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REVIEW

Insulators are fundamental components of the eukaryotic genomes

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The properties of *cis*-regulatory elements able to influence gene transcription over large distances have led to the hypothesis that elements called insulators should exist to limit the action of enhancers and silencers. During the last decades, insulators have been identified in many eukaryotes from yeast to human. Insulators possess two main properties: (i) they can block enhancer–promoter communication ('enhancer blocker activity'), and (ii) they can prevent the spread of repressive chromatin ('barrier activity'). This review focuses on recent studies designed to elucidate the molecular mechanisms of the insulator function, and gives an overview of the critical role of insulators in nuclear organization and functional identity of chromatin.
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Introduction

Precise control over the expression of a gene is exerted through interactions between the basic transcriptional machinery at the gene promoter and specific protein complexes at enhancer or silencer elements. Enhancers and silencers exert long-distance effects independently of their position and orientation. Nevertheless, neighbouring genes potentially influenced by the presence of the same enhancer within a defined chromosomal locus may display independent transcription profiles. A fundamental question is then how to explain the limited range of the enhancer action. The formation of independent domains of gene function may depend upon a class of regulatory elements able to block the inappropriate action of enhancers or silencers. Such regulatory elements are called insulators (Kuhn and Geyer, 2003). Insulators are defined by two functional properties illustrated in Figure 1. First, an insulator is able to block interaction between an enhancer and a promoter when positioned in-between (Conte *et al*, 2002; Geyer and Corces, 1992; Kellum and Schedl, 1992). Second, an insulator (also called barrier) prevents the advance of nearby condensed chromatin and protects gene expression from positive or negative chromatin effects (Kellum and Schedl, 1991; Roseman *et al*, 1993; Saitoh *et al*, 2000). In this review, we discuss recent advances in our knowledge of the complexity of the mechanism underlying the insulator function and its role in gene regulation.

What are the mechanisms of action of insulators?

Insulators are regulatory elements that can shelter genes from inappropriate regulatory interactions. Transgenic assays have helped to dissect the exact sequences required for insulation and have shown that short sequences – if multimerized – can reconstitute the insulator effect (Scott *et al*, 1999). They have also helped to define the general properties of insulators such as their enhancer-blocker and/or barrier functions. However, we are at present unable to understand the molecular mechanisms underlying these functions or to integrate into a general scheme additional observations such as: (i) the enhancer-blocker and barrier activities are separable (Recillas-Targa *et al*, 2002); (ii) insulator effectiveness is influenced by its structure, and by the nature of the enhancer, promoter and genomic context (Scott *et al*, 1999; Walters *et al*, 1999); and (iii) insulators are not permanent and impassable elements (Cai and Shen, 2001; Muravyova *et al*, 2001). Two nonexclusive models are currently proposed: one of them is established according to a series of data reporting links between insulators and the higher-order chromatin structures, and the other integrate data reporting connections between the insulator properties and gene transcription.

Insulators and higher-order chromatin structures

A structural model proposes that the properties of the insulators result from their relationship with the organization of higher-order chromatin structures (Labrador and Corces, 2002). Experiments performed on a *Drosophila* insulator identified in a retroelement called gypsy help to illustrate this model. This insulator was identified just 3′ of the 5′ long terminal repeat of gypsy. This is a 340-bp fragment, which contains a cluster of 12 degenerate binding sites for a zinc-finger DNA protein, Su(Hw). This insulator is able to block the interaction between enhancers and promoters, and to protect a gene from nearby chromatin effects (van der Vlag *et al*, 2000). Both properties depend on Su(Hw), which recruits the Mod(mdg4) protein. The gypsy insulator is not specific to a single enhancer, but has been shown to act as enhancer-blocker to more than 20 enhancers. Even so,

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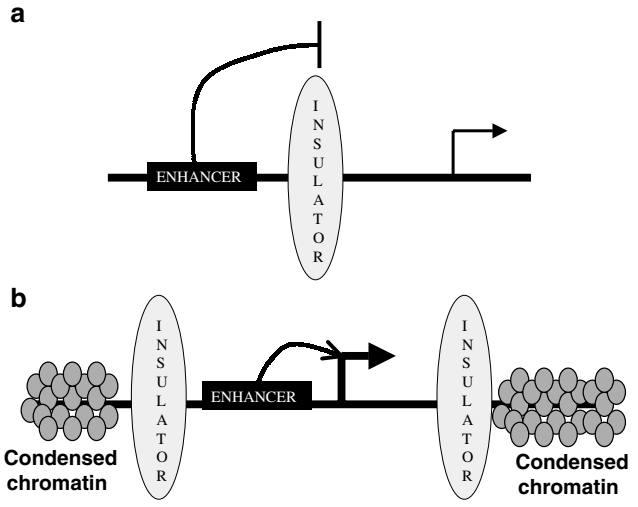

Figure 1 Insulators possess two main properties: (**a**) they can block enhancer–promoter communication (enhancer blocker activity), and (**b**) they can prevent the spread of repressive chromatin (barrier activity).

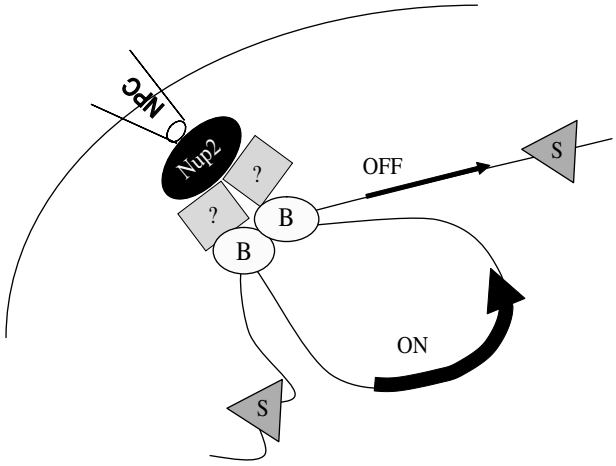

Figure 2 Boundaries interact with nuclear pore proteins by the nuclear pore complex (from Ishii *et al*, 2002). Triangles S represent silencer elements, the white circles B represent the boundary elements and the grey squares presumed unidentified proteins. The boundary elements interact with nuclear pore proteins via the nuclear pore complex (NPC). The authors propose that this nuclear organization allows the gene located between both boundaries to be isolated from the silencing effect. Its transcription is ON. The gene located outside the loop is not protected from the silencer effect, and its transcription is OFF.

this insulator does not establish an impassable barrier. In certain conditions, the insulator is bypassed, the enhancer-blocking effect is neutralized and enhancer–promoter communication is restored. Such a bypass is observed when two gypsy insulators are placed between an enhancer and a promoter. This loss of insulator activity has been proposed to result from intrachromosomal pairing between the two gypsy insulators, causing chromatin to fold and allowing the distal enhancer to contact the promoter. By extension, a single intervening gypsy insulator would block enhancer–promoter communication by interacting either with other insulators located at distant loci or at specific nuclear sites (Cai and Shen, 2001; Muravyova *et al*, 2001). Evidence that the gypsy insulator establishes chromatin domains is strengthened by the fact that Su(Hw) and Mod(mdg)4 associate with 500 sites in the *Drosophila* genome, but coalesce into only 25 large structures. These structures, named insulator bodies, are proposed to establish separate loop domains within the genome. The gypsy insulator sequences could then be genomic sites where such interactions are favoured, and thus be responsible for the generation of such loops. According to this model, Gerasimova *et al* (2000) have shown that the nuclear positioning of a sequence can be altered. If tethered to the gypsy insulator, this sequence is targeted to the nuclear periphery where the insulator bodies are mostly detected.

Furthermore, recent experiments have shown that pairing between two heterologous insulators such as the binding sites for the GAGA factor and the gypsy insulator may also occur in the genome and be a possible means to bypass the insulator activity (Melnikova *et al*, 2004).

Almost all vertebrate insulators described require binding of the regulatory protein CTCF for their activity. Some recent results show that CTCF is copurified with a nucleolar protein present at the nucleolar periphery, suggesting that it helps to displace insulators to the periphery of the nucleole. These interactions may generate similar loops described for the gypsy insulator element in *Drosophila* (Yusufzai *et al*, 2004). Taken together with the fact that CTCF is also associated with the nuclear matrix, these results suggest a functional connection between insulators, the nuclear matrix and nuclear organization.

A connection between insulator activities and their interaction with some nuclear structures is further supported by data obtained through a genetic screen performed in yeast and specifically addressed to isolate genes involved in a possible link between nuclear order and chromatin boundaries. Various proteins involved in nuclear-cytoplasmic traffic, such as the exportins Cse1p or Mex67p, have been identified in this screen and appear to block the propagation of heterochromatin by direct or indirect tethering of the insulator element to the nuclear pore (Ishii *et al*, 2002) (Figure 2).

Faswb, a notch mutation in *Drosophila*, disrupts a boundary element, which results in an alteration of the structural organization of the chromosome visualized by the elimination of a band observed in the giant larval polytene chromosomes (Vazquez and Schedl, 2000).

Although all these examples implicate 3D loops in the insulator function, some results do not fit well with a structural model as a unique model for insulation. As an example, the first insulators identified, the *Drosophila* specialized chromatin structures, *scs* and *scs′* (Kellum and Schedl, 1991, 1992), are boundaries surrounding the 87A7 locus where two hsp70 genes reside. As proposed above for the gypsy elements, interaction between scs and scs′ could explain their insulator function; however, this interaction fails to explain why interaction between *scs* and *scs′* is not a general property of these elements but depends on sequences located outside the specific domain bearing the insulator function (Kuhn *et al*, 2004). Additionally, Majumder and Cai have tested the effect of pairing on enhancer-blocking activity of 11 homologous

and heterologous insulator combinations. The results have shown that, unlike the homologous pairing of gypsy insulator or heterologous pairing of gypsy and binding sites for the GAGA factor (Melnikova *et al*, 2004), heterologous combinations of gypsy and other insulators, as well as homologous pairing with other boundary elements such as *scs* or SF1, do not always reduce their enhancer-blocking activity (Majumder and Cai, 2003). Further, some paired insulators exhibit a higher level of enhancer-blocking activity than either single insulator alone, suggesting that they can function independently or additively (Majumder and Cai, 2003).

Overall, the structural model proposes that insulators separate the chromatin fibre into loops attached to a fixed perinuclear substrate, perhaps the nuclear lamin, which serves as a scaffold to maintain the nuclear organization. However, if such 3D loops provoke special localizations inside the nucleus, they can also be a means to prevent *cis*-diffusion of some molecules necessary for the transcription machinery. Formation of loops could then act as the primary step of the transcriptional model.

Insulators and gene transcription

The transcriptional model advances that insulators have direct consequences on transcription (Geyer, 1997; Bell and Felsenfeld, 1999; Dorsett, 1999). Thus, this transcriptional model depends on the prevailing models of enhancer function and may be summarized in two different mechanisms. If it is assumed that a signal is propagated along the chromatin fibre from the enhancer to the promoter, then insulators assembled in nucleoprotein complexes might block the propagation of the enhancer signal along the DNA. In this case, they act as physical barriers able to stop the activation of a gene by its enhancer. Experiments performed on the transcription factor GAGA from *Drosophila melanogaster* illustrate this model. GAGA can stimulate transcription by linking an enhancer to its cognate promoter. It facilitates long-range activation by providing a protein bridge that mediates enhancer–promoter communication. Insulators could interfere with this property of GAGA, and restrict the recruitment of this factor to the promoter (Mahmoudi *et al*, 2002).

If it is assumed that the enhancer advances as an obligatory propagated signal toward the promoter, then an insulator could compete with the promoter for the enhancer, and trap it into a nonproductive liaison (Geyer, 1997). Supporting this model is the fact that a promoter has been detected within the *scs* and *scs′* elements (Glover *et al*, 1995; Avramova and Tikhonov, 1999), suggesting that these elements may not only be neutral structural elements as proposed by the structural model, but rather their promoter may titrate the enhancer function and keep it from activating transcription. A limit to the transcriptional model is that it fails to explain why boundary elements have to be between the enhancer and the promoter to function as enhancer blockers. In any case, it fails to explain how an enhancer blocked on one side by an insulator can activate a promoter on the other side. Thus, an alternative model involving proteins named facilitators that bring the enhancer and the promoter close to each other can be considered. Among these facilitators, the *Drosophila* Chip protein has been found to interact with Su(Hw) (Morcillo *et al*, 1997). Genetic evidence has shown that Su(Hw) becomes a more effective insulator when enhancer–promoter communication is weakened by mutations in Chip. It is proposed that formation of Chip–Su(Hw) complexes breaks the chain of interaction between Chip and homeodomain proteins, interfering with the process that brings the enhancer towards the promoter.

Recent analyses have shown that barrier elements might play a role in preserving the separation between a silenced and an active chromatin state. Repressive chromatin has been characterized by several molecular marks such as enrichment in methylation of histone H3 lysine 9, hypoacetylation of histones H3 and H4 as well as the binding of heterochromatin protein 1. On the other hand, transcriptionally active chromatin is associated with hyperacetylation of H3 lysine 9 and 14. Several observations suggest that barriers break the code of histone modifications necessary for the propagation of silencing along the chromatin fibre. For example, methylated nucleosomes around the HS4 insulator of the chicken β-globin locus have been proposed to recruit Suv39H1 and allow methylation of the adjacent nucleosomes. The 5′HS4 insulator of the β-globin locus would acetylate the adjacent upstream nucleosomes, which prevents methylation and thus terminates the propagation of the condensation signal (Burgess-Beusse *et al*, 2002). This modification state of nucleosomes within an insulated transgene suggests that another model may account for the position effect protection of insulators. Insulators might directly facilitate nucleosome acetylation. The resulting open chromatin structure would bind factors protecting the gene against DNA methylation (Recillas-Targa *et al*, 2002).

In conclusion, separate data obviously support one and/or the other of the structural and transcriptional models. It is then possible that insulators may utilize several of these mechanisms, although this remains to be demonstrated.

Role of insulators in nuclear function

From all the data reported so far, several roles can be attributed to insulators within the cell.

Partition of distinct chromosomal regions

In addition, to play a structural role in the organization of DNA within the nucleus, chromatin is also intimately involved in the regulation of eukaryotic gene expression (Felsenfeld *et al*, 1996). Barriers are fundamental actors, keeping adjacent domains of active and inactive chromatin distinct and preventing these regions from inappropriate interactions (Figure 3).

In the yeast *Saccharomyces cerevisiae*, a barrier is described at the junction between a heterochromatic region with hypoacetylated lysines of all core histones and an active euchromatic region with numerous acetylated histones (Kimura *et al*, 2002; Suka *et al*, 2002). These results suggest that insulators may establish a mark specifying the functional identity of adjacent chromatin domains.

In chickens, a folate receptor gene is separated from the upstream β-globin locus by a 16 kpb region of silent chromatin. At the 5′ boundary of the β-globin locus, the sequence 5′HS4 marked by a constitutive DNAse I-hypersensitive site acts as a barrier against the

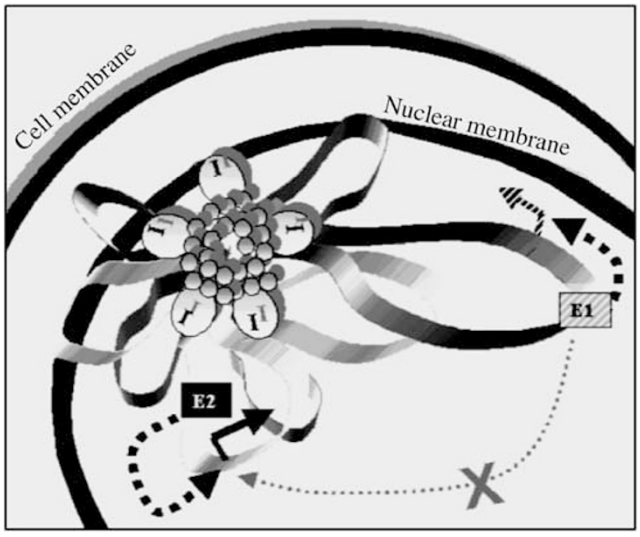

Figure 3 Schematic model of the insulator function in the nuclear organization of chromatin. Proteins (spheres) associated to insulators coalesce within the nucleus. These structures named insulator bodies establish separate loop domains. Located within such a loop, the enhancer E1 can activate transcription of a promoter located within the same loop. However, it is unable to activate a promoter located outside in another domain.

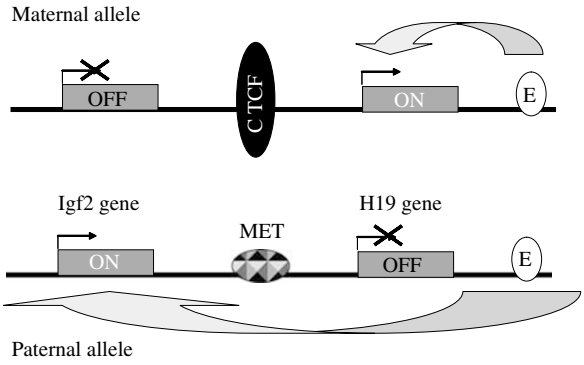

Figure 4 Connection between enhancer-blocking activity and imprinting: the mammalian insulator ICR taken as an example. In females, the endodermal enhancer, represented by a white circle E, is able to activate the H19 gene only because the CTCF binding site acts as an insulator able to block the enhancer effect on the downstream Igf2 gene. In males, the ICR is methylated, which prevents the binding of CTCF to its binding site. Activation of the Igf2 gene is then permitted. H19 is then off potentially due methylation spreading from ICR. ON: when transcription of the corresponding gene is allowed. OFF: when transcription of the corresponding gene is blocked.

incursion of the repressive chromatin immediately upstream (Prioleau *et al*, 1999).

Finally, in yeast telomeres, silent mating-type loci (HM) and rDNA repeats share many of the features of heterochromatic genes. This characteristic together with the compact organization of the genome suggests that yeast gene regulation has evolved efficient mechanisms for insulating genes from each other. Some sequences named STAR (subtelomeric antisilencing region) are able to counteract silencer-driven repression at the mating-type HML locus and act with antisilencing properties against the spreading of silenced chromatin (Singh and Klar, 1992; Fourel *et al*, 1999, 2001).

In all these cases, insulators would guarantee that transition from one domain to the next occurs at a fixed position.

Insulators facilitates complex gene regulations

In the euchromatin, insulators are able to block external enhancers and silencers (Akasaka *et al*, 1999). Thus, they play a fundamental role in blocking inappropriate action of these regulatory sequences on a gene, and in isolating independent transcriptional units from crossreaction with neighbouring regulatory sequences (Figure 4).

Another example taken from *D. melanogaster* concerns the Fab7 element. In the bithorax complex, BX-C, an array of parasegment-specific regulatory domains is separated by boundaries such as Fab7. These boundaries are responsible for the autonomous activity of IAB-6 and IAB-7, which control expression of the Abd-B gene in parasegments 11 and 12, respectively. Fab-7 is active in a wide range of tissues from early embryogenesis through the adult stage. The Fab-7 boundary contains separable regions that function at different stages of development (Schweinsberg and Schedl, 2004). This example illustrates how insulators can limit regulatory interactions at a defined locus, but it also exemplifies how such insulator elements may exhibit differential activities and orchestrate complex regulatory regions.

Finally, a connection between enhancer-blocking activity and imprinted loci has been found, suggesting a role of insulators in the establishment of epigenetic marks in chromatin. This function has been put forward through the analysis of the mammalian insulator ICR (imprinted control region), a functional element found at the endogenous locus IGF2/H19. Regulated by a parental-specific methylation, the insulator is implicated in the imprinting of this locus. When present on the maternal chromosome, its insulator function blocks access of the IGF2 promoter to endodermal enhancers, resulting in exclusive H19 expression. When present on the paternal chromosome, the ICR is methylated and impedes establishment of the insulation (Thorvaldsen *et al*, 1998; Webber and Tilghman, 1998). ICR, then, has two antagonistic roles depending on its parental origin: either it displays an insulator function or it is implicated in the maintenance of a methylated state of the chromatin (Engel *et al*, 2004) (Figure 4).

Insulators and higher-order nuclear organization of chromatin within the nucleus

As described above, several data indicate that insulators are involved in chromatin encroaching onto nuclear substructures. MARs (matrix attachment regions) have been observed close to several regions defined as insulators. One such example is the flanking MAR element of the human *apoB* gene locus presumed to represent the anchorage site for a chromosomal loop (Antes *et al*, 2001). In chicken, an MAR element was also identified at the 5′ boundary of the lysozyme locus, which is thought to mediate the organization of the *lysozyme* gene chromatin domain (Stief *et al*, 1989).

Thus, through their associated factors, insulators would play the fundamental cellular role to recruit

target genes to specific nuclear compartments as a way of maintaining a tissue or development-restricted pattern of expression.

Insulators may promote the interaction between distant regulatory elements and promoters

A possible model explaining distant interactions between enhancers and promoters implicates insulators. As reported above, two insulators may interact through complexes bound to them. This interaction may generate the looping-out of sequences separating an enhancer from its promoter, and bring enhancers and promoters in close proximity. Insulators could then facilitate interactions over large distances.

Recent studies on the active β-globin locus support this model. Indeed, they give evidence for long-range gene regulation *in vivo* involving interaction between transcriptional elements, with chromatin looping-out intervening. Additionally, the murine β-globin locus control region (LCR) is found in physical proximity to the active globin genes, although this LCR is located 40–60 kb away. This interaction and looping-out are only observed in expressing tissues and not in nonexpressing tissues (Tolhuis *et al*, 2002). These data provide the additional evidence that involvement of the insulators in such a regulation is a dynamic process that only occurs during transcription *in vivo*.

Conclusion

The prevalent mechanism leading to gene regulation operates via complex chromatin structures. In this context, insulators are fundamental components of the eukaryotic genomes because together with the chromatin structure, they act as crucial organizers of the genome dynamic. Since they have been identified in many eukaryotic genomes, they are supposed to have conserved roles in the organisms: they guarantee specificities of enhancer–promoter interactions, and define autonomous domains for transcription; they counteract regulatory communication between adjacent domains; they facilitate interactions between distant enhancers and promoters; and they act as genome organisers participating in nuclear organization. All these functions are not static as previously thought, but act as dynamic functions adapted to the transcriptional and/or developmental state of the cell. Thus, they provide the plasticity required to respond to developmental and environmental cues. As expected, in light of all these functions it is not surprising to find clear connections between insulator mutations and human diseases as illustrated in a congenital form of myotonic dystrophy associated with a loss of the function of the DM1 insulator (Filippova *et al*, 2001). Although all the roles reported above have been clearly attributed to insulators, it is nevertheless intriguing to find in some cases that deletion of some insulator sequences is not lethal and sometimes has no obvious phenotype. Genomic redundancies can certainly explain some of these results. Further, an as yet unsolved question is to understand why mutant alleles of genes implicated in the insulator function of a large number of sequences scattered in the genome, such as Su(Hw) for gypsy, are not lethal. It is obvious that further analyses of these functional elements of the chromosomes and their associated factors are necessary for the understanding of interactions linking large genomic regions into one regulatory, organizational and evolutionary unit.

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