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Genomic context modulates insulator activity through promoter competition

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SUMMARY

Chromatin insulators regulate gene expression by preventing inappropriate enhancer-promoter interactions. Our previous study showed that insulators do not merely function as rigid blockers, rather their activities are quantitative and selective. We have investigated the factors and mechanisms that determine the effectiveness of the suHw insulator in transgenic *Drosophila*. We show that the suHw-mediated blockage of the AE1 enhancer from a downstream promoter depends on the ability of the promoter to compete for AE1. Promoters that are highly competitive for the enhancer are blocked less effectively. Moreover, blockage of AE1 from its cognate *ftz* promoter can range from virtually complete to non-detectable, depending on the property of the neighboring upstream promoter. A highly competitive neighboring promoter enhances the suHw-mediated blockage, whereas a less competitive promoter reduces the insulator effectiveness. The influence on insulator effectiveness by both the interacting and the neighboring competing promoters correlates with their ability to compete for the enhancer, which was previously shown to depend on core promoter sequences. Our findings suggest a mechanism at the level of gene organization that modulates insulator effectiveness through promoter competition. The dependence of insulator function on its *cis* contexts may provide it with more regulatory flexibility while imposing organizational restraints on eukaryotic gene complexes.

Key words: Chromatin boundary, Enhancer specificity, Insulator, Promoter competition, suHw, *Drosophila*

INTRODUCTION

Tissue- and developmental stage-specific gene activation in higher eukaryotes depends on interactions between two classes of *cis*-regulatory DNA elements, the basal promoter where the transcription complex assembles, and the more distally located enhancers which interact with regulatory proteins (Burley and Roeder, 1996; Carey, 1998; Gray and Levine, 1996; Hansen et al., 1997; Small and Levine, 1991; Thanos and Maniatis, 1995; Verrijzer and Tjian, 1996). The relative independence of enhancer action regarding distance and orientation to the promoter presents a particular problem for the specificity of regulation among closely linked genes, such as those found in homeotic gene complexes (Gindhart et al., 1995; Karch et al., 1985; Krumlauf, 1994). Recent studies suggest that two distinct mechanisms specify promoter-enhancer interactions in eukaryotic gene complexes. Enhancer specificity may be determined by competition among multiple promoters through which the most preferred promoter(s) preclude others (Choi and Engel, 1988; Corbin and Maniatis, 1989; Foley and Engel, 1992; Sharpe et al., 1998; Walker et al., 1997). This is well illustrated by the developmental switch of the vertebrate β-globin genes in which two *cis*-linked ε and β globin promoters compete for a shared β/ε enhancer. During the embryonic stage, the ε gene promoter out-competes the β gene promoter and is preferentially activated by the β/ε enhancer. In adults, binding of adult-specific transcription factors near the basal promoter of the β gene augments the enhancer-β promoter interaction, resulting in activation of the β gene and concomitant shut-off of the ε gene (Foley and Engel, 1992).

Core promoter sequences have been shown to influence the ability of promoters to compete for a given enhancer (Merli et al., 1996; Ohtsuki et al., 1998). Specifically, the contribution of core sequences to the promoter competitiveness has been shown with two early *Drosophila* enhancers, AE1 and IAB5. The autoregulatory enhancer (AE1) of the *fushi tarazu* gene (*ftz)* directs the expression in seven transverse stripes during germ band extension in *Drosophila* embryogenesis. AE1 selectively activates *ftz* but not the neighboring homeotic gene *Sex combs reduced* (*Scr*), in spite of its intergenic position and comparable distance from both promoters (Hiromi et al., 1985; LeMotte et al., 1989; Ohtsuki et al., 1998; Pick et al., 1990; Schier and Gehring, 1993). The AE1 promoter specificity could be determined by the differences in the basal promoters of the two genes: whereas the *ftz* promoter contains a canonical TATA box, the *Scr* promoter contains no optimally defined core promoter motifs such as the TATA sequence, initiator (INI) or downstream promoter element (DPE; Burke and Kadonaga, 1996; Kutach and Kadonaga, 2000; Smale, 1997). The infra-abdominal 5 (IAB5) enhancer interacts specifically with the *Abdominal B* gene (*Abd-B*) and directs a broad band of expression in the presumptive abdomen of gastrulating embryos. Both AE1 and IAB5 contain binding sites for the FTZ activator. In transgenic *Drosophila* embryos, AE1 or IAB5 placed between two divergently transcribed reporter genes preferentially activates transcription from the TATA

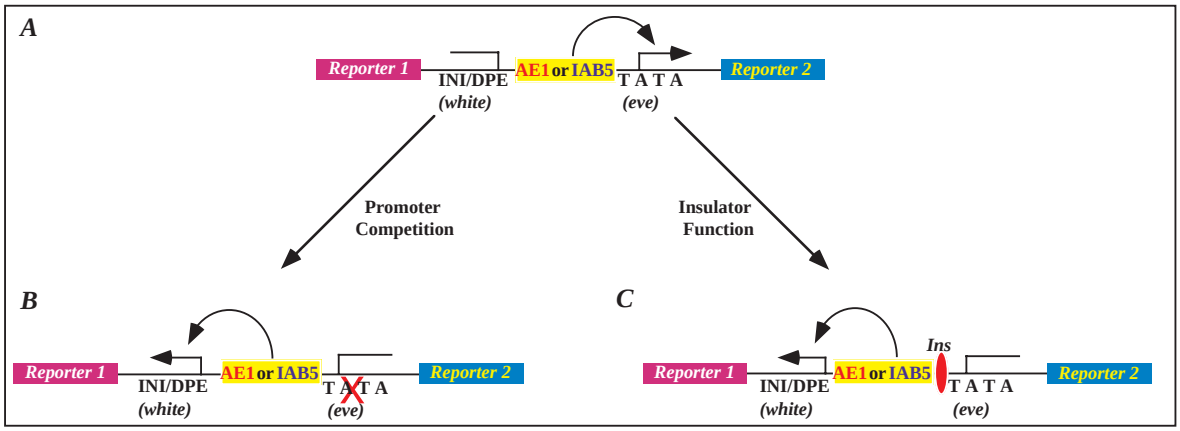

Fig. 1. Two mechanisms that specify enhancer and promoter interaction in transgenic *Drosophila*. Schematic diagram of transcriptional interactions between AE1 and IAB5 enhancers and promoters in transgenic *Drosophila*. Transgenes are shown to contain AE1 or IAB5 (yellow rectangle) between divergently pointed promoter-reporter fusion genes (red and blue boxes). Direction of transcription and the position of start site are indicated by the arrows. Activating interactions between promoter and the enhancers are represented by arched arrows. Core promoter elements of *eve* and *white* are indicated underneath each promoter. The size and distance of the DNA elements are not to scale. (A) AE1 and IAB5 preferentially activate transcription from the *evenskipped* (*eve*) promoter which contains TATA, but not from the *white* promoter which contain initiator (INI) and DPE, but not TATA sequences. (B) AE1 and IAB5 can activate the *white* gene if the TATA region in the *eve* promoter is changed to the corresponding region from the *white* promoter. (C) AE1-*eve* interaction is completely blocked by the suHw insulator (red oval), with a redirection of transcription activation to the *white* promoter.

containing *evenskipped* (*eve*) promoter, but not from the TATA-less *white* promoter (Fig. 1A, also see Construct 1 in Table 1; Ohtsuki et al., 1998). The promoter specificity of these two enhancers is due to the competition from the TATA containing *eve*, rather than to incompatibility with *white*, as the enhancers can activate the *white* gene if the *eve* promoter is replaced with a TATA-less promoter such as that from the *Transposase* gene; or if the TATA region in the *eve* promoter is changed to the corresponding region from *white* (Fig. 1B; Ohtsuki et al., 1998). The contribution of core sequences to promoter competitiveness is also supported by the observation that inserting an 8 bp TATA sequence into the *white* promoter enhances its competitiveness and results in its activation against *eve* (Ohtsuki et al., 1998). The competitive nature of promoter selection is further demonstrated by the observation that the AE1-*white* interaction can be restored by blocking the competing AE1-*eve* interaction with the suHw insulator (Fig. 1C; Ohtsuki et al., 1998).

Insulators represent the other important mechanism that regulates promoter-enhancer interactions in complex genetic loci in diverse organisms (Bell and Felsenfeld, 1999; Bi et al., 1999; Chung et al., 1993; Donze et al., 1999; Ellis et al., 1996). *Drosophila* insulators such as scs, scs' and suHw were identified as intergenic boundaries that are proposed to organize specialized chromatin structures or independent chromatin domains. Insulators are also found as intragenic regulatory elements (modulators), such as the Mcp-1, Fab-7 and Fab-8 elements in the *Abd-B* gene. They have been postulated to directly regulate *Abd-B* activity by modulating interactions between its promoter and upstream segment-specific enhancers (Galloni et al., 1993; Karch et al., 1994; Kellum and Elgin, 1998; Zhou et al., 1999).

The molecular basis of insulator action is not known. The best understood example is suHw, a 340 bp DNA element from the *Drosophila* retrotransposon *gypsy*. The suHw insulator causes mutations when transposed into regulatory regions of various genes. It also acts as a boundary by shielding chromosomal position effect when flanking a transgene (Hagstrom et al., 1996; Roseman et al., 1993; Sigrist and Pirrotta, 1997). Both the mutagenic effect and the boundary function of suHw are related to its ability to disrupt enhancer-promoter interactions. In fact, enhancer-blocking activity is observed in insulators either identified as enhancer modulators, or as chromatin boundaries (Cai and Levine, 1995; Chung et al., 1997; Kellum and Schedl, 1992; Milot et al., 1996; Scott and Geyer, 1995; Zhou et al., 1996). The insulator activity of suHw requires the function of two cellular proteins: SuHw, which directly interacts with the suHw DNA element, and Mod(mdg4), a chromosomal protein that interacts with the insulator element through SuHw (Cai and Levine, 1995; Gerasimova et al., 1995; Geyer and Corces, 1992; Parkhurst et al., 1988).

Previous studies have shown that insulators can function quantitatively and selectively. The strength and selectivity of their enhancer-blocking function depend on the qualitative and quantitative characteristics of the insulator and enhancers involved (Cai and Levine, 1995; Hagstrom et al., 1996; Scott et al., 1999; Zhou et al., 1996). In this study, we have investigated how promoter competitiveness and gene configuration affect insulator function. The suHw-mediated blockage of the AE1 enhancer was examined between two divergently transcribed fusion genes in transgenic embryos. We show that the properties of the core promoters that interacts with AE1 (interacting promoters) determine both their competitiveness for AE1 and their susceptibility to the suHw-mediated blockage. In addition, the suHw-mediated blockage of AE1 is also influenced by the neighboring promoters (competing promoters). Promoter-AE1 interactions strongly challenged by competing promoters are more susceptible to the suHw-mediated blockage. These observations provide evidence for a novel mechanism through which insulator function is modulated according to its regulatory contexts.

MATERIALS AND METHODS

P-element transformation and whole mount in situ hybridization

The y^1w^{67c23} *Drosophila* strain was used to generate all the transgenic lines reported here. P element-mediated germline transformation was carried out as described previously (Cai and Levine, 1997). Briefly, P-transposon DNA-containing fusion promoter/reporter genes was prepared using Qiagen Plasmid Midi Kit. The transgenic construct DNA and the helper plasmid containing the P-transposase gene (pUC-hsπΔ2-3) were co-injected into pre-cellularized embryos at a concentration ratio of 1.0: 0.1 mg/ml injection buffer (5 mM KCl and 0.1 mM K_2HPO_4, pH 6.8). Three or more independent w^+ lines were obtained and characterized for each transgene. Transgenic embryos were collected and fixed as described previously (Cai and Levine, 1997). Reporter gene expression in gastrulating embryos was detected using whole-mount in situ hybridization with digoxigenin-UTP labeled antisense RNA probes. Expression patterns were visualized by colorimetric reaction following incubation with anti-digoxigenin antibody conjugated to alkaline phosphotase (Genius Kit, Boehringer; Jiang et al., 1991; Tautz and Pfeifle, 1989). Same amounts of the anti-*Scr* probe were used in each in situ hybridization experiment. The anti-reporter probes were added in the following constant ratios to the anti-*Scr* probe: anti-*lacZ*: 0.2:1, anti-white: 1:1 and anti-CAT: 1:1. Hybridization was carried out at 55ºC for 18 hours in 60 μl final volume and colorimetric staining was developed at room temperature for 65 minutes. Thirty to 50 embryos at germ band extension stage were scored for number and level of AE1 expression, and categorized into weak (<20%), moderate (20-60%) and strong (>60%) levels, by comparing with maximal level of staining intensity (100%). For each transgene reported here, hybridization was carried out with two to four lines and repeated two to three times to ensure accurate representation.

Construction of AE1 fusion promoter constructs

All P-transposons used in this report are derivatives of pCaSPeR containing the mini-*white* marker/reporter gene. Construction of the *white*-AE1-*eve* and *white*-AE1-suHw-*eve* transgenes has been described previously (Ohtsuki et al., 1998). For constructs containing *ftz-Scr, ftz-white, ftz-eve, Scr-eve and Scr-white* promoter pairs, *Bam*HI-*Eco*RI fragments containing minimal promoter elements and a short coding region containing the first ATG codon were excised from pFEP (*eve*; Ohtsuki et al., 1998), and pFpSu3 (*ftz* and *Scr*; S. Ohtsuki, personal communication). Pairwise combinations of purified promoter fragments were then ligated and cloned into the *Bam*HI site in a pBluescript vector (KSΔEcoRI), resulting in dual-promoter subclones (KS-2Ps). *Eco*RI fragments containing AE1 and AE1-suHw, respectively, were inserted into the *Eco*RI site between the two promoters in each of the KS-2P plasmids. The position and orientation of the enhancer and insulator were determined by restriction digestions and DNA sequencing. *Bam*HI fragments containing dual-promoter combinations with AE1 or AE1-suHw between the two promoters were then ligated into pCaSPeR based injection vectors containing divergently pointed CAT and *lacZ* reporter genes. The resulting constructs contain AE1 or AE1-suHw between divergently transcribed promoters fused in-frame with reporter genes. The transgenic constructs were characterized extensively by restriction digestion and some by sequencing analysis before used for the micro-injection procedure as described above.

RESULTS

The *ftz*-AE1 interaction can overcome the suHw-mediated insulation

The AE1 enhancer activates the expression of *ftz* but not the neighboring *Scr* in spite of its intergenic position. Given the preference of AE1 for TATA promoters, its endogenous specificity between the TATA containing *ftz* and the TATA-less *Scr* may be determined by the competition between the two promoters (Ohtsuki et al., 1998). We tested whether the in vivo specificity of the AE1 enhancer between *ftz* and *Scr* could be replicated in transgenic embryos; and, if so, could suHw redirect AE1 to interact with *Scr* as predicted by the competition model. A 430 bp AE1 enhancer was inserted into the P-transformation vector between divergently transcribed *ftz* and *Scr* promoters fused to CAT and *lacZ* reporter genes, respectively (Fig. 2; Pick et al., 1990). Transgenic embryos were examined using whole-mount RNA in situ hybridization with antisense probes against the reporter mRNA (Tautz and Pfeifle, 1989). In order to quantitate the level of AE1 directed reporter expression, all in situ hybridization described in this report was carried out with an internal control probe that hybridizes to the endogenous *Scr* mRNA. Identical amounts of the anti-*Scr* probe were added to each anti-reporter probe (e.g., anti-*lacZ*, anti-CAT or anti-*white*) at a constant ratio, and all hybridization and staining reactions were carried out in the same volume and for the same length of time (see Materials and Methods for details). As shown in Fig. 2, AE1 activates the *ftz*-CAT fusion gene expression in seven transverse stripes (Fig. 2A) while no *Scr-lacZ* expression was detected (Fig. 2B).

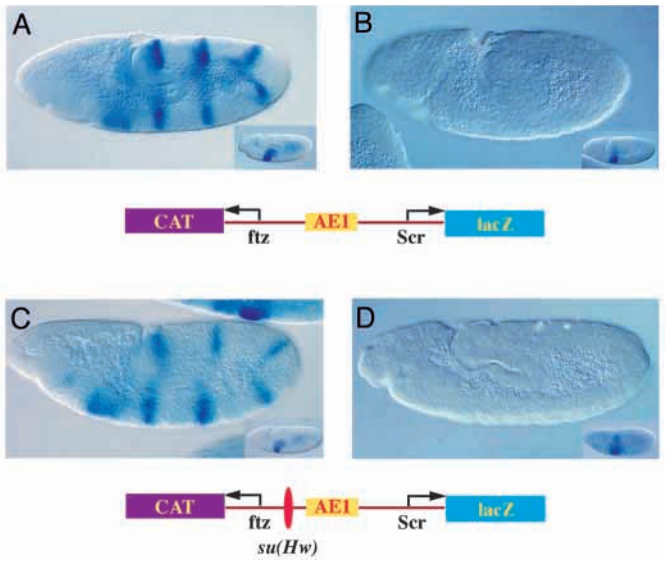

Fig. 2. The *ftz*-AE1 interaction overcomes the suHw insulator. Transgenic *Drosophila* embryos containing the AE1 enhancer between divergently transcribed promoter-reporter fusion genes were hybridized with antisense probes for reporter genes. The transgene is diagrammed below each pair of embryos with the reporter gene directly below the embryo showing the corresponding expression. Orientation and location of the promoters in the diagrams are indicated by arrows. The size and distance of the DNA elements are not to scale. Germband extension stage embryos are shown anterior to the left and dorsal up. (A,B) AE1 specifically activates *ftz*/CAT expression (A, CAT expression) but not the *Scr/lacZ* expression (B, *lacZ* expression). (C,D) AE1-*ftz*/CAT interaction overcomes the intervening the suHw insulator (C, CAT expression) and no AE1 specific expression is detected from the *Scr/lacZ* fusion gene (D, *lacZ* expression). The inset in each panel shows the endogenous *Scr* expression at or near the same stage as an internal control of the in situ staining (see Results, and Materials and Methods for details).

Table 1. AE1-directed reporter gene expression from transgenic embryos

Construct	Probe	AE1-directed reporter expression		
		Weak	Moderate	Strong
(1) WAE	lacZ	3	10	6
	white	1	0	0
(2) WAsuE	lacZ	0	0	0
	white	3	12	10
(3) FAS	lacZ	0	0	0
	CAT	0	8	9
(4) FsuAS	lacZ	0	0	0
	CAT	0	10	8
(5) WAF	lacZ	2	11	8
	white	3	1	0
(6) WAsuF	lacZ	14	3	0
	white	4	0	0
(7) FAE	lacZ	5	19	1
	CAT	10	14	1
(8) FsuAE	lacZ	0	0	0
	CAT	10	12	2
(9) EAS	lacZ	0	0	0
	CAT	0	18	0
(10) EsuAS	lacZ	0	0	0
	CAT	5	1	0
(11) WAS	lacZ	0	0	0
	white	2	24	1
(12) WsuAS	lacZ	0	0	0
	white	3	6	0

The levels and frequency of AE 1-directed reporter expression from tested promoters are scored from 30-50 germ band extension stage embryos for each transgene. Embryos were categorized into weak, moderate and strong groups, according to AE1-specific expression (see Materials and Methods for details). Names of the constructs indicate key regulatory elements contained between the reporter genes: A, the AE1 enhancer; su, the suHw insulator; W, the *white* promoter; E, the *eve* promoter; F, the *ftz* promoter; S, the *Scr* promoter.

Table 1 summarizes the AE1-directed reporter gene expression from each promoter, scored both in level and frequency among 30-50 embryos (see Materials and Methods for details). The most frequently observed pattern was chosen for the figures (Fig. 2A,B, Table 1 (Construct 3)). The internal control *Scr* expression is shown as an inset in each panel. Although both *ftz* AE1 enhancer and the endogenous *Scr* gene are active during the germ band extension stage, the dynamics of the two genes differ so that the *Scr* expression in the labial segment is often weak or undetectable when peak level AE1 activity is seen, and vise versa (see Fig. 2A,C, compare with Scr expression in the inset panel).

We next inserted a 350 bp suHw element between the AE1 enhancer and the *ftz* promoter in the above transgenic construct (Fig. 2C,D; Cai and Levine, 1995). To our surprise, suHw failed to block the *ftz*-AE1 interaction, as the level of CAT activity is comparable between embryos bearing the transgenes with and without suHw (compare *ftz*/CAT expression in Fig. 2A and 2C, Table 1 (Constructs 3 and 4)). The *Scr* promoter remains inactive in the embryos where AE1-*ftz* interaction is not blocked (*Scr/lacZ* expression, Fig. 2D). This result is in sharp contrast to our previous observation with AE1-*eve* interaction, which is completely blocked with a concomitant re-activation of the *white* promoter (Fig. 1C; Ohtsuki et al., 1998; Table 1 (Constructs 1 and 2)). It has been documented previously that changes in the characteristics of insulators or enhancers can influence the effectiveness of insulation (Cai and Levine, 1995; Hagstrom et al., 1996; Scott et al., 1999). However, our results here suggest that factors other than the insulator and the enhancer, which remained the same in both cases shown above (Figs 1C, 2C,D), can also dramatically influence the outcome of the insulator function.

suHw function is influenced by the interacting and neighboring promoters

The differential blockage of AE1 by suHw in the above two pairs of transgenes could result from different interacting promoters (*eve* in Fig. 1C, and *ftz* in Fig. 2C), which directly participate in the interactions the insulator impedes. Previous studies have shown that the promoter preference of AE1 depends on the presence of the TATA core motif and that *eve* and *ftz*, both containing TATA motif, can compete comparably for AE1 (Ohtsuki et al., 1998). However, the sequences immediately flanking the TATA motif diverge significantly between the *eve* and *ftz* promoters, so it is possible that AE1-*ftz* interaction is stronger and not blockable by the suHw insulator. Alternatively, the differential blockage of AE1 by suHw may result from the different neighboring competing promoters in the two transgenes (*white* in Fig. 1C, *Scr* in Fig. 2D), which could influence the *ftz*-AE1 interaction through promoter competition, thereby altering the effectiveness of suHw.

To distinguish between these two possibilities, we examined the effectiveness of suHw in blocking the same *ftz*-AE1 interaction against different neighboring promoters. For the following constructs, we used the *white* and *eve* promoters, whose core sequences have been shown to affect their ability to compete for enhancers such as AE1 and IAB5 (Fig. 1; Ohtsuki et al., 1998). The *white* promoter, which lacks TATA but contains an INI and a DPE, was first tested in place of the *Scr* promoter, which lacks any recognizable core promoter motif (Fig. 3A-D, Table 1 (Constructs 5, 6)). As in the *white*-*eve* promoter pairing, the TATA containing *ftz* promoter is preferentially activated by AE1 at the expense of the *white* promoter (Fig. 3A,B, compare with Fig. 1A; Table 1 (Construct 5)). However, when suHw was placed between the *ftz* promoter and AE1, their interaction is attenuated by the insulator, not completely blocked as seen with *eve*-AE1 (Fig. 3d, compare with Fig. 1C; Table 1 (Construct 6); Ohtsuki et al., 1998). Comparison of the suHw-mediated blockage of *ftz*-AE1 and *eve*-AE1 interactions, when opposed by the same *white* promoter, shows that the difference in the interacting promoters does affect the insulator effectiveness. It indicates that although *eve* and *ftz* both contain TATA elements, *eve*-AE1 interaction appears significantly weaker than *ftz*-AE1 interaction in terms of resistance to the suHw-mediated blockage. More interestingly, the suHw-mediated blockage of the same *ftz*-AE1 interaction is significantly enhanced when the competing promoter changed from *Scr* to *white* (Fig. 3C,D, compare with Fig. 2C,D; Table 1 (Constructs 4, 6)). This result confirms that the neighboring promoters, although out-competed by *ftz* and transcriptionally silent (Figs 1A, 2A), indeed influence the dynamics of AE1-*ftz* interaction and therefore the effectiveness of suHw. It is also worth noting that the attenuated *ftz*-AE1 interaction is not accompanied by an increase in the *white*-AE1 interaction (Fig. 3C,D, Table 1 (Construct 6)), although such redirection was seen when the *eve*-AE1 interaction was completely blocked (Fig. 1C; Table 1 (Construct 2); Ohtsuki et al., 1998).

The enhanced blockage of *ftz*-AE1 by suHw in the above experiment may be due to the increase in the competitiveness of the neighboring promoter from *Scr* to *white.* If so, an even more competitive neighboring promoter should further enhance the suHw-mediated blockage. To test this, we replaced *white* in the above transgene with *eve*, which is significantly more competitive for AE1 than *white* (Fig. 1; Ohtsuki et al., 1998). AE1 placed between *ftz* and *eve* promoters can simultaneously activate both reporter genes (Fig. 3E,F, Table 1 (Construct 7); Ohtsuki et al., 1998). As predicted, a suHw insertion between AE1 and the *ftz* promoter completely blocks their interaction (Fig. 3H, Table 1 (Construct 8)). The complete blockage of *ftz* is also accompanied by an increase in the *eve*-CAT activity, indicating a redirection of AE1 activation to *eve* (Fig. 3G). Similar redirection of AE1 to *white* was observed when the *eve* promoter was completely blocked (Fig. 1C, Table 1 (Construct 2); Ohtsuki et al., 1998), but was not seen when *ftz* was partially blocked (Fig. 3C, Table 1 (Construct 6)). More importantly, the dramatic difference in the effectiveness of the same suHw insulator in blocking the same *ftz*-AE1 interaction when the neighboring promoter changes from *Scr* (no blockage) to *white* (partial blockage), and then further to *eve* (complete blockage) illustrates the significant influence on insulator function by the neighboring promoters.

Our previous analyses have demonstrated that promoter competition, which depends on the core promoter sequences, is important for enhancer-promoter specification (Ohtsuki et al., 1998). The use of the AE1 enhancer and the same set of promoters in the current study further suggests that promoter competition is the underlying mechanism through which neighboring genes modify the outcome of insulator function. These findings provide the first evidence for a mechanism through which the effectiveness of an insulator is determined by regulatory context of neighboring genome. Our results further suggest that the greater the difference between the two competing promoters, the more difficult it is to insulate the enhancer from the interacting promoter (usually the more competitive one). Conversely, a given insulator would be more effective modulate enhancer specificity among more comparable promoters.

Modulation of the insulator function by the neighboring genome is not limited to the cognate *ftz*-AE1 interaction or TATA promoters

The ability of suHw to block the *ftz*-AE1 interaction is sensitive to the influence of neighboring promoters, as shown by the above three pairs of transgenes containing dual promoters (*Scr-ftz, white-ftz* and *eve-ftz*). To analyze if the effect of the competing promoters is unique to the cognate *ftz*-AE1 interaction, we constructed transgenes containing the *Scr-eve* promoter pair to complete a second series of transgenes with *eve* as the interacting promoter (*Scr-eve, white-eve* and *ftz-eve*). As seen in Fig. 4A,B, AE1 preferentially activated *eve* but not the *Scr* promoter (also see Table 1 (Construct 9)). However, when suHw is inserted in between *eve* and AE1, the interaction between the two elements is partially impeded without stimulation in the *Scr*-AE1 interaction (Fig. 4C,D, Table 1 (Construct 10)). The partial blockage suggests that the difference between *eve-Scr* is smaller than that of *ftz-Scr,* but greater than that of *eve-white,* which is consistent with our observations from previous and current studies of competitive interactions among these promoters. These result indicate that the competing influences from neighboring genomic context can modulate insulators effectiveness in blocking both cognate and non-cognate interactions.

The above two series of transgenes tested insulation of TATA-containing promoters as a function of the competing influences from the neighboring promoters. We further tested transgenes bearing AE1 between two TATA-less promoters, *white*, which contains INI and DPE, and *Scr,* which contains no optimal core elements. In our promoter competition and insulation tests, *white* consistently appears more competitive than *Scr* (compare Fig. 2C,D with Fig. 3C,D, and Fig. 1A,C with Fig. 4). In transgenic embryos, AE1 preferentially activates the *white* reporter expression, whereas the *Scr* promoter remains transcriptionally silent (Fig. 5A,B, Table 1 (Construct 11)). The suHw insulator can completely block the *white*-AE1 interaction, with a concomitant redirection of AE1 to *Scr* (Fig. 5C,D, Table 1 (Construct 12)). We have so far shown three pairs of transgenes containing the *Scr* promoter, opposed by *ftz, eve* and *white*, respectively. Again, *Scr* as the competing promoter shows the full range of influence on suHw function, from no blockage against *ftz,* to partial blockage against *eve* and full blockage against *white.* It demonstrates that insulator effectiveness can be affected by competition among various types of core promoters, both in competing and in interacting positions. This result also shows that AE1 indeed can activate the *Scr* promoter in the absence of the *ftz* promoter and that the AE1 specificity between the *ftz* and the *Scr* promoters in vivo could be determined by competition from the *ftz* promoter. The apparent weak interaction between AE1 and the *Scr* promoter could be due to competition from genes surrounding the transgene insertion site, possibly from the downstream direction.

DISCUSSION

Insulators play important roles in controlling gene activity and maintaining regulatory independence between neighboring genes. Recent studies suggest that the differences between the intergenic boundary function and the intragenic enhancer-modulator function of insulator elements may be quantitative and may reflect differences in the assays (Cai and Levine, 1995; Hagstrom et al., 1996; Scott et al., 1999; Zhou et al., 1996). The strength and selectivity of insulator activity could be influenced by the components involved: the insulator and its associated factors, and the enhancer-promoter interaction it intercedes. Indeed, enhancer-blocking studies in transgenic *Drosophila* have shown that the effectiveness of insulators depends on both the qualitative and quantitative characteristics of the insulator, as well as those of the enhancer (Cai and Levine, 1995; Hagstrom et al., 1996; Scott et al., 1999; Zhou et al., 1996).

Interacting promoters

In the current study we have further analyzed the suHw-mediated insulation as a function of the interacting promoters that differ in their core promoter sequences and in their ability to compete for AE1. Previous studies have shown that distinct *cis* elements in the core promoter such as a TATA, INI and DPE, and their associated *trans*-factors determine the ability of

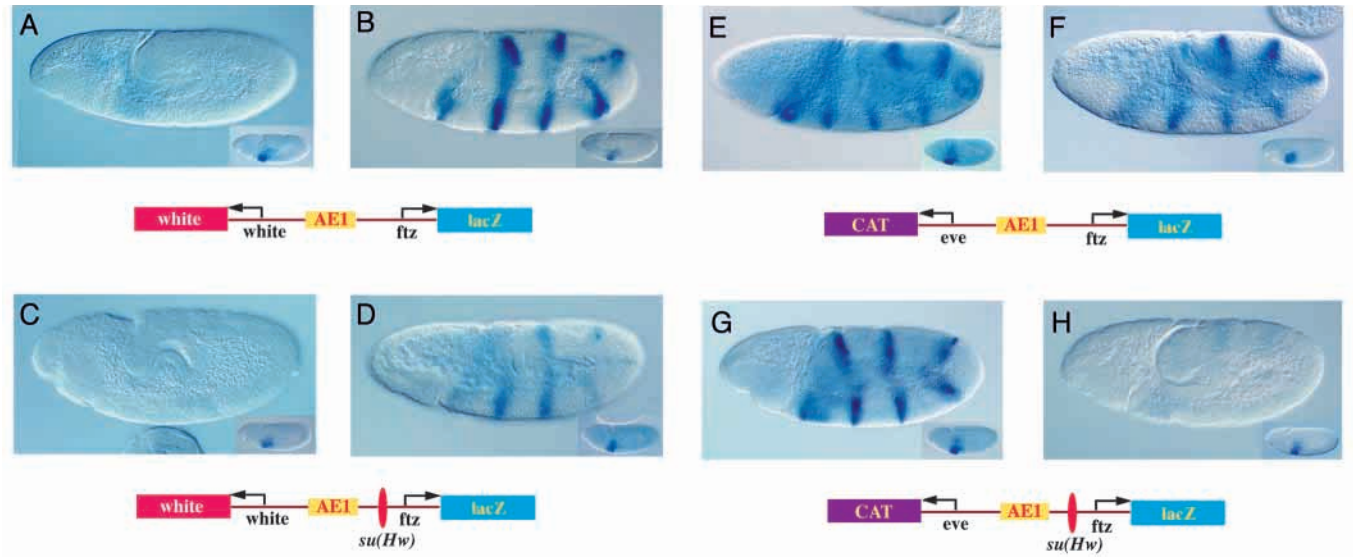

Fig. 3. Competing promoters can influence the suHw-mediated blockage of the *ftz*-AE1 interaction. Reporter gene expression are monitored in transgenic embryos containing the AE1 enhancer placed between two competing promoter-reporter fusion genes. Transgenes are diagrammed beneath each image pair (see Fig. 2 legend for general descriptions). (A-D) Transgenic embryos containing *ftz-white* promoter/reporters. (A,B) AE1 specifically activates *ftz*/*lacZ* expression (B, anti-*lacZ* probe) but not the *white* gene expression (A, *white* expression) in transgenic embryos. (C,D) AE1-*ftz*/lacZ interaction is partially attenuated by the intervening suHw insulator (D, *lacZ* expression) without concomitant restoration of AE1-*white* interaction (C, *white* expression). (E-H) Transgenic embryos containing *ftz-eve* promoter/reporters. (E,F) AE1 simultaneously activates *ftz*/lacZ (F, *lacZ* expression) and the *eve*/CAT expression (E, CAT expression) in transgenic embryos. (G,H) AE1-*ftz*/*lacZ* interaction can be completely blocked by the intervening suHw insulator (H, *lacZ* expression) with a concomitant increase in the level of AE1-*eve*/CAT interaction (G, CAT expression). The inset in each panel shows the internal control *Scr* expression (see Fig. 2 legend, and Materials and Methods for details).

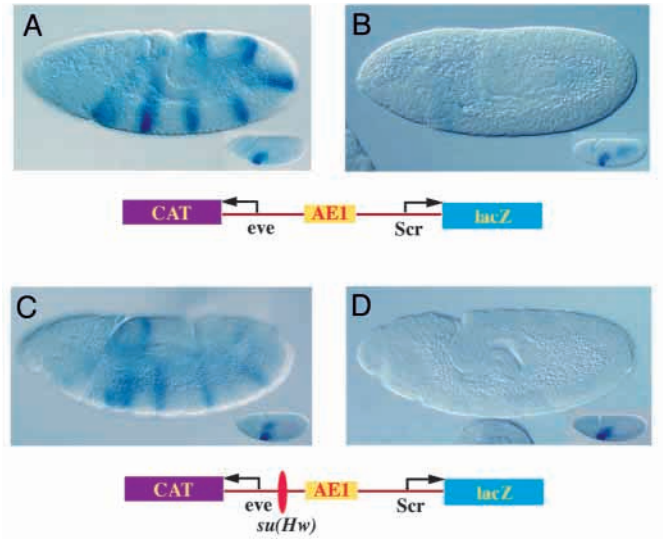

Fig. 4. Modulation of the suHw function by the regulatory context is independent of interacting promoters. Transgenic embryos containing AE1 between divergently transcribed *eve-Scr* promoter/reporter genes are hybridized with anti-reporter probes (A-D, see Fig. 2 legend for general descriptions). (A,B) AE1 activates *eve*/CAT expression (a, CAT expression) but not the *Scr*/*lacZ* expression (B, *lacZ* expression) in transgenic embryos. (C,D) AE1-*eve*/CAT interaction can only be partially blocked by the intervening suHw insulator (C, CAT expression) without concomitant restoration of AE1-*Scr*/*lacZ* interaction (D, *lacZ* expression). The insets show the internal control *Scr* expression (see Fig. 2 legend, and Methods for details).

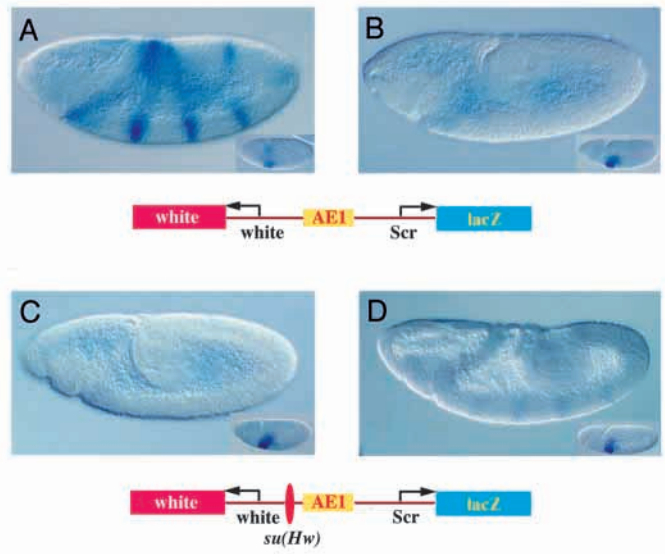

Fig. 5. AE1 specificity between *ftz* and *Scr* may be determined by promoter competition. AE1 can activate *Scr*/reporter expression in transgenic embryos containing divergently transcribed *white-Scr* promoter/reporter pairs (A-D, see Fig. 2 legend for general descriptions). AE1 activates *white* (A, *white* expression) but not the *Scr*/*lacZ* expression (B, *lacZ* expression). AE1-*white* interaction can be completely blocked by the intervening suHw insulator (C, *white* expression) with a moderate concomitant increase in the level of AE1-*Scr* interaction (D, *lacZ* expression). The insets show the internal control *Scr* expression (see Fig. 2 legend, and Materials and Methods for details).

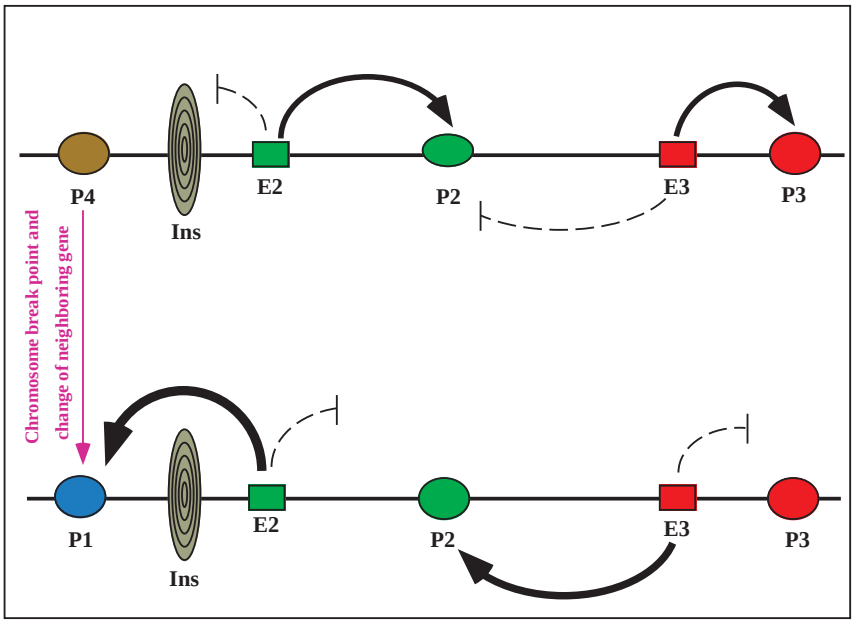

Fig. 6. Models for regulatory interdependence among closely linked genes. Regulatory interdependence 'relays' along neighboring genes and imposes organizational rigidity to closely linked genes or regions of chromosomes. Three closely linked neighboring genes (P2-P4) with their regulatory enhancers (E2-E3) are diagrammed. The specificity between the enhancers and their cognate promoters depends on the neighboring regulatory elements (E, enhancers; P, promoters; In, insulators), owing to the sharing and competing nature of the interactions between these elements (arrows). Changes in the relative positioning or regulatory capacity of any one component element, e.g. change of P4, a non-competitive promoter for E2 to P1, a highly competitive promoter for E2 (see purple arrow), will influence the regulatory outcome of neighboring interactions and in turn interactions further away thereby linking the entire genomic region into one regulatory, organizational and evolutionary unit.

the promoter to interact and compete for regulatory enhancers (Merli et al., 1996; Ohtsuki et al., 1998). We found that the suHw-mediated blockage of AE1 depends on the promoter with which it interacts, as shown by the complete blockage of AE1 from *white*, but not from *eve* or *ftz*, when opposed by *Scr*; or the complete blockage of AE1 from *eve*, but not from *ftz* when opposed by *white*. Our results suggest that the ability of a promoter to compete for an enhancer correlates with the ability of their interaction to resist insulator blockage. These abilities may reflect a characteristic of the enhancer-promoter interaction that is distinct from the one reflected in transcriptional activation. Our results further indicate that even promoters with the same core motifs, such as the TATA sequence, may differ significantly in their interactions with a given enhancers, suggesting a role for sequences outside of core motifs to also contribute to enhancer specificity. Recent studies indicate that different TATA-binding proteins (TBP, TRFs) and/or TBP associated factors (TAFs) may interact with distinct TATA promoters to confer gene and tissue specificity (Buratowski, 1997; Holmes and Tjian, 2000). The transcription complexes assembled at the these basal promoters may be different and so are the interactions they forge with upstream regulatory proteins.

Competing promoters

A key finding from our study is that insulator function is affected by the balance of promoter competition among neighboring genes. The *ftz*-AE1 interaction becomes more susceptible to the suHw-mediated blockage when challenged by neighboring promoters such as *eve*, which has been shown in our previous study to be highly competitive for AE1, owing to the presence of the TATA sequence. The same *ftz*-AE1 interaction is less susceptible when opposed by non-competitive promoters such as *white* or *Scr*. Our results indicate that the neighboring promoters, although out-competed by *ftz* and apparently transcriptionally silent, can nonetheless alter the dynamics of the *ftz*-AE1 interaction and the effectiveness of suHw in blocking it. This property, which we showed with different types of promoters both at interacting and at competing positions, afford insulators with a greater regulatory flexibility according to the integrated input from its genomic context.

It is not known how an enhancer interacts with multiple competing promoters. Previous studies indicate that an enhancer may form a large complex that includes multiple promoters (Freidman et al., 1996), or it may alternate between separate enhancer-promoter complexes (the 'flip-flop' model (Milot et al., 1996; Wijgerde et al., 1995). In our paired promoters configuration, the insulator function is not compatible with a complex formed between the enhancer and the interacting promoter, but is compatible or even synergistic with a complex formed between the enhancer and the competing promoter. This model predicts that a strong competing promoter enhances insulator function, which we observe.

A surprising observation from our data is that insulator function does not necessarily increase sharing of an enhancer among neighboring genes. For example, AE1 is not shared between promoters very different in their competitiveness (e.g. between *eve* and *Scr*, or *ftz* and *white*), even with the aid of an insulator. AE1 remains specific even when it is partially blocked. As the differences between the competing promoters reduce, the suHw-mediated insulation becomes complete, upon which the redirection of the AE1 interaction to the competing promoters occurs. The redirection occurs in an 'all or none' fashion: no expression was detected from the competing promoters in cases of partial blockage. The reason for such an 'all or none' switch is not clear. However, it is possible that the synergistic interaction between the formation of the complex among insulator components and the complex between the competing promoter and the enhancer could contribute to such abrupt transitions.

Selective insulation

Selective insulation (or differential blockage) has been documented for several insulator and boundary elements in

Drosophila (Cai and Levine, 1995; Hagstrom et al., 1996; Muller, 2000; Scott et al., 1999). It is now our understanding that insulators, including those identified as boundary elements and those identified as enhancer blockers, are not impenetrable 'walls' to transcriptional interactions. Rather they function with great flexibility depending on the regulatory context, including the characteristics of the interacting enhancer, promoter and competing interactions within the neighboring genome. The selectivity for any given insulator therefore reflect, in addition to the intrinsic characteristics of the insulator, but also the apparent effectiveness with which it respond to its *cis* and *trans* environment.

Integrated transcription regulation and genome organization

Promoter competition and insulator function are two important mechanisms that specify enhancer-promoter interaction in complex genetic loci. Our results demonstrate that these two mechanisms are interdependent, or may even be synergistic at times. The regulatory implication of such synergy is that enhancer specificity among different types of promoters may be determined mainly through promoter competition, and that insulators exert a greater influence among genes with more comparable promoters. Although our study focused on the effect of promoter competition, it is possible that other types of regulatory elements such as competing enhancers also exert similar influences on insulators through competition with promoters. The range and intensity of these influences may vary, but the regulatory interdependence could in principle 'relay' along neighboring genes through cross-interactions between promoters, enhancers and insulators (see Fig. 6). The functional interdependence of regulatory elements in such genomic contexts imposes organizational restraints on closely linked gene groups (see Fig. 6). Change in the relative positioning or regulatory capacity of any one component could influence the outcome of neighboring interactions, and in turn, interactions further away, thereby linking a larger genomic region into one regulatory, organizational and evolutionary unit. The observations from transgenic *Drosophila* may reflect aspects of regulatory principles in authentic gene complexes and provide a molecular explanation for the highly conserved genetic organization of the Hox genes during evolution.

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