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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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(or silencer) 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 1A2.

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', 1A2–1A2 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 Zw5, 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 deleted 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 2 684 773–2 683 995 bp (accession no. NC_004354.3) (52). This fragment was PCR-amplified with 5′-cgcaaggagtagcc gacatatat-3′ and 5′-ctttggagtacgaaatgcgtcg-3′ primers. The 454-bp sequence of the 1A2 insulator (1A2) numbered 255 315–255 768 bp (accession no. NC_004354.3) was PCR-amplified with 5′-ggagtactactaccaggc-3′ and 5′-caag aacatttccgatatg-3′ primers.

The sequences of the Su(Hw), dCTCF and Zw5 binding sites are shown in Figure 1B. The plasmid containing four reiterated Su(Hw)-binding sites ($S^{\times 4}$) was made by tetramerization of the third Su(Hw) binding site, as described (66). The synthetic dCTCF-binding region ($C^{\times 4}$) was made by multiplication of the dCTCF binding site from Fab-8, as described (51). The synthetic Zw5-binding region ($Z^{\times 8}$) 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′-ctcgaggttaccgcttcgctgcgaatgacaaaacg gg-3′ (sence) and 5′-gtcgacccgttttgtcattcgcagcgaagc ggtaa cc-3′ (antisense). The desired concatamers ($Z^{\times 8}$, $C^{\times 4}$, $S^{\times 4}$) 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 ($C^{x4}S^{x4}$) was made by cloning the $S^{\times 4}$ blunt-end fragment into C^{x4}–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, 1A2, $Z^{\times 8}$, $S^{\times 4}$, $C^{\times 4}$, $C^{\times 4}S^{\times 4}$) flanked by frt sites (frt(X)) was inserted in the direct or reverse orientation into the G4-Δyr plasmid cleaved by KpnI at –343 relative to the *yellow* transcription start site (G4-Δyr-frt(X)). In these constructs, the *yellow* enhancers were deleted.

A fragment X (scs, scs', Wari, 1A2, $Z^{\times 8}$, $S^{\times 4}$, $CTCF^{\times 4}$) flanked by lox sites (lox(X)) was cloned into yc-C2Δ (yc-C2Δ- lox(X)) or into yc-C2 (yc-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 yc-C2Δ- lox(X) or into yc-C2-lox(X) plasmids cleaved by XbaI–BamHI.

Generation and analysis of transgenic lines

The construct and P25.7wc plasmid were injected into *yacw*^{1118} preblastoderms (60). The resultant flies were crossed with *yacw*^{1118} 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 *yacw*^{1118} balancer stock carrying dominant markers, *In(2RL),CyO* for chromosome 2 and *In(3LR)TM3,Sb* for chromosome 3.

Lines with DNA fragment excisions were obtained by crossing the flies bearing the transposons with the Flp (*w*^{1118}*; S2CyO, hsFLP, ISA/Sco; +*) or Cre (*yw; Cyo, 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 2 h 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′-tagatcaaataaagtcccta-3′ and 5′-gtttggtatgatttttggccttc-3′), and the insertion site between the *yellow* and *white* genes (5′-ttttcttgagcggaaaa agcgga-3′ and 5′-atctacattctccaaaaaagggt-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 *yw*^{1118}*; P[w*$^{-}$*, tubGAL4]117/TM3,Sb* 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 constrains. 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

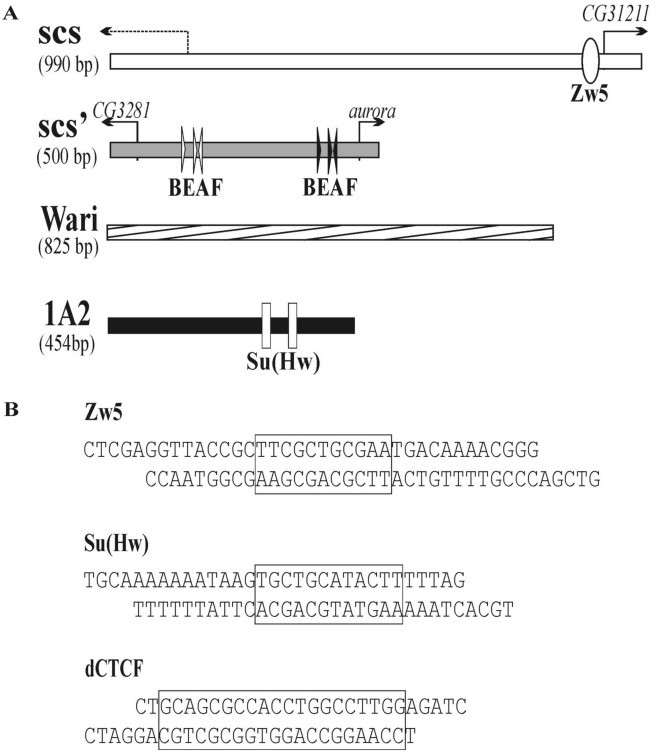

Figure 1. (**A**) Schemes of the scs, scs', Wari and 1A2 insulators. The scs insulator contains a cryptic promoter (59) and the promoter of the *CG31211* gene. The scs' insulator contains promoters of the *CG3281* and *aurora* genes. Arrows indicate gene promoters. The Zw5 binding site within scs is shown as a white oval. Positions of the CGATA motifs within scs' are shown as arrowheads, with the arrow indicating the direction of the motif (5′-CGATA-3′). Clusters of three CGATA motifs form low-affinity (white arrows) and high-affinity (black arrows) binding sites for the BEAF protein (28). Su(Hw) binding sites in the 1A2 insulator are shown as white rectangles. (**B**) Sequences of the oligonucleotides used to produce the Zw5, Su(Hw) and dCTCF synthetic binding regions. The core binding sites are boxed.

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 (scs^{R}) 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.

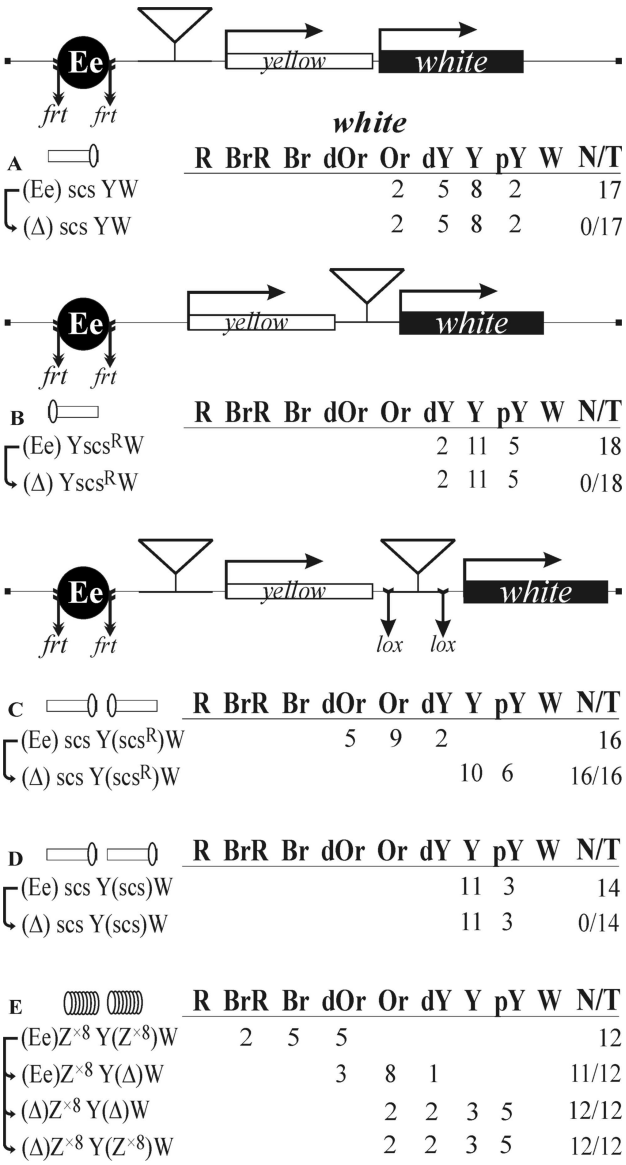

Figure 2. Insulator bypass depends on the relative orientation of two scs insulators inserted between the eye enhancer and the *white* promoter. (**A** and **B**) Experimental evidence that one copy of scs in both orientations can effectively insulate the eye enhancer. (**C** and **D**) Experimental 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 *white* expression. *N* is the number of lines in which flies acquired a new *white* (w) phenotype upon deletion (Δ) of the specified DNA fragment (the eye enhancer or scs); T is the total number of lines examined for each particular construct.

Next, we examined the activity of the eye enhancer in transgenic flies carrying two copies of the scs insulator inserted near the eye enhancer and the *white* promoter in either opposite or the same orientation relative to each other (Figure 2C and D). In the case of the construct carrying the scs insulators inserted in opposite orientations (Figure 2C), we obtained 16 transgenic lines in which flies had eye pigmentation ranging from dark 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 (30,63). It was shown that four Zw5 binding sites could partially block the eye enhancer (30). 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 ($Z^{\times 8}$), which insulated the eye enhancer better than oligos with four Zw5 binding sites (data not shown). In the construct (Figure 2E), we inserted the $Z^{\times 8}$ oligo (proximal) near the eye enhancer flanked by frt sites and $Z^{\times 8}$ flanked by lox sites (distal) near the *white* promoter. Eye pigmentation was compared in transgenic lines before and after the deletion of the distal $Z^{\times 8}$ 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 $Z^{\times 8}$ 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 (49). 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 (49).

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

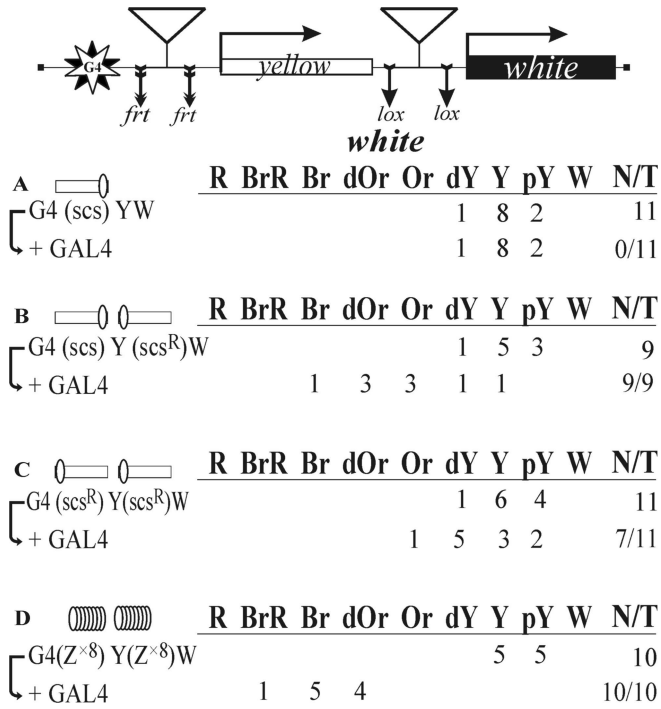

Figure 3. Testing the functional interaction between scs insulators or Zw5 binding sites in the GAL4/*white* model system. The GAL4 binding sites (indicated as G4) are at a distance of ~5 kb from the *white* promoter. A reductive scheme of transgenic construct used to examine the functional interaction between the insulators is presented in the upper part of the figure. '+GAL4' indicates that eye phenotypes in transgenic lines were examined after the induction of GAL4 expression. *N* is the number of lines in which flies acquired a new w phenotype upon induction of GAL4. For other designations, see Figure 2.

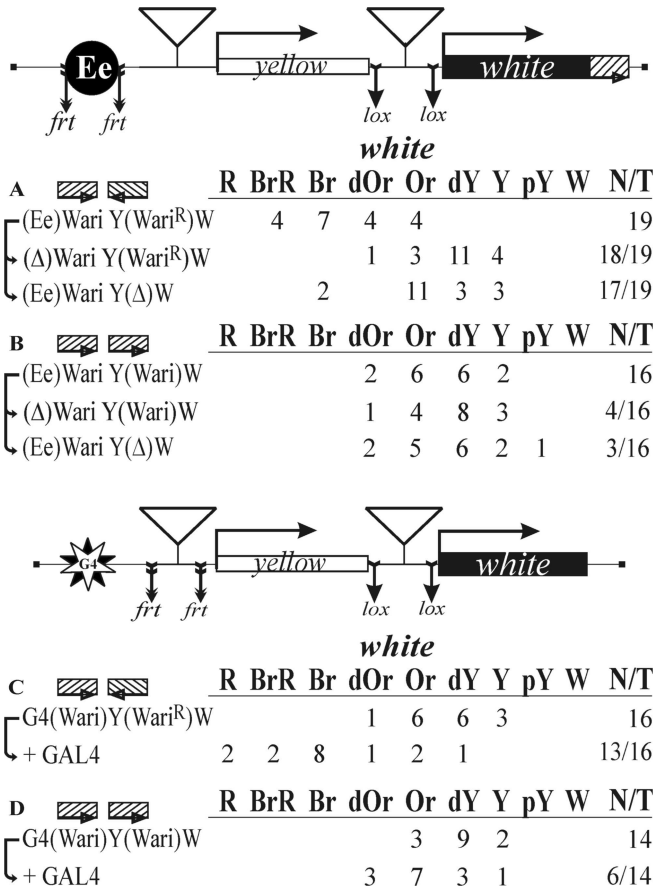

Figure 4. Testing the functional interaction between Wari insulators (**A** and **B**) in the eye enhancer/*white* model and (**C** and **D**) in the GAL4/*white* model. The Wari insulator is shown as a hatched box. For other designations, see Figures 2 and 3.

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 ($Z^{\times 8}$), 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* promoter 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 frt 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.

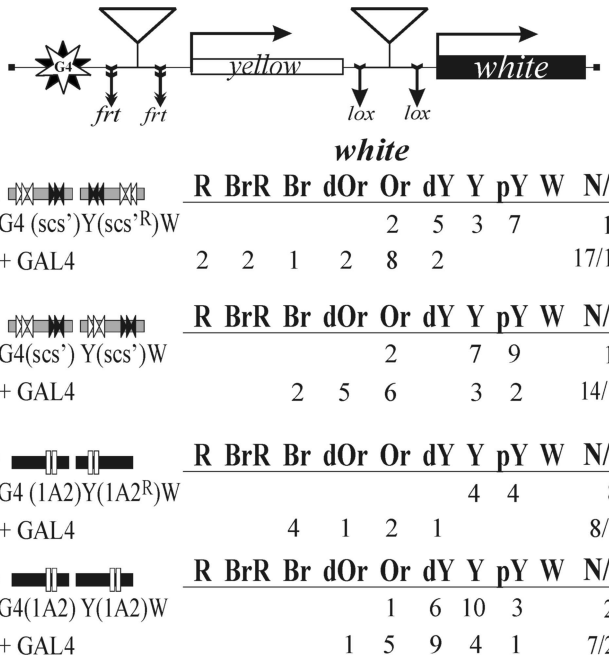

Figure 5. Testing the functional interaction between (**A** and **B**) two scs' or (**C** and **D**) two 1A2 insulators. The scs' insulator is shown as a gray box with the black and white arrows indicating binding sites for the BEAF protein. The 1A2 insulator is shown as a black box with white rectangles indicating Su(Hw) binding sites. For other designations, see Figures 2 and 3.

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 ($C^{\times 4}$) supported long-distance activation of *white* by the GAL4 activator (51) (Figure 6A). In this study, a functional interaction was revealed between DNA fragments $Z^{\times 8}$ 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 ($S^{\times 4}$) from the *gypsy* insulator (66). Here, we found that the functional interaction between the $S^{\times 4}$ 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: $Z^{\times 8}$ and $C^{\times 4}$ (Figure 6D), $C^{\times 4}$ and $S^{\times 4}$ (Figure 6E), and $Z^{\times 8}$ and $S^{\times 4}$ (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 ($S^{\times 4}C^{\times 4}$) could functionally interact in an orientation dependent manner. Such fragments were

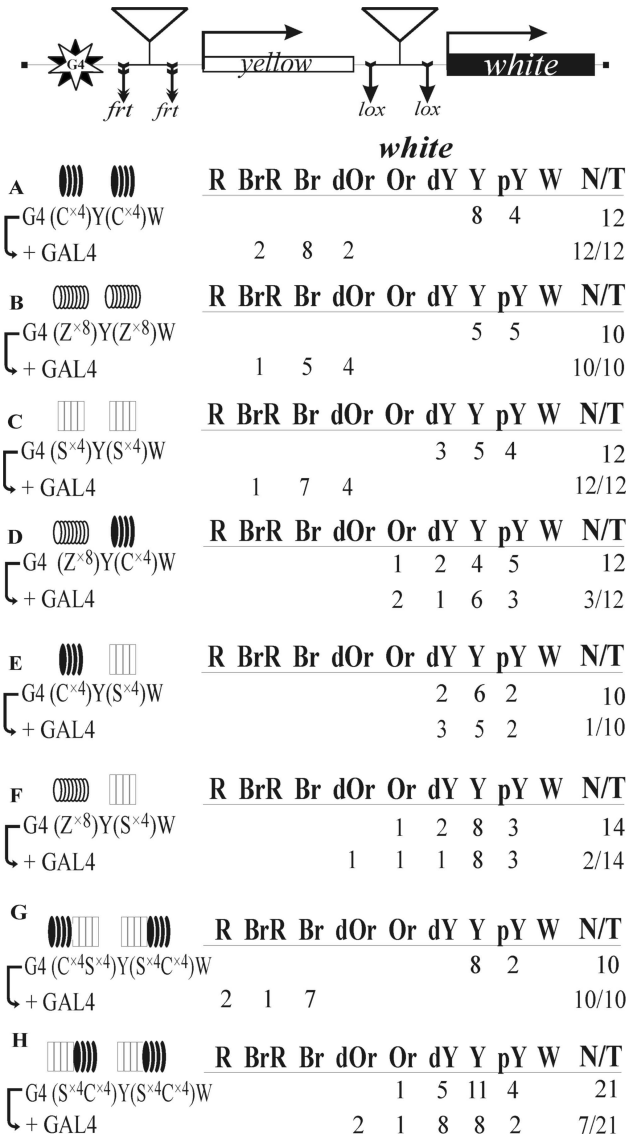

Figure 6. Testing the functional interaction between DNA fragments containing binding sites for different insulator proteins, dCTCF (black ovals), Zw5 (white ovals), Su(Hw) (white rectangles) or composite DNA fragments containing dCTCF and Su(Hw) binding sites (black ovals and white rectangles). For designations, see Figures 2 and 3. The results with G4(C^{x4})Y(C^{x4})W were taken from Kyrchanova *et al.* (51).

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

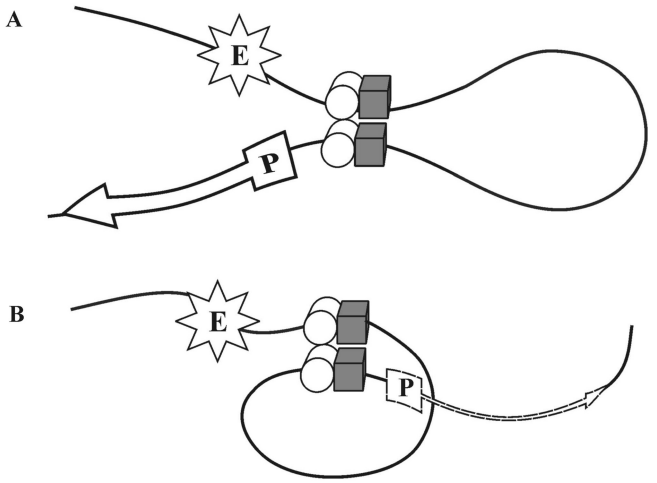

Figure 7. Two models of pairing between the insulators. Presumptive proteins responsible for insulator pairing are shown as a white cylinder and a gray cube. Solid and dotted arrows indicate high and low levels of transcription, respectively. Other designations: (P) promoter, (E) enhancer.

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).

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/Nf1 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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