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Review

Protecting against promiscuity: the regulatory role of insulators

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Abstract. Eukaryotic genomes contain transcriptional regulatory elements that alter promoter activity through long-range interactions. Many control elements show a broad range of promoter interactions, suggesting that these elements are capable of inappropriate transcription. The identification of a novel class of directing regulatory elements, called insulators, has provided clues into mechanisms used in eukaryotic genomes to maintain transcription fidelity. Insulators contribute to the organization of independent domains of gene function by restricting enhancer and silencer function. This review describes the properties of insulators and related elements that have been isolated from several eukaryotic genomes. Two classes of models of insulator function are considered. These models provide insights into possible mechanisms used by these diverse elements to provide regulatory autonomy.

Key words. Chromatin; CTCF; enhancer; *gypsy*; HS4; insulator; silencing; transcription.

Eukaryotes contain thousands of genes whose unique patterns of expression establish distinct cellular identities. These processes require coordinate transcriptional regulation of genes randomly distributed in the genome. In eukaryotes, the template for transcription is chromatin, representing the complex of DNA, histones, and non-histone proteins that comprise chromosomes.

Chromatin is not uniformly organized along the length of a chromosome. In most cells, chromosomes are organized into two types of chromatin domains that can be cytologically distinguished [1–3]. Euchromatin encompasses chromosomal regions that are decondensed in interphase cells. These regions are gene rich, are associated with disorganized nucleosomal arrays enriched in specific histone modifications, such as acetylation, and show increased protein accessibility, as assayed using nucleases. In contrast, heterochromatin encompasses chromosomal regions that remain condensed in non-dividing cells. These regions are gene poor, are associated with regular nucleosomal arrays that are hypoacetylated, and show a compact structure, observed as a decreased accessibility to nuclease probes. Heterochromatin represses transcription of euchromatic genes that are translocated into these domains [4–7]. Interestingly, genes that normally reside in heterochromatin require this chromatin environment for expression, as translocation of heterochromatic genes into euchromatin causes a loss in expression [8–11]. These position effects imply that appropriate patterns of gene expression require the correct establishment and maintenance of independent chromatin domains.

Gene-specific transcription factors can alter chromatin structure. These factors work through several classes of DNA control elements. Short-range regulation of RNA production depends upon promoter regions that contain both core elements that bind the general transcriptional machinery and promoter-proximal sequences that bind general activators. In contrast, enhancers and silencers act bi-directionally over long distances to control spatial and temporal patterns of transcription. In many cases, long-distance transcriptional effects depend upon recruitment

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of chromatin-modifying proteins by DNA-binding proteins [12, 13]. Long-range elements, such as enhancers and silencers, show limited promoter specificity [14, 15], suggesting that they have the capacity to modulate transcription of inappropriate genes. This possibility is underscored by recent observations that chromosomes are highly mobile in the nucleus. Such extensive motion of chromosomes may allow illegitimate interactions between enhancers, silencers, and promoters [16]. Mechanisms must exist to prevent inappropriate regulatory interactions between elements so that long-range control is maintained.

Insulators and related elements

Insulators represent a novel class of DNA sequences that constrain regulatory interactions within eukaryotic genomes. These elements restrict enhancer and silencer function and contribute to the generation of independent gene regulation within heterochromatic and euchromatic domains. Insulators are operationally defined by two functional properties. First, they protect gene expression from positive and negative chromatin effects caused when transgenes are integrated at random positions within a genome. Second, insulators block enhancer-activated transcription when the insulator is interposed between an enhancer and promoter, but not when the insulator is positioned upstream of the enhancer. Disruption of enhancer-activated transcription does not interfere with promoter function, since basal transcription of the target promoter is not affected [17–19]. Similarly, enhancer blocking does not result in inactivation of enhancers [18, 20]. These data suggest that insulators interfere with mechanisms used in signalling between long-distance regulatory elements and promoters.

Insulators and related sequences have been identified in several organisms (table 1). In many cases, an element has been classified as an insulator if it possesses at least one of the two functional properties. However, this classifica-

Table 1. Summary of insulator and insulator-like sequences identified in many organisms.

Name	Organism	Size (kb)	Origin	Classification	References
gypsy	fruit fly	0.35	*gypsy* retrotransposon	insulator	4, 17, 19, 75
scs	fruit fly	0.90	*87A7* locus, promoter region *CG14732*	insulator	55, 56
scs′	fruit fly	0.50	*87A7* locus, promoter region of *aurora* and *CG3281*	insulator	55, 56
eve promoter	fruit fly	0.03	promoter of *even skipped* gene	anti-enhancer	185
Fab-7	fruit fly	1.20	3′ regulatory region of *Abd-B*.	insulator	43, 112
Fab-8	fruit fly	0.59	3′ regulatory region of *Abd-B*.	insulator	116, 117, 193
Fa^{swb}	fruit fly	1.3	5′ region of *Notch* gene	insulator	165
BE76	fruit fly	0.32	upstream of *IMPdH* gene at the *raspberry* locus	anti-silencer	66
BE28	fruit fly	0.27	present at 400 chromosomal sites	insulator	65, 66
Idefix U3	fruit fly	0.47	LTR of *Idefix* retrotransposon	anti-enhancer	189
Alu2	human	0.32	SINE element	insulator	23
Apolipoprotein B 5′ boundary	human	1.8	5′ end of *apolipoprotein B* gene	insulator	34, 123
Apolipoprotein B 3′ MAR	human	0.79	3′ end of *apolipoprotein B* gene	anti-silencer	123
α1-ATR MAR	human	4.1	*α-1-antitrypsin-like* gene	anti-silencer	37
Kcnq1 ICR	human	3.6	intron of *Kcnq1* gene	anti-enhancer	51
BEAD-1	human	2.5	between *TCRα* and *Dad 1* genes	anti-enhancer	30
HS2–6	human	2.8	between *TCRα* and *TCRδ* genes	anti-enhancer	124
DM1	human	0.40	at CAG repeat between *DMPK* and *SIX5*	anti-enhancer	139
H19 ICR	mouse	2.0	5′ region of *H19* gene	anti-enhancer	49, 50, 144
5′ A element	chicken	2.9	5′ region of *lysozyme* gene	anti-silencer	25, 38, 39
HS4	chicken	0.25	upstream of *β globin* cluster	insulator	26, 33, 35
3′ HS	chicken	0.40	between *β globin* cluster and *OR* gene	anti-enhancer	22
RO	frog	1.3	rDNA cluster	anti-enhancer	126
sns	sea urchin	0.26	3′ end of *H2A* gene in histone gene cluster	anti-enhancer	194
Ars	sea urchin	0.57	5′ region of *arylsulphatase* gene	insulator	29, 40, 195
HMR $tRNA^{Thr}$	budding yeast	0.30	boundary of *HMR* silent mating type locus	anti-silencer	153, 159
CHA1 promoter	budding yeast	ND	gene near *HML* silent mating type locus	anti-silencer	153
UAS_{rpg}	budding yeast	0.10	control regions of *TEF* genes and ribosomal protein genes	anti-silencer	158
X-STAR	budding yeast	0.30	subtelomeric repeat	anti-silencer	161, 196
Y′-STAR	budding yeast	0.14	subtelomeric repeat	anti-silencer	24, 161
IR-L and IR-R	fission yeast	2.0	inverted repeat flanking silent mating type locus	anti-silencer	196

tion strategy should be used cautiously. Recently, functional dissection of two insulators demonstrated that the enhancer-blocking and protection against position effects are conferred by distinct sequences, uncoupling these activities [21–23]. These data argue that not all elements that possess one insulator property will possess both. Further, they indicate that insulators may utilize many mechanisms to impart regulatory autonomy.

We propose that a new terminology be adopted to clarify the properties of a given modulator element. We suggest that the term insulator be reserved for sequences that both block enhancer-activated transcription and confer position-independent expression. We propose that sequences only capable of blocking enhancer function be called anti-enhancer elements. Finally, we propose that the term anti-silencer, first introduced by Gilson and colleagues [24], be adopted for elements that have only been demonstrated to block the action of transcriptional repressors or are only known to protect against position effects, as the majority of position effects appear to be negative [see for example refs 25, 26]. In some cases, anti-silencers have been called barriers, to reflect their distinction from insulators [27, 28].

Several insulators and anti-enhancer elements prevent enhancer function when tested in different organisms [22, 29–33]. Similarly, many insulators and anti-silencers have the ability to block chromatin-mediated repression in different organisms [23, 26, 33–41]. Based on these observations, insulators and insulator-like sequences appear to be essential components of eukaryotic genomes that are required for establishment of appropriate levels of gene expression.

Insulators are not permanent, impassable elements. Certain conditions have been identified where insulators may be overcome or bypassed. Insulator effectiveness is influenced by the nature of the enhancer, promoter, and genomic context [42–47]. Furthermore, insulator function can be regulated [48–51] and can show tissue specificity [52]. These observations imply that insulators participate in diverse ways in the regulation of transcriptional activation and repression.

The discovery of insulators

The *Drosophila* specialized chromatin structure insulators, scs and scs′, were the first identified (table 1). These insulators are located in the *87A7* region of chromosome 3, flanking a pair of divergently transcribed *heat shock protein* (*hsp*) *70* genes. Upon heat shock, a high level of *hsp70* transcription occurs that causes a reversible decondensation of the local chromatin structure, visualized as a puff in the larval salivary gland polytene chromosomes. At the boundaries of the puff are regions of unusual chromatin structure that define the scs and scs′ insulators [53–56]. These observations led to the proposal that insulators are domain boundaries that control concerted changes in chromatin organization, such as those associated with puffing [54–56].

The chromatin structure of the scs and scs′ insulators is similarly organized, including two sets of strong nuclease-hypersensitive sites that separate a nuclease-resistant core. However, these insulators do not share significant DNA sequence identity [57]. The scs insulator is a modular element, with sequences from both hypersensitive regions contributing to the enhancer-blocking function [44, 58]. Enhancer blocking by monomers of each hypersensitive region is reduced compared to the complete scs insulator, but is restored by multimerization. These observations suggest that scs contains different sequences that are functionally equivalent and that contribute additively to insulator function [44]. The scs′ insulator also appears to have a modular structure. The two scs′ Dnase-I-hypersensitive regions share a cluster of CGATA sequences [59]. A partial block of enhancer function is conferred by a subfragment of scs′ containing one CGATA cluster, while multimerization of the CGATA clusters reconstitutes blocking [59]. Taken together, these data suggest that the full-length scs and scs′ insulators assemble multiple protein complexes on a single insulator that cooperate to confer enhancer blocking.

One component of the scs nucleoprotein complex is scs binding protein, SBP, that contains eight zinc fingers and binds a 24-bp region of scs (table 2) [58]. Multimers of an oligonucleotide containing an SBP-binding site partially reconstitute enhancer-blocking activity [58]. Chromatin immunoprecipitation studies demonstrated that SBP is associated with scs in vivo [58]. SBP is encoded by the *zeste-white (zw) 5* gene whose function is essential for cell proliferation and differentiation [60]. For this reason, testing the effects of mutations in *zw5* on the function of a complete scs insulator is difficult. Instead, effects of the reduction in *zw5* activity were tested on a compromised insulator that contained low-affinity SBP-binding sites. Results from these studies support a role for SBP in scs function [58].

The scs′ insulator binds two proteins produced by alternative splicing, boundary-element-associated factors (BEAF32 A and BEAF32 B; table 2). These proteins share a carboxy-terminal domain that has similarities with leucine zipper motifs that promote protein-protein interactions [59, 61]. The unique amino-terminal domains of BEAF32 A and 32 B mediate DNA binding with slightly different sequence specificities [61]. BEAF binds DNA as a trimer, with specificity for the CGATA sequence [61]. Immunolocalization studies showed that BEAF binds to many sites throughout euchromatin, with distinct combinations of BEAF isoforms at different genomic sites. These sites include a number of puff boundaries and scs′, but not scs [59]. No mutations in the gene encoding BEAF

Table 2. Proteins implicated in the function of insulator and insulator-like sequences.

Protein	Associated region	Protein motifs	References*
Su(Hw)	*gypsy*	12 zinc fingers, two acidic domains, leucine zipper	90, 91, 96, 97
Mod(mdg4)67.2	*gypsy*	BTB/POZ oligomerization domain, acidic domain	99, 100, 106, 107
SBP/Zw5	scs	8 zinc fingers, acidic domain	58
BEAF32A, BEAF32B	scs′, BE76, BE28	BED finger DNA-binding domain, dimerization domain	59, 61
DREF	scs′, BE76, BE28	BED finger DNA-binding domain	48
GAGA	*eve* promoter	BTB/POZ, zinc finger domain	185
D1	BE28	HMG DNA-binding domain (AT hook)	65
CTCF	HS4, 3′ HS, BEAD-1, H19 ICR, DM1, RO, Tsix, *Kcnq1* ICR	11 zinc fingers	22, 49–51, 130, 139, 140, 150
TFIIIB	*HMR tRNA^{Thr}*	three subunits: TBP, TFC5, BRF	28
TFIIIC	*HMR tRNA^{Thr}*	Pro-rich repeats, acidic domain	28
Gcn5p	*HMR tRNA^{Thr}*	histone acetyltransferase domain, bromodomain	153
Sas2p	*HMR tRNA^{Thr}*	MYST family histone acetyltransferase, zinc finger domain	153
Scm1p/Scm3p	*HMR tRNA^{Thr}*	coiled-coil domain	153
Rap1p	*UAS$_{rpg}$*	BRCT domain, myb-like domain	161
Reb1p	*STAR-X,Y*	two myb-like domains	161

* References refer to paper(s) that describe the role of the protein in the function of the insulator or insulator-like sequence

are known, precluding analysis of the effect of the loss of this protein on scs′ insulator function. Recently, a third protein, called DREF, was identified as an scs′ binding protein (table 2) [61]. DREF has weak affinity for the BEAF binding sites in scs′. The overlapping DNA-binding specificity of DREF and BEAF suggests that these proteins may compete for DNA binding and regulation of target gene expression [61].

The *87A7* region is transcriptionally complex. In addition to the divergent pair of *hsp70* genes, three transcription units have been identified, including *aurora* that encodes a kinase involved in cell cycle regulation, and two genes of unknown function (*CG14732, CG3281*) [62–64]. The relationship between the transcriptional regulation of the non-*hsp70* genes and insulator function is unclear. Interestingly, the scs and scs′ insulators contain the promoters of these genes, with SBP- and BEAF-binding sites located within 100 bp of the *CG14732* and *CG3281* promoters, respectively. These data suggest that SBP and BEAF may play a direct role in the expression of genes in the *87A7* locus.

Genomic sites of BEAF association were isolated to identify other potential insulators (table 1). Two sites, BE28 and BE76, have been characterized [65, 66]. BE28 is an insulator, shown to protect against position effects and positionally block enhancer activated transcription [65]. BE28 represents a subfragment of a 1.2-kb moderately repetitive sequence that contains binding sites for BEAF and a second protein, D1 [65]. D1 is an AT hook DNA-binding protein that binds satellite DNA [67]. BE28 elements map to pericentric regions of several *Drosophila* chromosomes, indicating that BE28 may help define the boundary between heterochromatin and euchromatin [65]. The second BEAF-containing sequence, BE76, represents the –350 to –670 region of the *inosine monophosphate dehydrogenase* gene, and confers position-independent expression in vivo [66]. The role that BE76 plays in the regulation of gene expression has not been explored.

The *gypsy* insulator

A second well-characterized *Drosophila* insulator is the *gypsy* insulator (table 1). This element was identified as the region of the *gypsy* retrotransposon (also known as *mdg4*) responsible for causing tissue-specific mutations of several genes [68–72]. The *gypsy* insulator resides within the 5′ untranslated region of the *gypsy* retrotransposon [73]. This region contains 12 copies of a degenerate sequence, with a core of TGCATA embedded in AT-rich sequences.

The *gypsy* insulator can affect the function of a large number of *Drosophila* enhancers that are active in many different tissues throughout *Drosophila* development [4, 17–20, 52, 69, 71, 74–79]. The *gypsy* insulator also protects against several types of repressive transcriptional effects, including silencing by Polycomb group proteins and heterochromatin [4, 41, 80–83]. Finally, the *gypsy* insulator protects a chromosomal DNA replication origin from repression [84–86]. These data suggest that the *gypsy* insulator is a very versatile modulator of regulatory interactions.

The Suppressor of Hairy-wing [Su(Hw)] protein is essential for *gypsy* insulator function (table 2). This discovery followed from the observation that mutations in the *su(Hw)* gene reverse the tissue-specific phenotypes of

gypsy-induced alleles [69, 87–89]. The Su(Hw) protein is expressed throughout *Drosophila* development in most, if not all, tissues [90, 91], consistent with the observation that the *gypsy* insulator interferes with the function of a variety of transcriptional modulators. Null *su(Hw)* alleles are female sterile, suggesting a specific function for this protein in oogenesis [87, 92].

The role of the Su(Hw) protein in the regulation of the *gypsy* retrotransposon is unclear. This protein may be an activator of *gypsy* transcription, as levels of *gypsy* RNA decrease in *su(Hw)* mutants [93, 94]. The recent demonstration that the insertion of a *gypsy* insulator close to a core promoter increased levels of transcription supports this contention [95]. Alternatively, the lowered level of *gypsy* RNA accumulation in *su(Hw)* mutants may reflect the loss of an insulator that prevents silencers, yet to be identified, in the body of the *gypsy* retrotransposon from acting on the *gypsy* promoter residing in the LTR.

Several structural motifs have been identified in the Su(Hw) protein (table 2). These include amino- and carboxy-terminal acidic domains, a 12-zinc-finger DNA-binding domain, a leucine zipper region, and three additional regions that are conserved among other *Drosophila* species [90, 91]. The zinc finger domain is essential for Su(Hw) function [91, 96, 97]. In addition to its DNA-binding function, this domain interacts with Chip, a proposed facilitator protein [98]. The carboxy-terminal region of the Su(Hw) protein, including the conserved B and C regions and the leucine zipper, is essential for enhancer blocking by the *gypsy* insulator [91, 96, 97]. Interestingly, these motifs are dispensable for blocking repression caused by Polycomb group proteins and centric and telomeric heterochromatin [R. R. Roseman and P. K. Geyer, unpublished results], suggesting that different regions of the protein are required to block silencers.

A second protein, Mod(mdg4)67.2, is required for some functions of the *gypsy* insulator (table 2) [99, 100]. Mod(mdg4)67.2 is the most abundant isoform encoded by the *mod(mdg4)* locus, which gives rise to an additional 20 proteins [101]. This protein complexity underscores a wide diversity of functions associated with the *mod(mdg4)* locus that includes regulation of synapse specificity [102], apoptosis [103], position effect variegation [100, 104], and homeotic gene expression [104, 105]. All Mod(mdg4) isoforms share an amino-terminal 402-amino-acid domain that includes a BTB/POZ motif that is involved in homodimerization and interactions with other proteins [101]. The Mod(mdg4) BTB/POZ domain interacts with Chip, suggesting that both of the known *gypsy* insulator proteins may interfere with the function of this facilitator [106]. The carboxy-terminal domain of Mod(mdg4)67.2 contains a highly acidic region that is unique among the isoforms [100, 101, 104]. This domain interacts with the Su(Hw) protein, specifically linking the Mod(mdg4)67.2 isoform with *gypsy* insulator function [106, 107].

Mutations that affect only Mod(mdg4)67.2 have diverse effects on *gypsy* insulator function. The loss of Mod(mdg4)67.2 suppresses enhancer blocking by the *gypsy* insulator at some genes, while enhancer blocking remains intact at others [52, 77, 99, 106, 108]. Furthermore, the absence of Mod(mdg4)67.2 enhances some *gypsy*-induced phenotypes as a result of promoter silencing [77, 99, 108]. Mod(mdg4)67.2 is not required for prevention of position effects by the *gypsy* insulator or for protection against silencing by Polycomb group proteins, consistent with the observation that the Mod(mdg4) interaction domain of the Su(Hw) protein is also not required for these processes [81; R. R. Roseman and P. K. Geyer, unpublished observations]. These data suggest that while Mod(mdg4)67.2 is involved in some *gypsy* insulator functions, under certain conditions, its activity is either not needed or can be provided by other *gypsy* insulator-associated proteins. Additionally, the Mod(mdg4)67.2 isoform may not be required for all functions of the Su(Hw) protein, as a loss of this isoform does not cause female sterility [99].

Insulators impart functional autonomy to complex regulatory domains

Insulators play a critical role in defining domains of gene function within eukaryotic genomes. This is illustrated by the regulation of the *Drosophila Abdominal B* (*Abd-B*) gene, one of the three genes in the bithorax complex. The *Abd-B* gene has an extensive 3′ regulatory region, which contains at least two insulators (Fab-7 and Fab-8) and a region (Mcp) that has some insulator properties [109–117]. The *Abd-B* insulators are associated with the borders of independent regulatory domains, a property that has led to their classification as boundary elements [109, 111]. For example, the Fab-7 insulator separates regulatory domains that contain the *infra-abdominal* (*iab*) enhancers, *iab-6* and *iab-7*. Deletion of Fab-7 changes *Abd-B* gene expression in a complex manner, reflecting both a gain and loss of *Abd-B* activity in specific regions of the developing embryo [109–111, 117, 118]. These effects imply that the Fab-7 insulator maintains the autonomy of two control regions, with its loss generating a new domain with a distinct function.

An apparent paradox is presented by the location of the Fab-7 and Fab-8 insulators, as they reside between functional enhancers and the *Abd-B* promoter [111, 119]. These observations suggest that the *Abd-B* locus contains sequences that allow enhancers to overcome or bypass intervening insulators. Several studies have uncovered sequences that may be responsible for the differential effectiveness of the *Abd-B* insulators. For example, the *Abd-B* promoter contains a large (> 7.6 kb) ‘tethering’ region that mediates communication with *iab* regulatory sequences,

even when the *Abd-B* promoter and the *iab* elements are on separate chromosomes [120–122]. This tethering region may be optimized to capture enhancers, thereby reducing the blocking ability of insulators. In addition, a 0.6-kb region, known as the promoter-targeting sequence or PTS prevents enhancer blocking by the Fab-8 and the *gypsy* insulators when tested in transgenes [116]. The PTS has been suggested to stabilize interactions between enhancers and certain promoters, thereby permitting the *iab* enhancers to overcome an insulator-mediated block. Each autonomous regulatory domain within the large *Abd-B* regulatory region may contain a PTS-like element that allows enhancers to bypass an insulator, without interfering with the definition of an independent regulatory domain. Alternatively, Fab-7 and Fab-8 may be weak insulators that have a limited capacity to attenuate enhancer function, a suggestion that is supported by the transgene assays [43, 112, 117]. This possibility is further strengthened by the finding that substitution of Fab-7 with strong insulators, like *gypsy* and scs, caused a loss of *Abd-B* activation by upstream enhancers [52]. Regardless, these data imply that insulators are not interchangeable within the genome. Instead, they provide specific functions for gene regulation [52].

A common player in vertebrate insulators

Vertebrate genomes possess a large collection of insulator and insulator-related sequences (table 1). As seen in *Drosophila*, some of these elements reside in complex regulatory regions and may help define independent domains of gene function [30, 34, 123–125]. Others are associated with repetitive DNA sequences, including rDNA repeats and SINES [23, 126]. Taken together, these observations imply that insulators have a broad genomic distribution in vertebrates.

One of the best-characterized vertebrate insulators is the chicken *β-globin* hypersensitive site 4 (HS4). This insulator was identified as a constitutive HS site that demarcates the 5′ boundary of a 30-kb domain that contains four developmentally regulated *β-globin* genes [33]. The HS4 insulator separates an open chromatin domain, containing the *globin* genes, from an upstream 16-kb region of condensed chromatin [21, 22, 125, 127–129]. Based on this location, HS4 has been suggested to protect the *globin* locus from silencing spreading from the repressed domain and/or block inappropriate cross-regulation from enhancers associated with the folate receptor gene that resides upstream of the repressed domain [125]. Tests of these ideas await studies where the HS4 insulator has been deleted from this region. Examination of histone modifications across the 54-kb domain that included the *globin* locus and the two neighboring genes demonstrated that the HS4 insulator represents a strong constitutive focus of histone hyperacetylation [21, 129]. These observations support the view that HS4 prevents heterochromatic spreading by recruiting histone acetylases that direct histone tail modifications that terminate the propagation of repressive chromatin [21, 129].

The HS4 insulator has a modular structure. Footprinting studies using human erythroleukemia nuclear extracts demonstrated that the HS4 core insulator contains several protein-binding sites (footprints I–V) [35]. FII contains a single binding site for the CCCTC-binding factor (CTCF) that is necessary and sufficient for enhancer blocking [130]. As mentioned above, CTCF sites do not protect genes from chromosomal position effects [discussed in refs 21, 22, 130], which depends upon other DNA sequences within the HS4 core insulator. These data demonstrate that these properties of the HS4 insulator can be uncoupled, implying that they may be conferred in mechanistically distinct ways.

CTCF is a ubiquitously expressed, 11-zinc-finger DNA-binding protein that is highly conserved (table 2) [131]. This protein is a versatile regulator of transcription, acting as an activator [132, 133], a repressor [134–138], or as an insulator protein at different target genes [50, 51, 130, 139, 140]. These diverse transcriptional effects are proposed to occur because CTCF uses different zinc fingers to bind gene-specific regulatory elements [131, 135, 141]. As the CTCF zinc finger domain appears to recruit partner proteins, such as histone deacetylases, the read out of CTCF association at a given gene may depend upon which fingers are available for protein-protein interactions after DNA binding [136].

The enhancer-blocking activity of CTCF can be regulated. This was discovered in studies of the *H19-Igf2* pair of imprinted genes [50, 130]. Genomic imprinting is an epigenetic modification that causes parent-of-origin-specific expression of genes. *H19* and *Igf2* share a set of enhancers located downstream of *H19* [142–145]. Expression of *H19* and *Igf2* is monoallelic, such that *Igf2* is only expressed from the paternally inherited allele and *H19* is expressed only from the maternally inherited allele [146, 147]. Chromosome-specific expression patterns are regulated by differential DNA methylation of the imprinting control region (ICR) that resides between *Igf 2* and *H19*, in a region 2–4 kb upstream of the *H19* promoter [148, 149]. This ICR contains four CTCF-binding sites [49, 50, 150]. On the maternal allele, the ICR is unmethylated, allowing CTCF association and the formation of an anti-enhancer element. As a result, the action of the downstream enhancers is limited to *H19* and no activation of *Igf2* occurs. In contrast, the ICR on the paternal allele is hypermethylated at CpG dinucleotides. When methylated, these sequences do not bind CTCF, enhancer blocking is lost, and activation of the *Igf2* gene occurs. Further, as methylation spreads from the ICR into the *H19* promoter region, a loss of *H19* expression is observed [151]. These data im-

ply that the effectiveness of anti-enhancers, and by extension, insulators may be modulated. The activity of other insulator and insulator-like sequences is also likely to be regulated [48, 51, 152, 153].

CTCF plays a major role in the enhancer-blocking activity of many vertebrate insulators and anti-enhancer elements (table 2). For example, CTCF sites are found in the 5′ boundary of the chicken lysozyme gene [130], the *Xenopus* repeat organizer [130], the myotonic dystrophy DM1 element [139], the human T cell receptor BEAD element [130], the promoter of the anti-sense *X*-inactive specific transcription gene, *Tsix* [140] and the *Kcnq1* imprinting control region [51]. This broad involvement of CTCF suggests that it plays an important role in defining autonomous expression domains in vertebrates.

Small genomes contain insulator-like sequences

In budding yeast, the genome is compact, with nearly 75% of the DNA sequences representing genes. This organization places regulatory sequences in close proximity, suggesting that inappropriate regulatory interactions may be possible. However, the majority of activating sequences, collectively referred to as upstream activating sequences (UASs), are distance limited, indicating that insulators may not be needed to maintain activator fidelity. In contrast, long-range silencers have been identified in budding yeast [154–157]. Interestingly, anti-silencers have been found that confine the spread of repression emanating from these silencers [24, 153, 158–161].

One anti-silencer, $tRNA^{Thr}$, was identified at the edge of the repressed domain associated with the *HMR* locus. This domain contains copies of the mating-type-specific *MATa* genes that are inactive due to the action of the flanking *HMR-E* and *HMR-I* silencers. Silencing requires recruitment of a complex of Sir proteins and other factors that propagate along nucleosomes, forming condensed chromatin that shares some features with metazoan heterochromatin [157, 162]. *HMR* silencing is limited by the *tRNA* gene, $tRNA^{Thr}$ [153, 159]. This *tRNA* gene is required for regulation of gene expression in the neighborhood of the *HMR* locus, as deletion of the $tRNA^{Thr}$ gene causes silencing of the downstream *GIT1* gene [153]. Interestingly, the anti-silencer action of $tRNA^{Thr}$ depends upon the internal core promoter elements for RNA polymerase III, suggesting that mitigation of repression may require recruitment of histone-modifying enzymes, such as acetylases. This proposal is further supported by observations that mutations in two genes that encode histone acetylases, *SAS2* or *GCN5*, reduce anti-silencer activity of the $tRNA^{Thr}$, whereas direct targeting of acetylases to this region reconstitutes the HMR chromatin boundary [153]. Similarly, DNA sequences that bind activators limit the spread of repression established by silencers at the related *HML* locus. These sequences include the *TEF2* and *CHA1* UAS sequences [153, 158]. Mutations in the *SMC1* and *SMC3* genes that encode proteins involved in chromosome condensation and cohesion also diminish $tRNA^{Thr}$ anti-silencer function, indicating that chromosome architecture plays a role in these processes [159].

The telomeres represent a second region of silent chromatin in budding yeast. Telomeric repression is established by the Rap1 and Sir proteins [155]. The boundary between active and silent telomeric chromatin is defined by repetitive sequences within the subtelomere, called X and Y′, that protect reporter genes from silencer-dependent repression [24, 160]. These anti-silencers are known as STARs, for subtelomeric anti-silencing regions. STARs are modular in nature, being composed of several DNA elements, including binding sites for the Reb1 and Tbf1 proteins that each reconstitute anti-silencer activity when multiple proteins are bound [161]. Reb1p is a weak activator [163], connecting anti-silencer action with transcriptional processes (table 2). Similar to findings at *HMR*, targeting of transcriptional activators to an array of reiterated binding sites positioned adjacent to the telomere prevents the propagation of telomeric repression [161]. These anti-silencer effects do not depend upon the transcriptional activation of a reporter gene [161], implying that protection from silencing is due to modifications of the local chromatin that interfere with the propagation of repressive chromatin.

Models of insulator function: insulators as domain boundaries

Early models of insulator action linked observations of the physical organization of chromosomes with the functional demonstration that insulators protect gene expression from influences of the surrounding chromatin. Such structural models propose that insulators have a primary effect on the organization of higher-order chromatin structures, with secondary effects on transcription [44, 105, 164, 165]. In this context, insulators have been suggested to assemble specialized nucleoprotein complexes that interact with other insulator complexes or nuclear substructures to demarcate looped chromatin domains (fig. 1 A). Independence of gene function results from topological constraints imposed by the organization of higher-order chromatin structures within each defined domain. As such, structural models suggest that insulators are equivalent to elements that form domain boundaries. A related version of this model hypothesizes that protein complexes assembled on insulators direct genes to the nuclear matrix, with the concomitant change in gene location being responsible for the imposition of regulatory isolation [166]. Structural models account for both properties of insulator action, as prevention of positive and negative regula-

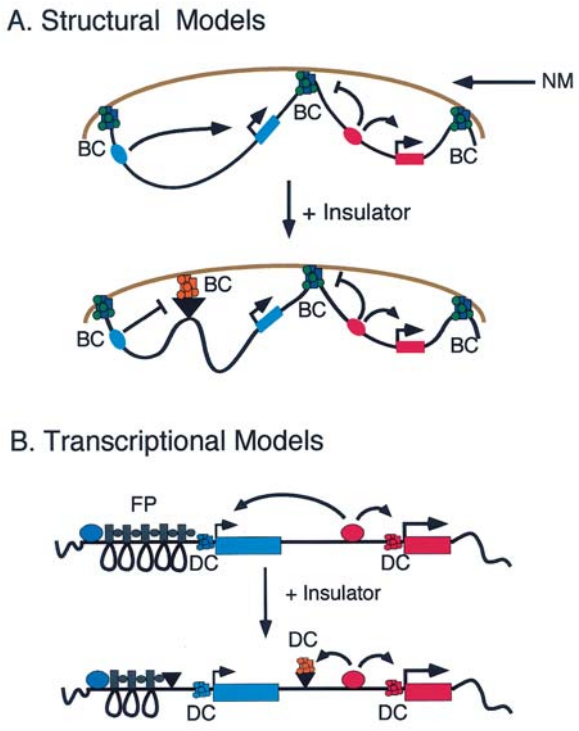

Figure 1. Models of insulator function. (*A*) Structural models of insulator function. Chromosomes are divided into independent functional units by specialized nucleoprotein complexes called boundary complexes (BC) that associate with the nuclear membrane (NM) or other nuclear structures, such as the matrix. Each chromatin domain is assembled into higher-order chromatin structures that prevent regulatory interactions between transcriptional components in different domains. An insulator (triangle) prevents enhancer (oval) activation of transcription by assembling a complex of proteins that resembles domain boundary complexes. Insertion of an insulator between an enhancer and promoter subdivides a single domain into two, resulting in a separation of the enhancer and promoter and a loss of enhancer-activated transcription. Furthermore, an insulator may change the nuclear compartment of a gene through its interaction with the nuclear periphery. (*B*) Transcriptional models of insulator function. Enhancer-activated transcription is proposed to require sequences near core promoter elements, docking sites, that bind nucleoprotein complexes (DC) that stabilize interactions between enhancer-binding proteins and the basal transcriptional machinery. Very long range interactions may require additional, facilitator (FP), proteins that link up with each other to decrease the apparent distance required for transmission of an enhancer signal. An insulator may prevent enhancer-activated transcription by assembling a nucleoprotein complex similar to the docking complex, decoying the enhancer away from the basal transcriptional machinery (right insulator). Alternatively, the insulator may disrupt the ability of facilitator proteins to shorten the enhancer-promoter distance (left insulator).

tory interactions is accommodated by the same mechanism.

Several lines of evidence support structural models. First, some insulators, such as scs/scs′, Fa^{swb}, and HS4 are located at the boundaries of distinct chromatin domains. In at least one case, changes in chromosome morphology are associated with insulator loss; deletion of Fa^{swb} results in the elimination of a band in the giant larval polytene chromosomes [165], supporting its role in establishing higher-order chromatin structures. Second, mutations in genes encoding proteins involved in higher-order chromatin organization affect the function of the *gypsy* insulator and the *tRNA^{Thr}* anti-silencer [99, 105, 159]. Third, at least one insulator, *gypsy*, appears to be localized within special subregions in the nucleus. Immunolocalization of the *gypsy* insulator proteins shows a punctate nuclear distribution pattern in diploid cells, indicating that *gypsy* insulators coalesce to form structures termed insulator bodies [97, 105]. These data led to the proposal that the *gypsy* insulator bodies generate looped chromatin domains that preclude interactions between regulatory elements residing in distinct domains (fig. 1) [166]. However, the formation of *gypsy* insulator bodies may not be essential for all insulator functions. Surprisingly, mutations in *mod(mgd4)* that disrupt aggregation of *gypsy* insulators do not reverse all of the enhancer-blocking or protection-from-position effects [52, 99, 107]. Finally, support for structural models comes from observations that the *gypsy* insulator alters the nuclear positioning of the *gypsy*-associated DNA sequences. The targeting of loci to the nuclear periphery requires the Su(Hw) and Mod(mdg4) proteins [166]. Findings that the *gypsy* insulator contains consensus binding sites for topoisomerase II and associates with nuclear matrices isolated from *Drosophila*, murine, and human cells provides insights into mechanisms for the repositioning of *gypsy*-associated sequences [167]. These data imply that the *gypsy* insulator is a matrix attachment region (MAR). However, a synthetic *gypsy* insulator corresponding to reiteration a single binding site that lacks the MAR motifs blocks enhancer-activated transcription, suggesting that enhancer blocking may be separable from matrix attachment [45]. Furthermore, the nuclear position of transgenes that contain only the *gypsy* insulator, not the intact retrotransposon, did not change when the Su(Hw) protein was lost, even though this protein provided for protection of the usage of a replication origin housed within the transgene [85]. These studies suggest that some *gypsy* insulator effects can be conferred without changing nuclear positioning.

Much support for structural models of insulator function has been derived from studies of the *Drosophila* scs, scs′, *gypsy*, and Fa^{swb}, and the vertebrate HS4 insulators. Yet, some experimental data obtained using these insulators do not fit with this class of models. For example, several insulators, including *gypsy*, scs, scs′, and HS4, do not require chromosomal integration to block enhancer-activated transcription [23, 31, 32, 75, 79, 168, 169]. In all of these cases, enhancer blocking was reconstituted within small episomes. These observations suggest that higher-order chromatin structures are not required to impart regulatory isolation. Furthermore, a single HS4 insulator present on a linearized episome prevented enhancer-promoter interactions, implying that the formation of a looped do-

main is not essential for the function of this insulator [168]. Second, insulators may be more effective at preventing interactions between transcriptional proteins than proteins involved in other nuclear processes. Interactions between the yeast FLP recombinase complexes were not prevented by the *gypsy*, scs, or scs′ insulators [31, 169], demonstrating that insulators are not impassable blocks for all protein interactions. Third, the effectiveness of the *gypsy* insulator can be altered by topology effects imposed by a paired, homologous chromosome [46]. If insulators organize structural domains within chromosomes, then the pairing of homologous chromosomes carrying structurally altered alleles would not be expected to change this domain organization.

Models of insulator function: insulators as transcriptional decoys

The transcriptional class of insulator models presents an alternative view of insulator function [95, 169, 170]. These models suggest that insulators have a primary effect on transcriptional processes, with secondary consequences on chromatin organization. In this context, insulators are proposed to assemble protein complexes that intercept or interfere with transmission of regulatory signals before they reach a promoter. These models couple the mechanism of insulator action to that used by enhancers and silencers to modulate promoter activity.

Enhancer activation of transcription has been proposed to involve a direct interaction between enhancer-bound transcription factors and the basal transcriptional machinery produced by looping out of intervening DNA. This enhancer-promoter interaction may increase the recruitment of RNA polymerase II and/or facilitate formation of productive elongation complexes [171, 172]. The looping model of enhancer function is supported by observations that some enhancers show promoter specificity, suggesting that enhancers identify specific proteins bound at promoters for transfer of a transcriptional signal [14, 78, 173]. Furthermore, some genes contain sequences within the promoter proximal regions, termed docking sites, that are required for promoter responsiveness to an enhancer [174]. These docking sites may stabilize enhancer-promoter interactions, allowing promoter identification by random collision [175].

One version of the transcriptional class of models suggests that insulators evolved from promoter sequences that are responsible for enhancer capture (fig. 1B). Separation of such docking sequences from core promoter elements would generate an element capable of intercepting enhancer signals, without the capacity for transcriptional activation. In this model, as enhancers loop to a promoter, insulators decoy the enhancer, as these sequences are encountered first. This model invokes a ‘first come, first served’ rule for enhancer capture, as suggested by studies at the *globin* locus [176, 177]. Enhancer capture by the insulator results in a loss of most or all of the enhanced signal. Stronger enhancers may bypass the insulator, as the insulator may not be able to diffuse all of the enhancer signal. Interactions between insulators and enhancers are predicted to be dynamic, as most interactions between enhancers and promoters are transient.

Random looping interactions between enhancers and promoter-associated transcription complexes may not provide efficient promoter activation from long distances. The large size of many eukaryotic control regions has been suggested to necessitate participation of a specialized class of proteins, called facilitator proteins, for the passage of the enhancer signal to the promoter [170, 178]. Facilitator proteins are proposed to support enhancer function by organizing chromatin between the enhancer and the promoter into a series of intermediate loops that bring the enhancer complex closer to the promoter (fig. 1B). To date, two candidate facilitator proteins have been identified, Chip and Nipped B [179–181]. These proteins are required for activation of the *Drosophila cut* gene by an enhancer located 80 kb from the promoter. Chip and Nipped B are believed to be distinct from general transcription factors regulating *cut* expression, because mutations in these genes show genetic interactions specifically with *cut* alleles caused by the *gypsy* insulator [179–181]. However, as Chip is a LIM domain protein that appears to interact with the LIM homeodomain transcription factor Apterous [182, 183], Chip may participate in activation of the *cut* gene by enhancing the action of general *cut* transcription factors. Based on ideas of long-distance enhancer action, a second version of the transcriptional class of insulator models was proposed that suggests that insulators interfere with the function of facilitator proteins, thereby preventing the enhancer from gaining promoter proximity (fig. 1B) [170].

Several lines of evidence support transcriptional models of insulator function. First, enhancer and promoter strength influence insulator effectiveness. Increasing the potency of an enhancer reduces the blocking capacity of insulators [44, 45]. Similarly, the effectiveness of the insulator can be increased by a strong promoter located upstream of an enhancer that is blocked by an insulator, while the presence of a weak upstream promoter can support enhancer bypass of the insulator [42, 95]. These data reinforce the idea that insulators and promoters compete for the enhancer signal. Second, several insulator proteins function as short-range transcriptional activators, including the Su(Hw) and CTCF proteins [94, 95, 132, 133, 184]. Third, many insulators contain promoter regions, including promoter-proximal sequences that may include enhancer docking sites (table 1). In *Drosophila*, six of the ten known insulator and insulator-like elements contain promoter regions (table 1). In one case, the *even-skipped*

gene, a GAGA-factor-binding site, located between the TATA box and the transcription start site, prevents promoter activation by certain enhancers [185]. These data provide direct evidence that promoter-proximal sequences can selectively block enhancer communication with core promoter elements. GAGA interacts with chromatin remodelling complexes [186–188], suggesting that enhancer blocking may involve alterations of nucleosome organization in such a way as to impact regulatory interactions. Many *Drosophila* promoters may possess an intrinsic insulator activity, as GAGA-binding sites are located in many promoter-proximal regions [62, 189]. Fourth, the *gypsy* insulator proteins, Su(Hw) and Mod(mdg4), directly interact with the putative facilitator protein, Chip [98, 106]. Fifth, at least one insulator, HS4, directs a highly localized peak of histone acetylation [21], which could both serve to capture enhancers and block the propagation of specific chromatin structures. Finally, in certain contexts, insulators are not transcriptionally inert. For example, the *gypsy* insulator can stimulate transcription from some promoters [94, 95].

Although transcriptional models of insulator function are supported by many studies, two observations are difficult to reconcile with this class of models. If insulators interact with enhancers to capture the regulatory signal, then arguably, insulators should affect transcription when inserted upstream of an enhancer. However, two aspects of this objection need to be considered. First, if insulators act similarly to promoter docking sites, then insulators should not act as static sinks for enhancer interactions, as enhancer-promoter interactions are dynamic. Second, the position-dependent block of enhancer action reflects a bias in the assay system. An insulator positioned upstream of an enhancer does affect the function of the enhancer, if the enhancer is shared by divergently transcribed promoters [18, 20]. Such observations support the notion that activation of a promoter depends on a directional signal that is sent from an enhancer. A second observation that is hard to fit in the context of transcriptional models is the recent finding that enhancer-blocking activity is lost when two *gypsy* insulators are placed between an enhancer and promoter [42, 190]. If the insulator and promoter compete for the enhancer signal, then increasing insulator number should improve, not diminish enhancer blocking. These data are claimed to support structural models that propose that *gypsy* insulators organize chromatin into looped domains. However, enhancer bypass of a pair of *gypsy* insulators also challenges structural models of insulator function, because these studies demonstrate that enhancers and promoters residing in two structurally and topologically distinct domains can interact. The simplest view of these data is that *gypsy* insulators located in close proximity have a propensity to interact. Whether this is a requirement for enhancer blocking remains unclear. Of note is that this property of the *gypsy* insulator does not appear to be shared by other insulators or anti-enhancer elements. In the case of scs, HS4 and the *H19/Igf2* ICR, multimerization of a complete insulator or specific subregions establishes a stronger block of enhancer-activated transcription than conferred by a single element [33, 35, 44, 144].

Transcriptional models of insulator function are not restricted to explaining the blocking of activators. Mechanisms of silencer action suggest that repressive chromatin structures spread into affected genes [5, 26, 191, 192]. In this context, insulators may protect against repression by recruiting nucleoprotein complexes that block the spread of repressive chromatin. Two mechanisms have been proposed [153, 158]. First, insulator-binding proteins may generate a region of chromatin devoid of nucleosomes, thereby interfering with the propagation of silencing complexes [158]. However, the observation that DNA-binding proteins inert for transcriptional activation cannot prevent silencing implies that anti-silencer effects involve more active processes. Alternatively, insulator-binding proteins may recruit histone-modifying proteins, such as histone acetyltransferases, that modify histone tails in a manner that interferes with formation of silencing complexes [21, 153, 158, 161]. This suggestion is consistent with the decoy model for insulator function, as promoter docking sites are likely to possess the capacity to recruit chromatin-modifying complexes as one means for enhancer capture.

Conclusions

The organization of chromatin domains is an important facet of the regulation of gene expression. Insulators appear to be universal components of eukaryotic genomes that play critical roles in defining independent domains of gene regulation. Recent demonstrations that insulator activity is regulated increase the repertoire of mechanisms that may be used to modulate transcription.

The diversity of insulator and insulator-like elements poses a challenge in formulating models of insulator function. While two broad classes of models have been considered, several pieces of data are not easily accommodated by either model. Furthermore, not all insulators are likely to use the same mechanism of action. Studies of the proteins bound at insulators, their interactions and properties, will advance our understanding of this interesting class of elements.

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