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16. Serum concentrations of corticosterone were measured with a commercial radioimmunoassay kit (Coat-A-Count, Diagnostic Products, Los Angeles). One rat showed 207.9 and 41.0 ng/ml and another showed 105.8 and 68.9 ng/ml at 3 hours after lights were turned on ("prefeeding") and 9.5 hours after lights were turned on ("basal"), respectively. The difference between our results and those reported in (*13*) may be due to the fact that our animals were just weaned and growing rapidly, so that any restrictions in food access may be stressful. Aging markedly reduces the prefeeding corticosterone secretion in rats exposed to RF [S. Honma *et al.*, *Am. J. Physiol.* **271**, R1514 (1996)].
17. Five milligrams of corticosterone (Sigma) dissolved in 0.2 ml of dimethyl sulfoxide (DMSO) was given daily as intraperitoneal injections for 7 days. Control animals received 0.2 ml of DMSO.
18. On the seventh day of treatment, the serum level of corticosterone, 30 min after injection, was 581 ± 174 (SEM) ng/ml (n = 6) and 39 ± 17 ng/ml (n = 6) in animals receiving corticosterone and DMSO injections, respectively.
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28. We thank M. Quigg for measuring corticosterone concentrations and K. M. Greene and S. C. Miller for technical assistance. This work was supported in part by the NSF Center for Biological Timing, NIH grant MH 56647 (to M.M.); by travel grant 130173/410 from the Norwegian Research Council (to K.-A.S.); and by a research grant from the Japanese Ministry of Education, Science, Sports and Culture and the Japanese Ministry of Health and Welfare (to H.T.).

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Effects of *cis* Arrangement of Chromatin Insulators on Enhancer-Blocking Activity

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Chromatin boundary elements or insulators are believed to regulate gene activity in complex genetic loci by organizing specialized chromatin structures. Here, we report that the enhancer-blocking activity of the *Drosophila* suHw insulator is sensitive to insulator copy number and position. Two tandem copies of suHw were ineffective in blocking various enhancers from a downstream promoter. Moreover, an enhancer was blocked more effectively from a promoter by two flanking suHw insulators than by a single intervening one. Thus, insulators may modulate enhancer-promoter interactions by interacting with each other and facilitating the formation of chromatin loop domains.

Insulators regulate gene activity in diverse organisms (*1–8*). The defining feature of insulators as a class of regulatory elements is their ability to block enhancer-promoter interactions when positioned interveningly. One of the best characterized insulators is suHw, a 340–base pair (bp) element from the *Drosophila* gypsy retrotransposon. It protects transgenes from chromosomal position effects and blocks various enhancer-promoter interactions (*9–13*). SUHW, a zinc-finger DNA binding protein, and MOD(MDG4), a BTB domain protein, are essential for suHw function (*13–16*). Using divergently transcribed reporter genes in transgenic *Drosophila* embryos, we have shown that an enhancer blocked from the downstream promoter by suHw is fully competent to activate an upstream promoter (*12*).

To probe the insulator mechanism, we tested the effect of suHw copy number on its insulator strength in *Drosophila* embryos. The *zerknullt* enhancer VRE (ventral repression element) has been shown to be partially blocked by suHw (*12*). In blastoderm embryos, the *V2* transgene containing VRE and E2, an *even-skipped* stripe 2 enhancer, directs reporter expression in a composite pattern of broad dorsal activation and dominant ventral repression of the E2 stripe (Fig. 1, A and D) (*13, 17, 18*). A single 340-bp suHw insulator element in the *VS2* transgene partially blocked the upstream VRE enhancer (Fig. 1, B and D). Two tandem suHw elements (arranged as direct repeats) were inserted between VRE and E2, resulting in *VSS2*. Instead of enhanced blockage, *VSS2* embryos exhibited a loss of suHw insulator activity (Fig. 1, C and D). This was observed in most *VSS2* embryos (Fig. 1D) and in all 10 independent *VSS2* lines, indicating that it is unlikely to be caused by chromosomal position effects. Genomic polymerase chain reaction (PCR) analysis of independent *VS2* and *VSS2* lines further verified the structural integrity of the transgenes in vivo (Fig. 1E) (*19*).

To determine whether the loss of insulator function in *VSS2* embryos is enhancer-specific, we constructed transgenes using a *rhomboid* neuroectodermal enhancer (NEE) and a *hairy* stripe 1 enhancer (H1) (*13*). The *NLH* embryos containing NEE and H1 enhancers separated by a 1.4-kb neutral spacer (L) exhibited a composite *lacZ* pattern directed by both enhancers (Fig. 2, A and H). A single suHw element in the *NSH* transgene blocked the upstream NEE enhancer (Fig. 2, B and H), whereas two tandem suHw elements (*NSSH*) did not block the NEE enhancer (Fig. 2, C and H). A second group of transgenes uses a *twist* mesoderm enhancer (PE) and an *evenskipped* stripe 3 enhancer (E3) (*13*). Both enhancers are active when separated by the L spacer (*PL3*) (Fig. 2, D and H). Insertion of a suHw element in the *PS3* transgene blocked the upstream PE enhancer (Fig. 2, E and H), whereas two tandem suHw elements (*PSS3*) did not block the PE enhancer (Fig. 2, F and H). Replacing one of the two suHw elements in *PSS3* with a spacer of comparable size (A) restored the enhancer-blocking activity of the remaining suHw in *PSA3* embryos (Fig. 2G), indicating that loss of insulator activity with two suHw elements is not due to the spacing change but to the presence of the additional insulator. Genomic PCR with individual *NSH*, *NSSH*, *PS3*, and *PSS3* lines indicated that the transgenes were structurally intact (Fig. 2I). These results suggest that the loss of insulator activity with tandemly arranged suHw is independent of the enhancer tested.

The enhancer-blocking activity of suHw may require its interaction with other sites (or insulators) within the nucleus. A second suHw nearby may compete dominantly for the existing suHw and affect the neighboring enhancer-promoter interactions, depending on the *cis* arrangement of these elements. To test this hypothesis, we constructed the *SVS2* transgene in which the VRE enhancer is flanked by two suHw elements. In contrast to the loss of insulator function seen in *VSS2* embryos, the VRE enhancer is more effectively blocked in *SVS2* embryos than in *VS2* embryos (Fig. 3, A, B, and D). Thus, it is the tandem arrangement rather than physical proximity that causes the loss of insulator activity. VRE-mediated dorsal activation of the divergently transcribed *miniwhite* is also diminished in *SVS2* embryos (*19*), indicating that VRE is blocked from promoters on either side. suHw-mediated blockage of VRE is significantly reduced in *SVS2/mod(mdg4)^{u1}* embryos (Fig. 3C), indicating that a MOD-(MDG4)-mediated complex is required for the enhanced insulator activity (*13, 16, 20*). *VSS2*,

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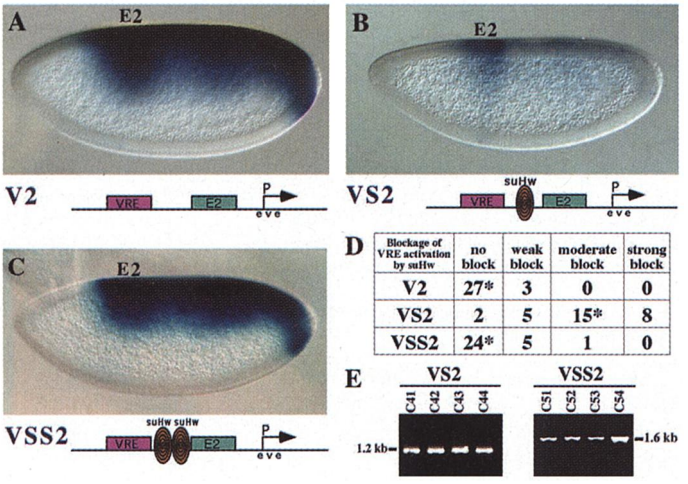


Fig. 1. suHw-mediated blockage of the VRE enhancer depends on copy number of the insulator. *eve-lacZ* reporter expression was detected in transgenic embryos (anterior is left, dorsal is up) (*19*). In transgene diagrams (not drawn to scale), boxes represent enhancers, ovals represent suHw insulators, and arrows represent the *eve-lacZ* promoter. (**A**) A *V2* embryo showing composite *lacZ* pattern consisting of VRE-directed broad dorsal stain and the anterior E2 stripe, repressed in the ventral region. (**B**) An intervening suHw in *VS2* greatly reduced the VRE-directed dorsal activation but did not affect ventral repression of E2. (**C**) Two tandem copies of suHw in *VSS2* failed to block VRE-mediated activation, shown by the intense *lacZ* expression in the dorsal region. (**D**) Thirty transgenic embryos from three random insertion lines (10 embryos per line) were categorized for blockage of VRE-mediated activation by visual inspection. The most frequently observed staining patterns (asterisks) are those shown in (A) to (C). (**E**) Independent *VS2* and *VSS2* lines (four each) were analyzed by genomic PCR using transgene-specific primers. Expected product sizes for intact transgenes are indicated.

NSSH, and *PSS3* transgenes were also examined in a *mod(mdg4)^{u1}* background, and no change in the staining patterns was seen (*19*). The structural integrity of the *SVS2* transgenes was confirmed by genomic PCR (Fig. 3E).

Insulators block enhancer-promoter interactions only when positioned between them. How can this occur without inactivating the enhancer or promoter (*12*)? It has been hypothesized that insulators may interact with each other to form chromatin loop domains, restricting interactions among neighboring regulatory elements (*5, 21, 22*). We showed that the enhancer-blocking activity mediated by suHw is abolished when insulators are in tandem, and is enhanced when they flank the enhancer. Thus, suHw does not seem to block distal enhancers by locally capturing the enhancer complex or its associated proteins, because two tandem elements abolished rather than enhanced the insulator function. Instead, a single intervening suHw insulator may interact with other insulators or chromosomal/nuclear sites (Fig. 4A) (*21*), separating the enhancer and the promoter into topologically distinct chromatin domains. Two tandem suHw elements may preferentially interact with each other, excluding other interactions necessary to sequester the enhancer from the promoter, and may even augment the enhancer-promoter interaction by "looping out" the intervening DNA (Fig. 4B) (*23*). In contrast, suHw elements flanking an enhancer may readily interact as a result of their proximity, leading to better blockage of the enhancer (Fig. 4C). Loss of insulator function was seen when the distance between the two tandem suHw elements is 50, 150, and 170 bp (*VSS2*, *PSS3*, and *NSSH*, respectively). It has also been observed with spacers ranging from 200 bp to 5 kb in length (*23*). Therefore, it is unlikely to be caused by nonspecific steric hindrance due to the close juxtaposition of the insulators. DNA looping has been observed between interacting regulatory elements as close as 100 bp apart (*24*). Insulator assembly may induce alternative chromatin structure, resulting in DNA bending or nuclease-hypersensitive sites, which often indicate nucleosome-free DNA, to facilitate loop formation (*4, 25, 26*). Insulators or chromatin boundaries are frequently found in multiple copies, flanking enhancers or the genetic locus they regulate, such as the scs and scs′ elements, the Mcp-1 and Fab boundaries, and the chicken β-globin 5′ and 3′ boundaries (*22, 27–29*). Selective interactions between neighboring insulators may regulate the access of

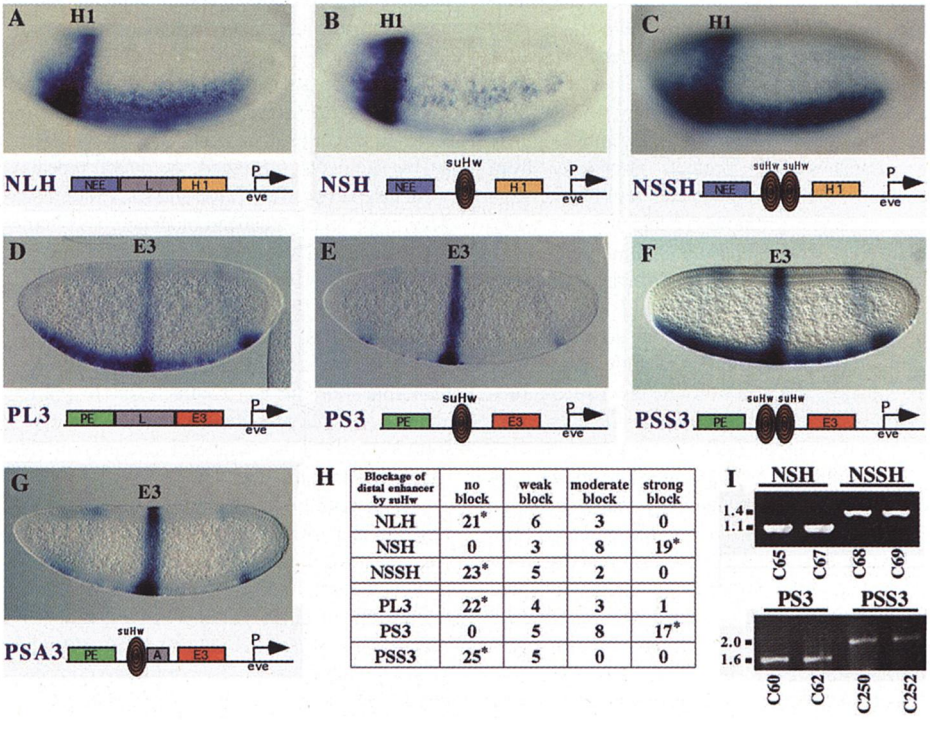
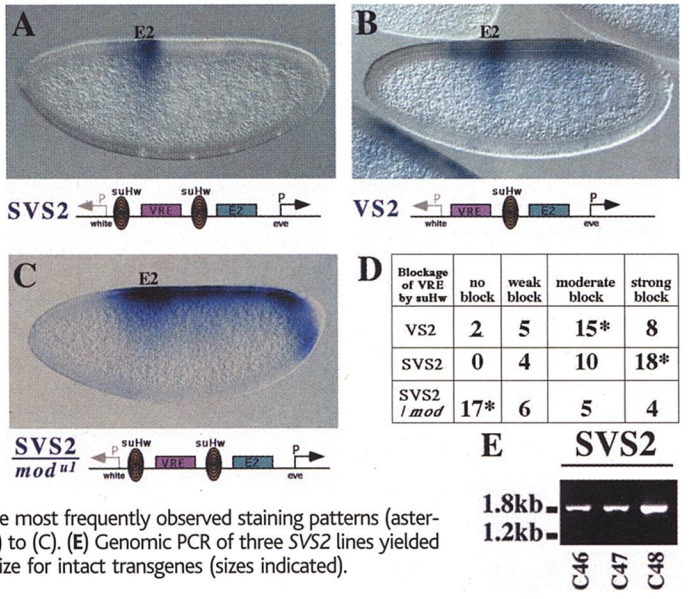


Fig. 2. Loss of insulator activity with tandem suHw is independent of the enhancer tested. (**A**) *NLH* embryos exhibit ventrolateral expression directed by NEE and an anterior transverse stripe directed by H1. (**B**) NEE-directed ventrolateral expression is blocked by suHw in the *NSH* embryos, whereas H1 expression is unaffected. (**C**) NEE-directed expression is not blocked by two tandem suHw elements in *NSSH* embryos. (**D**) *PL3* embryos showing PE-directed *lacZ* expression in the ventral region and an E3-directed mid-embryo stripe. PE expression is more intense in the anterior, possibly as a result of repressors bound to E3. (**E**) *PS3* embryos exhibit greatly reduced PE-directed ventral expression. The E3 stripe was unaffected. (**F**) Two tandem copies of suHw failed to block the upstream PE enhancer in *PSS3* embryos, as shown by the strong *lacZ* staining in the ventral region. (**G**) Replacing one of the tandem suHw elements with a 320-bp neutral spacer in *PSA3* restored the suHw insulator function, resulting in the blockage of the PE-directed ventral expression, whereas the E3 stripe was unaffected. (**H**) suHw-mediated blockage of the upstream enhancer was categorized in 30 embryos from three random lines (10 embryos per line) for each transgene. The most frequently observed staining patterns (asterisks) are those shown in (A) to (F). (**I**) Genomic PCR of *NSH*, *NSSH*, *PS3*, and *PSS3* lines (two each) yielded products of the expected size for intact transgenes (sizes indicated).



Blockage of VRE by suHw	no block	weak block	moderate block	strong block
VS2	2	5	15*	8
SVS2	0	4	10	18*
SVS2 / *mod*	17*	6	5	4

Fig. 3. Two flanking suHw elements can block VRE more effectively. VRE-mediated dorsal staining is blocked more effectively in *SVS2* embryos (**A**) than in *VS2* embryos (**B**). E2 expression and VRE-mediated repression of E2 are unaffected. (**C**) suHw insulator activity was reduced in embryos hypomorphic for *mod(mdg4)^{u1}*, shown by the intense dorsal stain. (**D**) suHw-mediated blockage of VRE was categorized in three random lines of *VS2*, *SVS2*, and *SVS2/mod(mdg4)^{u1}* embryos. The most frequently observed staining patterns (asterisks) are those shown in (A) to (C). (**E**) Genomic PCR of three *SVS2* lines yielded products of the expected size for intact transgenes (sizes indicated).

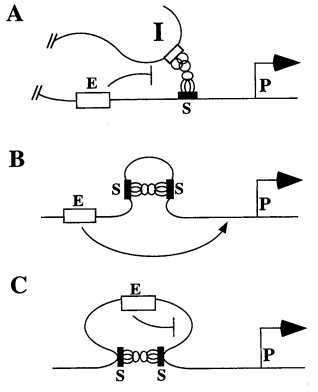


Fig. 4. Insulator-mediated loop formation. (**A**) A suHw insulator (S) may interact with other nuclear sites/insulators (I), separating the enhancer (E) and the promoter (P) into distinct domains and blocking their interaction. (**B**) Interactions between two tandem suHw insulators fail to sequester the enhancer and may even facilitate enhancer-promoter interaction by "looping out" the intervening DNA. (**C**) Enhancer blocking may be strengthened by the preferred interactions between two suHw insulators flanking the enhancer.

tissue-specific enhancers to target promoters by forming alternative chromatin loop domains. It is conceivable that these domains not only block inappropriate enhancers but also facilitate interaction between distant enhancers and the target promoter.

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19. Transgenes shown are derived from pCaSPeR-containing enhancers and insulators inserted between divergently transcribed *miniwhite* and *lacZ* reporter genes. The cloning of suHw and *V2*, *VS2*, *NLH*, *NSH*, *PL3*, and *PS3* transgenes was as described (*13*). A 360-bp fragment from the chloramphenicol acetyltransferase (CAT) gene coding region was PCR-amplified to produce *PSA3*. Details of transgene construction are available upon request. Copy number, position, and orientation of enhancers and insulators were characterized by restriction digestions and also, in some cases, by DNA sequencing. P-element transformation using y^1w^{67c23} *Drosophila* and whole-mount RNA in situ staining was done as described (*13*, *30*). *mod(mdg4)^{u1}* virgin females were mated with transgenic males to produce transgene/*mod(mdg4)^{u1}* embryos (*13*). Genomic PCR analysis of transgenes was performed according to standard protocols (Promega). *VS2*, *VSS2*, and *SVS2* PCR products were further digested with Eco R1 and Bam H1.
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Loss of Insulator Activity by Paired Su(Hw) Chromatin Insulators

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Chromatin insulators are regulatory elements that block the action of transcriptional enhancers when interposed between enhancer and promoter. The *Drosophila* Suppressor of Hairy wing [Su(Hw)] protein binds the Su(Hw) insulator and prevents enhancer-promoter interaction by a mechanism that is not understood. We show that when two copies of the Su(Hw) insulator element, instead of a single one, are inserted between enhancer and promoter, insulator activity is neutralized and the enhancer-promoter interaction may instead be facilitated. This paradoxical phenomenon could be explained by interactions between protein complexes bound at the insulators.

The *Drosophila* gypsy retrotransposon contains a chromatin insulator that consists of cluster of 12 binding sites for the Su(Hw) zinc-finger protein (*1*–*6*). In the presence of Su(Hw) protein binding, the insulator blocks the activity of an enhancer separated from the promoter by an Su(Hw) binding region. However, this insulator action fails in certain genetic rearrangements that introduce more than one *gypsy* retrotransposon in the region of the *yellow* gene (*7*). The loss of insulator activity might result from intrachromosomal pairing between the two *gypsy* retrotransposons, causing chromatin to fold and allowing the enhancer to contact the promoter. Alternatively, interaction between the pro-