



Two insulators are not better than one

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Chromatin insulators seem to control eukaryotic gene expression by regulating interactions between enhancers and promoters. Mounting evidence suggests that these sequences play a complex role in the regulation of transcription, perhaps mediated by changes in chromatin structure and nuclear organization.

The expression of eukaryotic genes in specific tissues and at particular times of development is determined by regulatory sequences called enhancers and silencers. Enhancers bind transcription factors, which then help initiate transcription of the RNA at the promoter. Enhancers accomplish this independently of their distance and location with respect to the gene. This flexibility in enhancer function also gives these sequences the potential of being highly promiscuous. Since this promiscuity is rarely observed, eukaryotes must have developed mechanisms to insure that genes are not activated in the wrong tissue or at the wrong time of development by enhancers from a neighboring gene. Chromatin insulators may play the role of gatekeepers that curtail enhancer promiscuity.

Boundaries or insulator elements have been experimentally identified based on two defining characteristics. Insulators interfere with interactions between enhancers and promoters and inhibit enhancer-activated transcription, specifically when interposed between the affected enhancer and the promoter. Insulators also buffer transgenes, inserted in the genome at random sites, from chromosomal position effects¹. Based on the first property, insulators can be viewed as an additional class of regulatory sequences in the arsenal of controlling elements at the service of genes to ensure their proper spatial and temporal regulation. But the latter property seems to suggest a more complex role, perhaps involving the organization and compartmentalization of the chromatin fiber within the nucleus. Two papers recently published in *Science* appear to support this interpretation^{2,3}.

Properties of an insulator

Insulator elements have been described in many organisms, including yeast, *Drosophila* and vertebrates¹. In yeast, insulators appear to delimit the boundaries of silenced chromatin at telomeres and the mating-type loci HML and HMR. *Drosophila* insulators were the first to be characterized and this organism displays

the largest collection of these sequences so far. The specialized chromatin structure (scs and scs' elements) flank one of the heat shock protein 70 (hsp70) gene loci, where they seem to delimit the region that becomes transcriptionally active (or puffed) in response to heat shock. Other *Drosophila* insulators include those found in the so-called gypsy retrovirus and the *Fab-7* and *Fab-8* elements of the bithorax complex, a complex containing three of the Hox genes that encode important developmental control proteins in flies. Insulators have also been studied in vertebrates, where they have been found in the ribosomal RNA genes of *Xenopus*, the chicken β -globin genes and the human T cell receptor (TCR)- α/δ locus among others. The widespread distribution of insulators is suggestive of an important role for these sequences in the biology of the nucleus.

An idea of the role insulators could play in the cell can be gleaned from the analysis of their distribution in the bithorax complex of *Drosophila*. The various genes of this complex are expressed in a parasegment-specific pattern (a parasegment is a metameric unit along the anterior-posterior axis of flies that is out of phase with segments) by a complex set of regulatory sequences arranged in a linear fashion over 300 kb of DNA, corresponding to the order of expression of the genes along the anterior-posterior axis of the animal. These parasegment-specific regulatory sequences appear to be separated by insulator elements, one of which is called *Fab-7*. The *Fab-7* insulator is located between two enhancers named *iab-6* and *iab-7* that control expression of the *Abdominal-B* (*Abd-B*) gene in parasegments PS11 and PS12, respectively. Deletion of the *Fab-7* insulator eliminates the gatekeeper normally ensuring that the *iab-6* and *iab-7* enhancers activate transcription of the *Abd-B* gene in the appropriate tissues. Without the *Fab-7* insulator, the *iab-7* enhancer activates expression of *Abd-B* in the wrong cells, causing homeotic phenotypes in the adult fly. Homeotic phenotypes are those in which homologous

structures, such as legs and antennae, are changed into one another. These results suggest that the *Fab-7* insulator is required for proper gene expression in order to avoid interference among the different regulatory sequences of the *Abd-B* gene⁴⁻⁶. A second example that illustrates the role of insulators *in vivo* is that of imprinting in mice. Imprinting is an epigenetic trait often seen as differences between paternal and maternal inheritance. The parent-of-origin-specific expression of the mouse *H19* and *Igf2* genes is controlled by an insulator element located between the two genes^{7,8}.

Two possible models

Much of the work on chromatin insulators has centered on the characterization of associated proteins with the goal of explaining how these sequences affect enhancer function, however, progress in the field has been hampered by the lack of understanding of how enhancers activate transcription. Enhancers are currently viewed as entry points for transcription factors which, once bound, can interact with the transcription complex by looping out intervening sequences⁹. More recently, enhancer-bound transcription factors have been shown tethered to proteins at the promoter region that are involved in histone modification or other types of chromatin remodeling complexes¹⁰. Based on these findings, the effects of insulators on enhancers could be explained by assuming that the insulator acts as a barrier that inhibits the processivity of a signal traveling from the enhancer to the promoter, without inactivating either. The leitmotiv of this scenario, sometimes dubbed the 'transcriptional model', is that the insulator acts as a promoter decoy, confusing the enhancer-bound transcription factor into interacting with the insulator instead of the transcription complex¹¹. This would result in the insulator trapping the enhancer, which then would not be able to interact with the promoter, thus precluding transcriptional activation. Another explanation, termed the 'structural model' envisions insulators

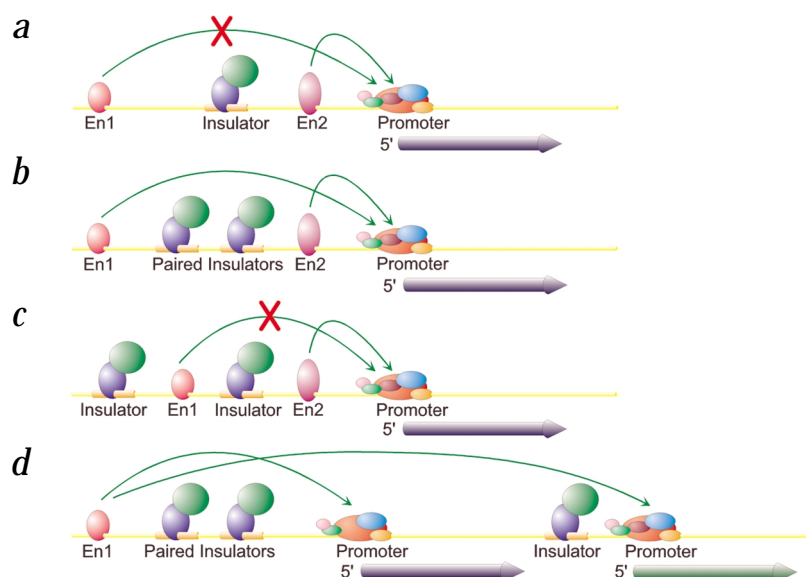


Fig. 1 Effects of insulators on enhancer–promoter interactions. The diagrams represent a generic gene, depicted in blue, with a series of proteins forming the transcription complex present in the promoter region. Two enhancers, named Enhancer1 and Enhancer2, serve as binding sites for transcription factors depicted in pink and purple, respectively. An insulator is shown in brown with two protein components represented as blue and green ovals. Green arrows indicate transcriptional activation by a specific enhancer. **a**, An insulator present between Enhancer1 and Enhancer2 affects the ability of the former to activate transcription (indicated by a red X) but not the latter. **b**, When an additional insulator is present between the two enhancers, the dual insulator cannot interfere with transcription activation by Enhancer1. **c**, The simple duplication of insulators is not responsible for their loss of function, since when Enhancer1 is present between the tandemly repeated insulator it is unable to activate transcription. **d**, In a more complex situation, a second reporter gene is present downstream from the first one. Transcription of this gene can also be activated by Enhancer1. When tandemly repeated insulators are present and an additional insulator is present between the two genes, both genes are transcribed.

as sequences that organize the chromatin fiber within the nuclear space, creating transcriptionally independent domains¹² by providing chromatin with anchoring points able to interact with some kind of fixed substrate, perhaps the nuclear matrix (see below). A way of explaining both the effect of insulators on enhancer–promoter interactions and their ability to buffer transgenes from position effects is to assume that the barrier is a consequence of the involvement of insulators in the establishment of domains of higher-order chromatin structure within the nucleus. The formation of these domains would then be the primary function of insulators, and their effect on enhancer–promoter interactions would be a secondary consequence of the establishment of these domains. Recent articles by Cai and Shen² and by Muravyova *et al.*³ seem to support this view.

Effects of insulators

Cai and Shen² used an elegant series of transgenic assays to assess the function of different combinations of enhancers and gypsy insulators in *Drosophila* embryos.

This insulator has been shown to contain a series of binding sites for a zinc finger protein called Suppressor of Hairly-wing [Su(Hw)], which in turn interacts with a second protein named Modifier of *mdg4* [Mod(*mdg4*)]. When positioned between the *zerknüllt* enhancer VRE (Enhancer 1 in Fig. 1) and the even-skipped E2 enhancer (Enhancer 2 in Fig. 1), the gypsy insulator exhibited the expected behavior, blocking only the promoter-distal VRE enhancer, while the promoter proximal E2 enhancer activated the *eve-lacZ* reporter gene (Fig. 1a). A striking result was obtained when a direct tandem repeat of insulators was used instead of a single copy. Not only was the insulating effect not reinforced, it was actually abolished, both enhancers being able to activate transcription (Fig. 1b). Control experiments involving other enhancers demonstrated that the loss of insulator activity is independent of the enhancer studied. Also, the distance between insulators can range from 50 to 170 base pairs with no impact on the result. Interestingly, when flanked by a pair of gypsy insulators, the activity of

the VRE enhancer is more efficiently blocked (Fig. 1c).

Similar observations were made by Muravyova *et al.*³ in their companion paper. Using *yellow* and *white* as reporter genes, they show that a tandem repeat of gypsy insulators does not have any insulating properties (Fig. 1b). Indeed, the authors note that enhancer–promoter communication in this configuration is improved. This holds true even when a 1.5 kilo base pair spacer was present between the two insulators, ruling out the trivial possibility of some steric hindrance between insulators. These authors contribute an additional important result. One of the constructs tested carried two copies of the gypsy insulator between the enhancers and the *yellow* gene, and an additional copy between *yellow* and *white*. In such a configuration both genes are fully active (Fig. 1d).

Role of insulators in chromatin domain organization

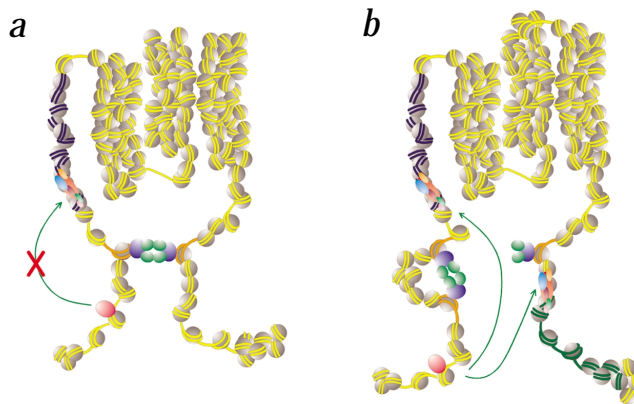
It is difficult to reconcile these observations with a transcriptional insulator model. For example, a reasonable prediction from the decoy model is that two insulators would be better than one. That is, a tandem repeat of insulators would reinforce the trapping of the enhancer. Similarly, if insulators were entry points for chromatin modifying enzymatic complexes, the presence of two insulators should result in a doubling of the efficiency with which chromatin remodeling complexes are brought to the promoter region. Rather, the results of both papers are more consistent with the structural model. In such a model, two insulators physically interact and promote the looping of the intervening sequence. As a consequence, the distance between enhancer and promoter is reduced, and transcription activation then takes place (Fig. 2). Similarly, an enhancer flanked by gypsy insulators may be looped out, resulting in inhibition of transcription. This demonstrates that the mere interaction between repeated copies of the insulator is not responsible for the inability to interfere with enhancer–promoter communication, but rather the special chromatin structure generated by the interaction of neighboring insulators.

Questions that remain

The ability of insulators to mediate the formation of loops in the chromatin fiber agrees with their possible role in the establishment of domains of chromatin

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Fig. 2 Formation of loop domains by an insulator. The diagrams show the chromatin fiber, with the histone octamers shown in gray and the DNA in yellow. As in Fig. 1, the coding regions of two putative genes are shown in blue green, the transcription complex at the promoter is shown by a series of multicolored ovals and enhancer-bound transcription factors are depicted in pink and purple. The insulator DNA is shown in brown and two insulator proteins are shown in blue and green. **a**, Interactions between insulator proteins result in the formation of a loop within the chromatin fiber, giving rise to a domain of higher-order chromatin organization. The enhancer present outside of this domain cannot activate transcription of the gene present inside of the domain (indicated by a red X). **b**, The presence of tandemly repeated insulators creates a small loop. An assumption of the experimental results is that the formation of this loop between adjacent insulators precludes interactions with other insulators. The enhancer is then present within the same domain as the gene and transcription activation takes place (green arrows).



organization. These type of loops have been observed experimentally¹³, and appear to be attached to a proteinaceous backbone by sequences known as SARs or MARs (scaffold and matrix attachment regions)¹⁴. Interestingly, the gypsy insulator has been shown to display SAR/MAR activity¹⁵. The structure of the loops generated by insulators, the dynamics of their assembly and the mechanism of their effects on the function of enhancers are yet to be understood. One intriguing possibility is that an enhancer/promoter pair has to reside within the same loop to interact (Fig. 2a). Two tandemly repeated insulators (Fig. 2b) may have a tendency to preferentially interact with each other at the exclusion of other insulators because of their physical proximity. The ability of the enhancer to activate a second distal gene, bypassing a third insulator, may be due to the inability of the third insulator to find an interacting partner. Conversely, if the third insulator is able to interact with the

paired one, the absence of insulator function could be explained by the proximity of the enhancer and the promoter region of the second gene, bypassing the requirement to reside within the same loop. These issues will need to be addressed before we have a clear picture of how insulators work.

The ability of insulators to confer transgenes position-independent expression makes these sequences extremely interesting from a practical point of view, as they can be used to construct transgenic animals and for gene therapy purposes, to ensure that genes are expressed at the proper levels and in the appropriate tissues. But equally enticing is the prospect of understanding how the DNA is organized in the nucleus. For too long the eukaryotic nucleus has been viewed as a black box where the cell carries the DNA. It is now time to unravel the mysteries of nuclear organization and bring this organelle to the same level of understanding as the rest of the cell.

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