histone acetylases/deacetylases or other chromatin remodeling complexes (6). Much of our knowledge on these issues comes from studies carried out in yeast, where upstream activating sequences are located relatively close to the promoter. But in most eukaryotes, including *Drosophila,* enhancer elements are located tens or even hundreds of kilobases away from the promoters of genes. How do eukaryotic enhancers activate transcription over such long distances? Since insulators regulate this interaction, studies on the mechanisms of insulator function should shed light into how enhancers activate transcription over long distances, and some of these studies are already giving important insights (21). Studies of the effects of the *gypsy* insulator on the regulation of the *cut* gene by the wing margin enhancer have led to the identification of Chip, a protein that appears to regulate enhancer-promoter interactions (56). Chip is a homolog of the mouse Nli/Ldb1/Clim-2 family and can also interact with nuclear LIM domain proteins. Chip is widely distributed on *Drosophila* polytene chromosomes and it is required for the expression of many

genes, although it does not participate directly in transcriptional activation. These results have led to the suggestion that Chip facilitates enhancer-promoter interactions by stabilizing the formation of chromatin structures that bring enhancers located far upstream in close proximity with the promoter (21). The analysis of Chip and Nipped-B (65), both of which affect insulator function, will shed light on the mechanisms of long-range interactions between enhancers and promoters.

A connection between insulators and other proteins involved in the establishment of particular chromatin structures was made by the observation that the Mod(mdg4) protein of the *gypsy* insulator has properties of E(var) and trx-G proteins. Interestingly, the function of the *gypsy* insulator is affected by mutations in *trx-G* and *Pc-G* genes. This genetic interaction correlates with changes in the ability of *gypsy* insulator sites to form aggregates in the nuclei of interphase diploid cells; in the background of mutations in *trx-G* and *Pc-G* genes, these aggregates fail to form and the insulator sites appear to be distributed throughout the nucleus. These observations have been interpreted in the context of a model in which trx-G and Pc-G proteins participate and help insulator proteins in the establishment and maintenance of higher-order chromatin domains (Figure 3) (31).

Other factors that are more directly involved in regulating insulator activity must be present in the nucleus. If insulators play a role in establishing higher-order domains of chromatin organization, their activity might be modulated during both cell division and cell differentiation. There must then be proteins that are either constitutive insulator components or are functionally linked to alter the properties of insulators by modifying their protein components. Such proteins have not yet been identified and their existence would lend support to the idea that insulators play important roles in global aspects of gene regulation.

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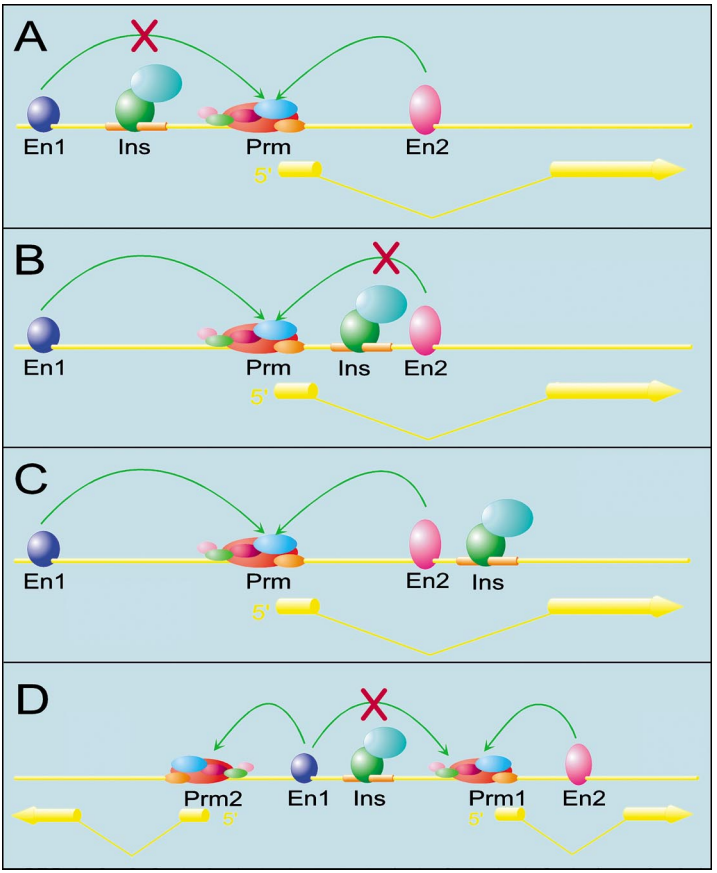

Figure 1 Polar effect of an insulator on enhancer-promoter interactions. The DNA and a hypothetical gene with two exons and one intron are shown in *yellow*. En1 and En2 represent two different enhancers and their associated transcription factors bound to nucleosomal DNA. Prm is the promoter of the gene where the different components of the transcription complex are present. Ins is an insulator element with its associated proteins. Solid arrows indicate a positive activation of transcription by the enhancer element; an X on the arrows indicates the inability of the enhancer to activate transcription. (*A*) An insulator (Ins), with two associated proteins, located in the 5′ region of the gene inhibits its transcriptional activation by an upstream enhancer (En1) without affecting the function of a second enhancer (En2) located in the intron of the gene. (*B*) When the insulator is located in the intron, expression from the downstream enhancer (En2) is blocked, whereas the upstream enhancer (En1) is active. (*C*) When the insulator is located in the intron but distal to the En2 enhancer, both enhancers are active and transcription of the gene is normal. This property distinguishes an insulator from a typical silencer. (*D*) If a second gene is located upstream of the En1 enhancer, although this enhancer cannot act on the Prm1 promoter, it is still functional and able to activate transcription from the upstream Prm2 promoter.

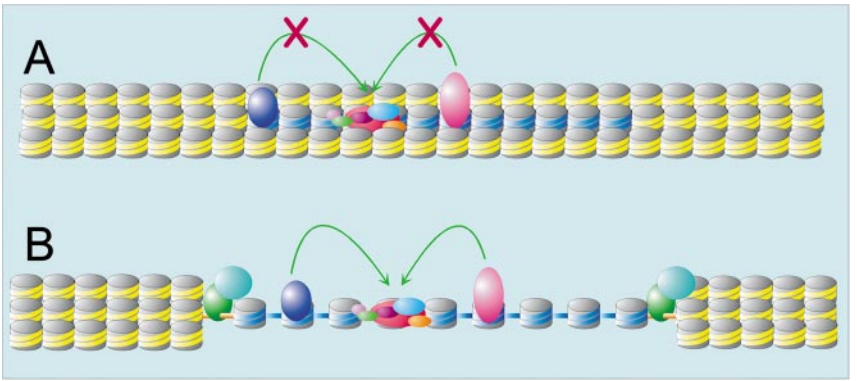

Figure 2 Insulator elements buffer gene expression from repressive effects of adjacent chromatin. Symbols are as in Figure 1. (*A*) A transgene (represented by the *blue* DNA) integrated in the chromosome in a region of condensed chromatin is not properly expressed; the repressive chromatin structure of the surrounding region presumably spreads into transgene sequences, inhibiting enhancer-promoter interactions. (*B*) If the transgene is flanked by insulator elements (in *brown*), these sequences inhibit the spreading of the repressive chromatin, allowing an open chromatin conformation and normal transcription of the gene.

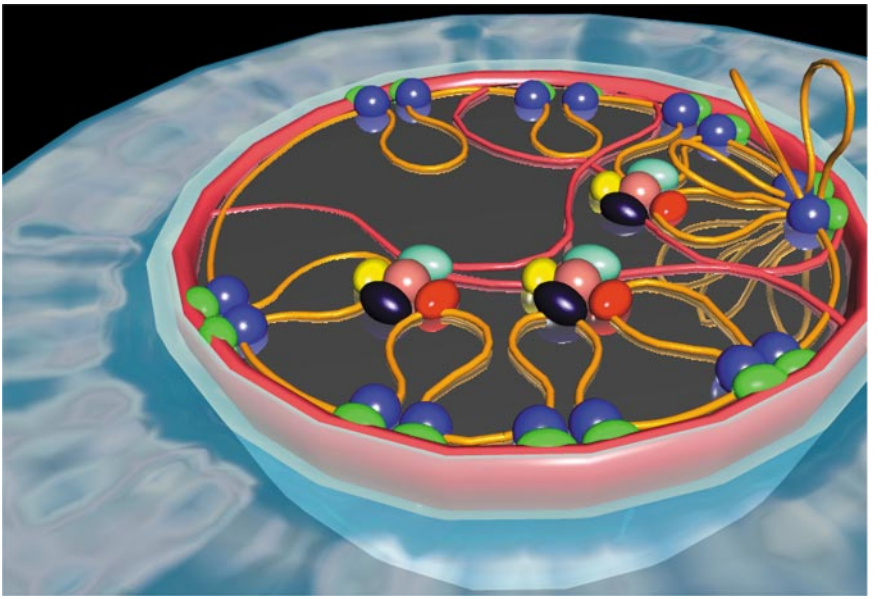

Figure 3 Schematic model explaining the role of trxG and PcG proteins in the function of the *gypsy* insulator. The diagram represents a section though a cell (*blue*) with a nucleus (*dark gray*) surrounded by the nuclear membrane (*light blue*) and the nuclear lamina, which is also located on the inside of the nucleus (*red*). The chromatin fiber is represented as a *gold* line and proteins are represented as ovals colored in *dark blue* [Su(Hw)] and *green* [Mod(mdg4)]; members of the trxG and PcG are represented as *dark purple*, *red*, *pink*, *yellow* and *light green* ovals.