Orientation-dependent interaction between Drosophila insulators is a property of this class of regulatory elements

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ABSTRACT

Insulators are defined as a class of regulatory elements that delimit independent transcriptional domains within eukaryotic genomes. According to previous data, an interaction (pairing) between some Drosophila insulators can support distant activation of a promoter by an enhancer. Here, we have demonstrated that pairs of well-studied insulators such as scs–scs, scs’–scs’, 1A2–1A2 and Wari–Wari support distant activation of the white promoter by the yeast GAL4 activator in an orientation-dependent manner. The same is true for the efficiency of the enhancer that stimulates white expression in the eyes. In all insulator pairs tested, stimulation of the white gene was stronger when insulators were inserted between the eye enhancer or GAL4 and the white promoter in opposite orientations relative to each other. As shown previously, Zw5, Su(Hw) and dCTCF proteins are required for the functioning of different insulators that do not interact with each other. Here, strong functional interactions have been revealed between DNA fragments containing binding sites for either Zw5 or Su(Hw) or dCTCF protein but not between heterologous binding sites [Zw5–Su(Hw), dCTCF–Su(Hw), or dCTCF–Zw5]. These results suggest that insulator proteins can support selective interactions between distant regulatory elements.

The term ‘insulators’ refers to the class of DNA sequence elements that contribute to organization of independent gene function domains by restricting the enhancer and silencer functions. Insulators have two distinctive properties. First, insulators block the enhancer and silencer functions in a position-dependent manner, producing this effect when inserted between these regulatory elements and a promoter but not when located upstream or downstream of them (1–6). Insulators do not inactivate enhancers, silencers or promoters, which indicates that insulators interfere with signaling between these classes of regulatory elements (3,7,8). Second, insulators protect gene expression from positive and negative effects of chromatin surrounding the gene (9–11) and confer the capacity for position-independent transcription on transgenes stably integrated into the genome (12–16).

The Drosophila genome contains many sequences with an insulator function (17–25). The first insulators to be identified were scs and scs’ located at the boundaries of two heat shock 70 genes (4,13,26). Multiple sequences within scs and scs’ are required for their insulator function (5,27–31). Two proteins, Zw5 and BEAF, bind to scs and scs’, respectively, and partially account for their insulator properties (28,30,32,33).

The best characterized insulator consisting of reiterated binding sites for the Su(Hw) protein was found in the gypsy retrotransposon regulatory region (2,3). The Su(Hw) protein associates with hundreds of non-gypsy regions that do not contain clustered Su(Hw) binding sites, with the vast majority of them carrying a single copy of the corresponding sequence (34–38).

Binding sites for a Drosophila homolog of vertebrate insulator protein CTCF were recently identified in several insulators (Mcp, Fab-8, etc.) separating regulatory domains of the bithorax complex (39–42). In vertebrates, almost all known insulator elements were shown to interact with CTCF, a DNA-binding protein that contains 11 zinc fingers (25). It was shown that dCTCF is required for the enhancer-blocking activity of the Fab-8 insulator (39,40).

The first experimental evidence for the functional interaction between insulators came from the fact that insertion of two gypsy insulators between an enhancer

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and a promoter allowed the enhancer (silencer) to bypass the insulators and activate transcription (43–45). The same was also reported for several other insulators (46–51). Recently, we identified an insulator, named Wari, from the 3'-side of the white gene (52). Although Wari contains no binding sites for known insulator proteins, it can equally well interact with another copy of Wari and with unrelated Su(Hw)-dependent insulators, gypsy or IA2.

On the other hand, pairs of scs or scs' insulators proved to have a higher enhancer-blocking activity than either of the insulators in a single copy (53,54). To explain different behavior of insulators in tandems, it was suggested that only some of them are capable to tandem interaction resulting in mutual neutralization of their enhancer-blocking activity. However, an alternative explanation arose from the recent observation that two Mcp insulators placed between the enhancers and promoters allowed effective bypass only when they were inserted in opposite orientations relative to each other (49).

Indeed, we have demonstrated here that paired scs or scs' insulators can functionally interact with each other, supporting distant activation of the white transcription by the eye enhancer and the yeast GAL4 activator. All insulator pairs tested (scs–scs, scs – scs', IA2–IA2 and Wari–Wari) display orientation-dependent pairing, which may account for the fact that functional interactions between the pairs of many other insulators have not yet been revealed. We also found that DNA fragments containing binding sites for either Z5, or Su(Hw), or dCTCF alone can support long-distance white activation by GAL4 only upon interaction with another copy of the same fragment, being incapable of interacting in heterologous pairs.

MATERIALS AND METHODS

Plasmid constructions

The constructs were made on the basis of the CaSpeR vector (55). The 5-kb BamHI–BglII fragment (56) containing the coding region (yc) was subcloned into CaSpeR2 (yc-C2). The 3-kb SalI–BamHI fragment containing the yellow regulatory region (yr) was subcloned into pGEM7 cleaved by BamHI–XhoI (yr plasmid). The eye enhancer (Ee) flanked by frt sites was then inserted into the yr plasmid cleaved by BglII at –1874 relative to the yellow transcription start site (yr-frt(Ee)). The pCaSpew15 (+ RI) plasmid was constructed by inserting an additional EcoRI site at +3291 bp of mini-white gene in the pCaSpew15 plasmid. An insulator located at the 3'-side of the mini-white gene (Wari insulator) was deleter from pCaSpew15 (+ RI) by digestion with EcoRI to produce the pCaSpeRΔ700 plasmid. The yellow gene coding region, a BamHI–Eco47III fragment from the yc-C2 plasmid, was subcloned into pCaSpeRΔ700 digested with BamHI and Eco47III to produce yc-C2Δ plasmid.

The scs' insulator corresponded to an ~500-bp fragment, numbered 1–501 in the scs' GenBank sequence (accession number X63732). This fragment contains high- and low-affinity BEAF binding sites and two promoters of the CG3281 and aurora genes (28,31,57). The scs insulator corresponded to a 990-bp PvuII–PvuII fragment numbered 510–1503 bp in the GenBank scs sequence (accession number X63731). This fragment has an enhancer-blocking activity similar to that of the full-length scs and contains Zw5 binding sites (27,30) and two promoters (58,59). The 825-bp sequence containing the white-abutting resident insulator (Wari) is numbered 2684–773–2683995 bp (accession no. NC_004354.3) (52). This fragment was PCR-amplified with 5'-cgcaagagtaagcc gacatat-3' and 5'-ctgagttagaagaagtcgg-3' primers.

The 454-bp sequence of the IA2 insulator (IA2) numbered 255315–255768 bp (accession no. NC_004354.3) was PCR-amplified with 5'-ggagttcaacacgc-3' and 5'-caagaacattc-3' primers.

The sequences of the Su(Hw), dCTCF and Z5 binding sites are shown in Figure 1B. The plasmid containing four reiterated Su(Hw)-binding sites (S4) was made by tetramerization of the third Su(Hw) binding site, as described (66). The synthetic dCTCF-binding region (C4) was made by multiplication of the dCTCF binding site from Fab-8, as described (51). The synthetic Z5-binding region (Z4) was created by concatamerization of oligonucleotides containing the 32-bp binding site of the natural scs insulator (30). Two pairs of single-stranded 37-bp oligonucleotides (corresponding to the sense and antisense strands) were synthesized so as to contain overhangs for either XhoI or SalI. The sequences of the oligonucleotides were 5'-ctgagttcaacacgc-3' (sense) and 5'-ggagttcaacacgc-3' (antisense). The desired concatamers (Z4,S4,C4,S4) were isolated, purified and cloned into the pBluSK plasmid. The resulting DNA fragment was verified by sequencing and inserted between two lox or two frt sites. The DNA fragment containing the dCTCF and Su(Hw) binding sites (C4,S4) was made by cloning the S4 blunt-end fragment into C4–PSK cleaved by Eco32I.

All constructs were made according to two general schemes. In the first scheme, a fragment X (scs or Wari or Zw5 binding sites) was inserted in the direct or reverse orientation into the yr-frt(Ee) plasmid cleaved by Eco47III at –893 relative to the yellow transcription start site. As a result, the frt-flanked eye enhancer in these constructs was placed between the enhancers required for yellow expression in the wing and body, respectively.

In the second scheme, a fragment X (scs, scs', Wari, IA2, Z8,S4, C4,S4,C4,S4) flanked by frt sites (frt(X)) was inserted in the direct or reverse orientation into the G4-yr plasmid cleaved by KpnI at –343 relative to the yellow transcription start site (G4-yr-frt(X)). In these constructs, the yellow enhancers were deleted.

A fragment X (scs, scs', Wari, IA2, Z8, S4, C4, CTCF) flanked by lox sites (lox(X)) was cloned into ye-C2Δ (ye-C2Δ-lox(X)) or into ye-C2 (ye-C2-lox(X)) at +4964 relative to the yellow transcription start site between the yellow and white genes. Next, yr-frt(Ee)-X or G4-Δyr-frt(X) fragments were cloned into the corresponding ye-C2A-lox(X) or into ye-C2-lox(X) plasmids cleaved by XbaI–BamHI.
Generation and analysis of transgenic lines

The construct and P25.7wc plasmid were injected into yacw1118 preblastodermers (60). The resultant flies were crossed with yacw1118 flies, and the transgenic progeny were identified by their eye color. Chromosome localization of various transgene insertions was determined by crossing the transformants with the yacw1118 balancer stock carrying dominant markers. In(2RL)Cyo for chromosome 2 and In(3LR)/TM3,Sc for chromosome 3.

Lines with DNA fragment excisions were obtained by crossing the flies bearing the transposons with the Flp (w1118; S2Cyo, hsFLP, ISA/Sco; +) or Cre (yw: Cy, P[w+cre]/Sco; +) recombinase-expressing lines (61,62). Cre recombinase induces 100% excisions in the next generation. A high level of FLP recombinase (almost 90% efficiency) was produced by daily heat-shock treatment for 2h during the first 3 days after hatching. All excisions were confirmed by PCR analysis with the pairs of primers flanking the insertion site located at –343 relative to the yellow transcription start site (5'-tagatacaaaagtgctcta-3' and 5'-gtaagcagtggctggctctc-3'), and the insertion site between the yellow and white genes (5'-ttttcttgagcggaaaa aaggtg-3' and 5'-ttctacattctccaaaaagggt-3'). Details of the crosses used for genetic analysis and excision of functional elements are available upon request.

To induce GAL4 expression, we used the modified yw1118, P[w-, tubGAL4]/TM3,Sc line (Bloomington Center #5138), in which the marker mini-white gene was deleted as described (49).

The white (w) phenotype was estimated from eye pigmentation in adult flies. Wild-type white expression determined the bright red eye color (R); in the absence of white expression, the eyes were white (W). Intermediate levels of white expression (in increasing order) were reflected in the eye color ranging from pale yellow (pY) to yellow (Y), dark yellow (dY), orange (Or), dark orange (dOr), and, finally, brown (Br) or brownish red (BrR).

RESULTS

Insulator bypass depends on the relative orientation of two scs insulators inserted between the eye enhancer and the white promoter

The scs insulator (Figure 1) was mapped in the 990-bp region including two promoters and a binding site for the Zw5 protein involved in enhancer blocking (27,30,63). Previously, it was found that the scs insulator activity was increased when two copies of this element were inserted in a tandem arrangement (53,54). In these experiments, however, two scs copies were inserted in the same direction between closely spaced enhancers and promoters, and the enhancer–promoter interaction across the insulators could be prevented due to spatial constraints. If so, increasing the distance between insulators and placing them in opposite orientations relative to each other might facilitate insulator bypass.

To check this assumption, we chose the regulatory region of the white gene that has been extensively used in insulator studies (4,13,14,34,44,52). The white gene determines eye pigmentation and is regulated by a specific enhancer (64). In our constructs, the yellow gene was inserted between the eye enhancer and the mini-white gene, with the eye enhancer being flanked by frt sites (Figure 2). Parentheses in construct designations indicate the elements flanked by frt or lox sites for in vivo excision by crossing, as described in Materials and methods section; such excisions are denoted by (Δ) in the primary (expression) data tables. Comparing eye pigmentation in flies from the transgenic line before and after the deletion of the eye enhancer, we could estimate its contribution to white expression.

To assess the enhancer-blocking activity of the scs insulator, we made constructs in which the scs insulator was inserted either in the direct orientation, according to its position in genome (scs) near the eye enhancer (Figure 2A), or in the reverse orientation (scsR) near the white promoter (Figure 2B). We obtained a total of 35 transgenic lines carrying these constructs. The eye color in these flies ranged from pale yellow to orange, and the deletion of the eye enhancer did not affect eye pigmentation. These results show that a single copy of scs completely blocked the eye enhancer.

![Figure 1](https://academic.oup.com/nar/article-abstract/36/22/7019/1198095/7021)
Figure 2. Insulator bypass depends on the relative orientation of two scs insulators inserted between the eye enhancer and the white promoter. (A and B) Experimental evidence that one copy of scs in both orientations can effectively insulate the eye enhancer. (C and D) Experimental evidence that scs insulators are capable of functional interaction. (E) Tests for the functional interaction between Zw5 binding sites. Reductive schemes of the transgenic constructs are shown (not to scale). The yellow and white genes are shown as boxes, with arrows indicating the direction of their transcription. The eye enhancer (Ee) is shown as a black circle. Downward arrows indicate target sites for Flp recombinase (frt) or Cre recombinase (lox). The same sites in construct names are denoted by parentheses. The scs insulator is shown as a white box, with a white oval indicating the Zw5 binding site. The superscript index 'R' indicates that the corresponding element is inserted in the reverse orientation in the construct. The 'white' column shows the numbers of transgenic lines with different levels of white expression. The wild-type white expression determined the bright red eye color (R); in the absence of white expression, the eyes were white (W). Intermediate levels of pigmentation, with the eye color ranging from pale yellow (pY) through yellow (Y), dark yellow (dY), orange (Or), dark orange (dOr) and brown (Br) to brownish red (BrR), reflect the increasing levels of pigmentation, with the eye color ranging from pale yellow to dark orange. The deletion of the eye enhancer resulted in a noticeable reduction of eye pigmentation in all transgenic lines, indicating that the eye enhancer was capable of stimulating white expression. When the scs insulators were placed in the same orientation (Figure 2D), eye pigmentation ranged from pale yellow to yellow in all 14 transgenic lines obtained, with the deletion of the eye enhancer having no effect on white expression. Therefore, the pair of scs insulators completely blocked the enhancer. These results indicate that relative orientation of the two scs copies is critical for the ability of the eye enhancer to stimulate the white promoter across the insulator pair.

The scs insulator contains a binding site for the Zw5 protein that is necessary for its enhancer-blocking activity. It was shown that four Zw5 binding sites could partially block the eye enhancer. Thus, it is possible that Zw5 participates in pairing between two scs insulators. To test this possibility, we prepared oligos containing eight binding sites for Zw5 (Zw5^8), which insulated the eye enhancer better than oligos with four Zw5 binding sites (data not shown). In the construct (Figure 2E), we inserted the Zw5^8 oligo (proximal) near the eye enhancer flanked by frt sites and Zw5^8 flanked by lox sites (distal) near the white promoter. Eye pigmentation was compared in transgenic lines before and after the deletion of the distal Zw5^8 oligo flanked by lox sites. This deletion resulted in a considerable reduction of eye pigmentation. In derivative transgenic lines with the deleted enhancer, eye pigmentation in flies with and without the distal Zw5^8 oligo was the same. These results support the model that the interaction between protein complexes bound to the Zw5 binding sites promotes white activation by the eye enhancer.

Pairing of scs insulators or Zw5 binding sites facilitates long-distance stimulation of the white promoter by the GAL4 activator

To confirm that the scs insulators can functionally interact at a distance, we used the GAL4/white model system based on the finding that the GAL4 activator cannot stimulate the white promoter across the yellow gene. To test whether the interaction between the scs insulators can facilitate white stimulation by GAL4, we inserted ten GAL4 binding sites (designated G4) at the 5′ side of the yellow gene. As a result, the distance between the mini-white gene and the GAL4 binding sites reached almost 5 kb. To express the GAL4 protein, we used a transgenic line carrying the GAL4 gene under control of the ubiquitous tubulin promoter.

In the control construct, a single copy of scs was inserted near the GAL4 binding sites (Figure 3A). In all 11 transgenic lines tested, GAL4 failed to stimulate
white activation. Next, we checked if the interaction between two scs insulators inserted in opposite orientations would facilitate white activation by GAL4 (Figure 3B). These transgenic lines showed strong white activation by GAL4, indicating that the pair of scs insulators in such an arrangement supported communication between the GAL4 activator and the promoter complex.

We then inserted two scs insulators in the same orientation (Figure 3C) and observed a relatively weak white activation by GAL4, compared to that in transgenic lines carrying the scs insulators inserted in opposite orientations. This is evidence that the scs insulators functionally interact in an orientation-dependent manner.

Finally, we checked whether Zw5 binding sites could support white activation by GAL4. Once again, we used the oligos containing eight Zw5 binding sites (Zw5/C2), which were inserted near the GAL4 binding sites and the white promoter (Figure 3D). In all 10 transgenic lines tested, GAL4 strongly stimulated white transcription, confirming our previous observation that Zw5 binding sites can functionally interact with each other.

Taken together, these results show that scs insulators can functionally interact in the orientation dependent manner and that the Zw5 protein may contribute to their pairing.

**Functional interaction between two Wari insulators depends on their relative orientation**

As shown in our previous study (52), pairing between two copies of the Wari insulator is required for the effective blocking of the enhancers. Hence, the question arose as to whether the relative orientation of the Wari insulators is of significance for their functional interaction.

At first, we examined the interaction between the Wari insulators in the eye enhancer/white model system. The first copy of Wari was inserted in the direct orientation near the eye enhancer that was flanked by the frt sites. The second copy, flanked by the lox sites, was inserted near the white promoter in either the opposite (Figure 4A) or the same orientation (Figure 4B). To improve the enhancer-blocking activity of these insulators, the endogenous Wari located at the 3' end of the white gene was left intact. Thus, the resulting constructs contained three copies of the Wari insulator.

When Wari insulators were placed in opposite orientations relative to each other (Figure 4A), we observed high levels of eye pigmentation, which decreased considerably upon deletion of one Wari insulator or the eye enhancer. Thus, the functional interaction between Wari insulators allowed the eye enhancer to stimulate white expression...
more effectively. In contrast, transgenic flies carrying two copies of Wari insulators in the same orientation (Figure 4B) had relatively weak eye pigmentation. The deletion of either eye enhancer or Wari changed the eye pigmentation only slightly and in a minor part of corresponding transgenic lines. These results show that the relative orientation of Wari insulators is important for eye enhancer/white promoter communication.

Next, we examined the interaction between Wari insulators in the GAL4/white assay. The insulators flanked by either lox or ftr sites were inserted near the GAL4 binding sites and the white promoter in either the opposite or the same orientation (Figure 4C and D). In this case, the Wari insulator was removed from the 3′-side of the mini-white gene. When they were placed in opposite orientations, GAL4 strongly activated white expression (Figure 4C), whereas insulators in the same orientation allowed only weak stimulation of white expression by GAL4 (Figure 4D). Thus, the relative orientation of the interacting Wari insulators determines the efficiency of white stimulation by GAL4.

**The pairing between two scs’ or 1A2 insulators supports long-distance white activation by GAL4**

To determine whether orientation-dependent pairing is a common property of *Drosophila* insulators, we tested two other well-studied endogenous *Drosophila* insulators, 1A2 (34,35) and scs’ (4,13,28,32), in the GAL4/white assay. The scs’ insulators were inserted either in opposite orientations (Figure 5A) or in the same orientation (Figure 5B). In both cases, the scs’ insulators markedly enhanced white activation by GAL4, confirming their ability to interact with each other. Once again, the relative orientation of the scs’ insulators proved to influence the level of white stimulation by GAL4.

In addition, two similar constructs were made with the 1A2 insulators inserted in either opposite or the same orientation (Figure 5C and D). We observed that white activation by GAL4 depended on the relative orientation of the 1A2 insulators. When the insulators were in opposite orientations, GAL4 strongly stimulated white expression (Figure 5C); when their orientation was the same, only relatively weak stimulation was observed (Figure 5D). Thus, in the model of white activation by GAL4, the 1A2 insulators appear to interact in an orientation-dependent manner.

**Su(Hw), Zw5 and dCTCF binding sites are capable of selective pairing only with their copies**

In several previous studies, no functional interactions between different insulators were observed (53,54). Likewise, our experiments with the eye enhancer/white and GAL4/white model systems also did not reveal any functional interactions between heterologous insulators such as gypsy [with 12 binding sites of Su(Hw)], scs (with one binding site for Zw5) and Fab-8 (with two binding sites for dCTCF) (data not shown).

However, the gypsy, scs and Fab-8 insulators have a complex structure and may contain binding sites for additional proteins involved in the insulator activity.

Hence, we decided to examine functional interactions between the oligos containing binding sites for Zw5, Su(Hw) and dCTCF proteins. Recently, we found that the functional interaction between two DNA fragments containing four consensus binding sites for the dCTCF protein (C×4) supported long-distance activation of white by the GAL4 activator (51) (Figure 6A). In this study, a functional interaction was revealed between DNA fragments Z×8 containing eight binding sites for Zw5 (Figures 3D and 6B).

Previously, it was shown that four Su(Hw) binding sites function as a strong insulator (65), and we made the oligos containing four copies of the third Su(Hw) binding site (S×4) from the gypsy insulator (66). Here, we found that the functional interaction between the S×4 DNA fragments can facilitate white activation by GAL4 (Figure 6C). This is evidence that all three insulator proteins can support long-distance interactions in the GAL4/white model system.

Next, we analyzed functional interactions between DNA fragments containing binding sites for different proteins: Z×8 and C×4 (Figure 6D), C×4 and S×4 (Figure 6E), and Z×8 and S×4 (Figure 6F). No white activation by GAL4 was observed in any of these variants, indicating that insulator proteins could selectively support interactions within the genome.

Finally, we tested if composite DNA fragments containing four consecutive binding sites for each of the dCTCF and Su(Hw) proteins (S×4C×4) could functionally interact in an orientation dependent manner. Such fragments were
inserted in the GAL4/white model system either in opposite orientations (Figure 6G) or in the same orientation (Figure 6H) relative to each other. Strong white activation by GAL4 was observed only when the DNA fragments were inserted in opposite orientations. This result showed that the relative orientation of the composite DNA fragments containing binding sites for two different insulator proteins determines the ability of the GAL4 activator to stimulate white expression.

DISCUSSION

The results of this and previous studies (43–46,49–52) confirm that most of well-studied insulators can functionally interact in pairs, but the functional effect of this interaction depend on their relative orientation. A probable explanation to this orientation-dependent effect is that there are at least two insulator-bound proteins involved in specific protein-protein interactions. If so, the pairing of insulators, depending on their relative orientation, may lead to the formation of two loop configurations (Figure 7). In our model system, when the insulators are in opposite orientations relative to each other, the configuration of the loop formed upon their pairing is favorable for communication between regulatory elements located outside the loop, as these elements are brought in close proximity to each other (Figure 7A). Such a loop configuration can provide for the observed strong white stimulation by GAL4 or effective bypass of the insulators by the eye enhancer. In contrast, pairing between two insulators located in the same orientation leads to the formation of the loop that spatially separates regulatory elements (Figure 7B), with the consequent weakening of white activation by the eye enhancer or GAL4.

It appears that most of Drosophila insulators contain binding sites for more than one insulator protein. For example, the scs insulator is assembled from a discrete number of functionally redundant DNA elements (27), and it is likely that Zw5 is only one of several proteins that are responsible for the activity of this insulator (27,30,63). The enhancer-blocking activity of the 1A2 insulator depends on the presence of not only two Su(Hw) binding sites but also of certain additional sequences, which indicates that at least one more transcriptional factor, in addition to Su(Hw), is necessary for its functioning (34,35,67). A direct test of other genome regions containing one or several endogenous Su(Hw) binding sites in the transgene assay shows that most of them effectively block enhancers, suggesting that additional proteins bound to non-gypsy regions contribute to the insulator function of Su(Hw) (36,37,67,68).

Figure 6. Testing the functional interaction between DNA fragments containing binding sites for different insulator proteins, dCTCF (black ovals), Zw5 (white ovals), Su(Hw) (white rectangles) or composite DNA fragments containing dCTCF and Su(Hw) binding sites (black ovals and white rectangles). For designations, see Figures 2 and 3. The results with G4(Cx4)Y(Cx4)W were taken from Kyrchanova et al. (51).

Figure 7. Two models of pairing between the insulators. Presumptive proteins responsible for insulator pairing are shown as a white cylinder and a gray cube. Solid and dotted arrows indicate high and low levels of transcription, respectively. Other designations: (P) promoter, (E) enhancer.
It is of interest that, as shown previously, the functional interaction between *gypsy* insulators, each containing 12 binding sites for the Su(Hw) protein alone, is less sensitive to their relative orientation (53,54). Here, we observed that the relative orientation of the scs' insulators had only a slight effect on white stimulation by GAL4. Note that the weak scs' insulator probably contains binding sites for only one protein, BEAF (8,32,33). On the other hand, stimulation of white by GAL4 in experiments with composite DNA fragments containing binding sites for two insulator proteins, dCTCF and Su(Hw), displayed striking dependence on their relative orientation. These results are in agreement with the proposed model that the binding of at least two different insulator proteins is essential for effective orientation-dependent interaction between insulators. However, more information about proteins bound to insulators is required to construct the model comprehensively explaining the phenomenon of insulator pairing.

Previously (53,54), no interaction between unrelated insulators was revealed. The results of this study show that DNA fragments containing binding sites for either of three different insulator proteins—Zw5, Su(Hw) and dCTCF—can effectively support long-distance interactions in pairs, with no functional interaction being observed between heterologous DNA fragments containing binding sites for different insulator proteins. Thus, insulator proteins can ensure selective long-distance interactions in chromosomes. For example, the interaction between *gypsy* insulators can support activation of the yellow promoter by enhancers separated by many megabases (69). In mammals, the interaction of the imprinting control region on chromosome 7 with the Wsb1/Nfl locus on chromosome 11 depends on the presence of the CTCF protein (70).

Interestingly, although no functional interaction is observed between binding sites for Su(Hw) and dCTCF, both these proteins interact with CP190, the protein required for the enhancer-blocking activity of dCTCF- and Su(Hw)-dependent insulators (42,71,72). CP190 contains the BTB/POZ domain involved in homodimerization and the additional domain that interacts *in vitro* with the Mod/mdg4 protein, another component of the Su(Hw) insulator complex (73). Thus, the presence of the same protein in two different insulator complexes does not ensure the functional interaction between them. Further extensive studies are required to elucidate the proteins and their domains that are involved in selective long-distance interactions.

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