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Selective interactions of boundaries with upstream region of *Abd-B* promoter in *Drosophila bithorax* complex and role of dCTCF in this process

Olga Kyrchanova, Tatiana Ivlieva, Stepan Toshchakov, Alexander Parshikov, Oksana Maksimenko and Pavel Georgiev*

Department of the Control of Genetic Processes; Institute of Gene Biology, Russian Academy of Sciences, 34/5 Vavilov St., Moscow, 119334 Russia

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ABSTRACT

Expression of the genes *Ubx*, *abd-A,* and *Abd-B* of the *bithorax* complex depends on its *cis*-regulatory region, which is divided into discrete functional domains (*iab*). Boundary/insulator elements, named Mcp, Fab-6, Fab-7 and Fab-8 (PTS/F8), have been identified at the borders of the *iab* domains. Recently, binding sites for a *Drosophila* homolog of the vertebrate insulator protein CTCF have been identified in Mcp, Fab-6 and Fab-8 and also in several regions that correspond to predicted boundaries, Fab-3 and Fab-4 in particular. Taking into account the inability of the yeast GAL4 activator to stimulate the *white* promoter when the activator and the promoter are separated by a 5-kb *yellow* gene, we have tested functional interactions between the boundaries. The results show that all dCTCF-containing boundaries interact with each other. However, inactivation of dCTCF binding sites in Mcp, Fab-6 and PTS/F8 only partially reduces their ability to interact, suggesting the presence of additional protein(s) supporting distant interactions between the boundaries. Interestingly, only Fab-6, Fab-7 (which contains no dCTCF binding sites) and PTS/F8 interact with the upstream region of the *Abd-B* promoter. Thus, the boundaries might be involved in supporting the specific interactions between *iab* enhancers and promoters of the *bithorax* complex.

INTRODUCTION

The large *cis*-regulatory region of the *bithorax* complex (*BX-C*) is divided into nine parasegment-specific chromatin domains that control the expression of three homeotic genes, *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*) (1). These genes are responsible for specifying the identity of parasegments 5–14 (PS5—PS14), which form the posterior half of the thorax and all abdominal segments of the adult fly (2–5). The PS-specific expression patterns of *Ubx*, *abd-A* and *Abd-B* are determined by a complex *cis*-regulatory region that spans a 300-kb DNA segment (5–7). Genetic analysis has indicated that this large regulatory region can be divided into nine discrete segment-specific domains, which are aligned on the chromosome in the same order as the body segments in which they operate (2,5,8–10). For example, *Abd-B* expression in PS10, PS11, PS12 and PS13 is controlled by the *iab-5*, *iab-6*, *iab-7* and *iab-8* *cis*-regulatory domains, respectively (2,11–16). Each *iab* domain appears to contain at least one enhancer that initiates *Abd-B* expression in the early embryo, as well as a PRE silencer element that maintains the expression pattern throughout development (9,16–24).

Boundary elements of a specific class are proposed to exist between each *iab* domain to allow their autonomy in properly specifying segmental identity (4,16,24–26). Only four out of the nine postulated boundaries have been identified genetically. The Miscadastral (Mcp), Frontadominal-7 (Fab-7) and Frontadominal-8 (Fab-8) elements have been functionally identified by deletion analysis within the *bithorax* complex (24,25,27). In addition, characterization of genomic deficiencies covering part of the *iab-5* and *iab-6* *cis*-regulatory domains (16) and experiments with transgenic enhancer-blocking assays (28,29) provided evidence for the existence of the Fab-6 boundary. All these boundaries display insulator activity. Generally, insulators are defined by two properties: enhancer-blocking activity, preventing communication between an enhancer and a promoter separated by the insulator, and boundary function (barrier activity), preventing repressive chromatin spreading (30–35). It has been shown that boundaries from BX-C are capable of

*To whom correspondence should be addressed. Tel: +7 499 1359734; Fax: +7 499 1354105; Email: georgiev_p@mail.ru; georgiev_p@genebiology.ru

suppressing reporter gene expression when placed between an enhancer and a promoter in a transgenic insulator assay (18,23,24,28,29,36–41).

Recently, binding sites for the *Drosophila* homolog of vertebrate insulator protein CTCF were identified in the *bithorax* complex (29,42–44), and dCTCF was suggested to be the key protein involved in organization of chromatin domains within this complex (43,44). Binding sites for dCTCF were found in the Mcp, Fab-6 and Fab-8 insulators (29,42,43). At the same time, the Fab-7 boundary proved to be devoid of dCTCF binding sites. Strikingly, the distribution of dCTCF protein within the BX-C coincides almost perfectly with the regions in which boundary elements were predicted (Figure 1). In particular, dCTCF binding sites were found in the regions mapped as putative Fab-3 and Fab-4 boundaries (5,16,43,45). Fab-6, Fab-7 and Fab-8 flank the *iab* domains that activate the *Abd-B* gene, while Fab-3 and Fab-4 belong to the regulatory system of the *abd-A* gene. Mcp is located at the boundary of the *Abd-B* and *abd-A* regulatory regions.

In mammals, CTCF supports long-distance interactions (35). Previously, we have shown that dCTCF can support the distant interaction between two Fab-8 boundaries (46). However, Fab-8 failed to interact with the Fab-7 boundary (lacking dCTCF binding sites), whereas the composite element consisting of two neighboring regulatory elements Fab-8 and PTS (promoter-targeting sequences) (15) could interact with the Fab-7 boundary and the upstream region of *Abd-B* promoter A (46).

The main purpose of this study was to examine interactions between dCTCF-containing boundaries and to reveal the role of dCTCF in such interactions. We also tested whether the boundaries other than Fab-7 and PTS/F8 are capable of interacting with the upstream promoter region of the *Abd-B* promoter A.

MATERIALS AND METHODS

Plasmid construction

The 3-kb SalI—BamHI fragment containing the *yellow* regulatory region was cloned into BamHI–XhoI-cleaved pGEM7 (yr plasmid). Ten binding sites for GAL4 (G4) were cloned into the yr plasmid cleaved by NcoI and Eco47III (G4-Δyr). The pCaSpew15(+RI) plasmid was constructed by inserting an additional EcoRI site at +3291 of the *mini-white* gene in the pCaSpew15 plasmid. An insulator located on the 3′ side of the *mini-white* gene (Wari insulator) was deleted from pCaSpew15(+RI) by digestion with EcoRI to produce the pCaSpeR700 plasmid. The 5-kb BamHI–BglII fragment of the *yellow* coding region was cloned into pCaSpeR700 (C700-yc).

Fragments PTS/F8 (64 038 to 64 374), Fab-8 (63 683 to 64 291), PTS (64 292 to 64 916), Fab-7 (83 647 to 84 504), A^{CTCF} (48 350 to 48 724), Fab-6 (100 464 to 100 888), Mcp (113 993 to 114 332), Fab-4 (125 859 to 126 642) and Fab-3 (148 340 to 148 965) were obtained by polymerase chain reaction (PCR) amplification and sequenced. The coordinates are from the sequences of the *bithorax* complex presented in (6).

The PCR-amplified fragments (X or Y) were cloned between either two frt (frt(X)) or two lox (lox(Y)) sites.

All constructs were made by the same general scheme. A fragment flanked by frt sites (frt(X)) was inserted in the direct or reverse orientation into the G4-Δyr plasmid cleaved by KpnI (G4-Δyr-frt(X)). A fragment flanked by lox sites (lox(Y)) was cloned into C700-yc between the *yellow* and *white* genes (C2-lox(Y)-yc). Next, G4-Δyr-frt(X) fragments were cloned into the corresponding C700-lox(Y)-yc plasmids.

To mutate both dCTCF binding sites in the 425-bp F6 fragment ($F6^{m}$), two pairs of oligonucleotides carrying the

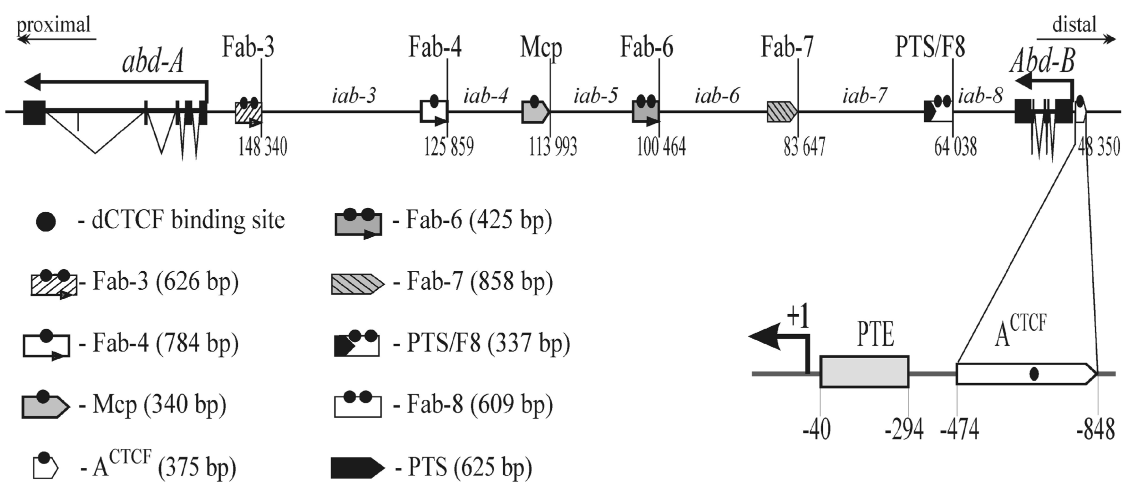


Figure 1. Schemes of the distal part of the *bithorax* complex including the *abd-A* and *Abd-B* loci. The horizontal line represents the *bithorax* DNA sequence marked off in kilobases according to the coordinates given in (6). The only class A *Abd-B* transcript that is required for morphogenesis in PS 10 to 13 is drawn above the DNA line. Arrows marked 'Proximal' and 'Distal' point toward the centromere and the telomere, respectively. Positions of the boundaries and dCTCF-containing regions are indicated by vertical lines. The *Abd-B* promoter region is shown below the DNA line. Locations of regulatory elements are shown relative to the *Abd-B* transcription start site (+1). The PTE identified previously (61) is located at –40 relative to the *Abd-B* transcription start site. The A^{CTCF} region is located at –474 relative to the *Abd-B* transcription start site. The DNA fragments tested are shown as differently marked boxes. Black circles represent functional binding sites for dCTCF.

desired mutant sequences (5′-cgagcgcagatctttttctaa ccggc-3′)–(5′-cggccggagatcttttttttgtgccg-3′) within the EcoRI restriction site for mutation in F6 CTCF1 and (5′-gcagtccgccaagctttttcgcgtgc-3′)–(5′-gcacgcgagcgaagcttt ttctttttc-3′) within the HindIII restriction site for mutation in F6 CTCF2, were used to amplify PCR products. Three mutated parts of Fab-6 were then assembled in pBluSK ($F6^{m}$). The resulting DNA fragment was sequenced to confirm that the intended mutant sequences had been introduced and that other PCR-induced mutations were absent. As a result, the first site (cgagcgccgcctgccggccg) was changed to (aaaaaaagatattttttct), and the second (gcagtccgccagggggcgct), to (gaaaaagaaaaagctttttt).

To inactivate the dCTCF binding site within Mcp, a small deletion was made by amplifying the plasmid containing Mcp between primers 5′ggtttggatattggct3′ and 5′ctttcgcagctcatgc3′. To obtain PTS/$F8^{m}$ we used two pairs of oligonucleotides carrying the desired mutant sequences described previously (46). To mutate dCTCF binding site within the A^{CTCF} we used two oligonucleotides carrying the desired mutant sequences (5′-ctcggacat agatagatctttttttgcg-3′) and (5′-caccacagatctatttaagctttata tttcg-3′) with BglII restriction site. As a result, the dCTCF binding site (ctcggacatagatggcgctgtgg) was substituted by (ataaagcttaaatagatcttttt).

Generation and analysis of transgenic lines

The construct and P25.7wc plasmid were injected into *yacw^1118^* preblastoderm embryos (47). The resultant flies were crossed with *yacw^1118^* flies, and the transgenic progeny were identified by their eye color.

The lines with DNA fragment excisions were obtained by crossing transposon-bearing flies with the Flp (*w^{1118}; S2CyO, hsFLP, ISA/Sco*; +) or Cre (*yw; Cyo, P[w+,cre]/ Sco;* +) recombinase-expressing lines. The Cre recombinase induces 100% excisions in the next generation. A high level of Flp recombinase was produced by heat shock treatment for 2 h during the first 3 days after hatching. All excisions were confirmed by PCR analysis. Details of the crosses and primers 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 in ref. 40.

To test role of dCTCF in the interaction of the F8/PTS boundary and the upstream region of the *Abd-B* promoter (A^{CTCF}), we used three transgenic lines carrying the G4(A^{CTCF})Y(PTS/$F8^{R}$)W construct (designated here as *P*) on the second chromosome. The *yw^{1118}; P/CyO; $CTCF^{y+1}$/TM6,Tb* and *yw^{1118}; P/CyO; $CTCF^{y+6}$/ TM6,Tb* lines were constructed. *$CTCF^{y+1}$* and *$CTCF^{y+6}$* are null mutations in the *dCTCF* gene, resulting in late pupal lethality (48). For this reason, we examined *white* stimulation by GAL4 in transgenic lines heterozygous for the *dCTCF* mutation. The *yw^{1118}; P/CyO; $CTCF^{y+1}$*(or *$CTCF^{y+6}$*)*/TM6,Tb* and *yw^{1118}; P/CyO* (control) females were crossed to *yw^{1118}; P[w^{-}, tubGAL4]117 /TM3,Sb* males. In the progeny, eye pigmentation in *yw^{1118}; P/+; $CTCF^{y+1}$*(or *$CTCF^{y+6}$*)*/ P[w^{-}, tubGAL4]117* males was compared with that in *yw^{1118}; P/+; +/ P[w^{-}, tubGAL4]117* males.

The *white* (*w*) phenotype was determined 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 brown (Br) or brownish red (BrR).

Electrophoretic mobility shift assay

For the purpose of synthesizing dCTCF *in vitro*, the cDNA of dCTCF (kindly provided by J. Zhou) was cloned into the pET 23a plasmid (Novagen). The dCTCF protein was synthesized *in vitro* in the TNT-coupled transcription/translation reticulocyte lysate (Promega) from a T7 promoter. *In vitro* translated protein (6 µl) was added to 25 fmol of a radioactively labeled DNA probe in 20 µl (final volume) of binding reaction mixture in a phosphate-buffered saline (PBS) buffer containing 5 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 mM DTT, 0.1% Nonidet P-40 and 10% glycerol. The mixture was incubated at room temperature for 30 min and then resolved in 5% nondenaturing polyacrylamide gel with 0.5× TBE buffer at 5 V/cm.

Chromatin Immunoprecipitation

Chromatin was prepared from mid-late pupae. A 500-mg sample was ground in a mortar in liquid nitrogen and resuspended in 10 ml of buffer A (15 mM HEPES-KOH, pH 7.6; 60 mM KCl, 15 mM NaCl, 13 mM EDTA, 0.1 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, 0.5% NP-40, 0.5 mM DTT) supplemented with 0.5 mM PMSF and Complete (EDTA-free) Protease Inhibitor Cocktail V (Calbiochem, United States). The suspension was then homogenized in a Dounce homogenizer with pestle B and filtered through Nylon Cell Strainer (BD Biosciences, United States). The homogenate was transferred to 3 ml of buffer A with 10% sucrose (AS), and the nuclei were pelleted by centrifugation at 4000*g*, 4°C for 5 min. The pellet was resuspended in 5 ml of buffer A, homogenized again in a Dounce homogenizer, and transferred to 1.5 ml of buffer AS to collect the nuclei by centrifugation. The nuclear pellet was resuspended in wash buffer (15 mM HEPES-KOH, pH 7.6; 60 mM KCl, 15 mM NaCl, 1 mM EDTA, 0.1 mM EGTA, 0.1% NP-40, protease inhibitors) and cross-linked with 1% formaldehyde for 15 min at room temperature. Cross-linking was stopped by adding glycine to a final concentration of 125 mM. The nuclei were washed with three 10-ml portions of wash buffer and resuspended in 1.5 ml of nuclei lysis buffer (15 mM HEPES, pH 7.6; 140 mM NaCl, 1 mM EDTA, 0.1 mM EGTA, 1% Triton X-100, 0.5 mM DTT, 0.1% sodium deoxycholate, 0.1% SDS, protease inhibitors). The suspension was sonicated on ice with a Branson Sonifier 150 (Branson Instruments, United States) for 5 × 20 s at 1-min intervals. Debris was removed by centrifugation at 14 000 *g*, 4°C for 10 min, and chromatin was pre-cleared in protein G agarose (Pierce, Unites States) blocked with BSA and salmon sperm DNA. Aliquot of such pre-cleared chromatin was used as the input samples. These samples were incubated overnight, at 4°C, with rat antibodies against dCTCF (1:200) and nonspecific IgG purified from rat pre-immune serum, and chromatin–antibody complexes were collected using blocked protein G agarose at 4°C over 5 h. After several rounds of washing with lysis buffer (as such and with 500 mM NaCl), LiCl buffer (20 mM tris–HCl, pH 8; 250 mM LiCl, 1 mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate, protease inhibitors) and TE buffer, the DNA was eluted with elution buffer (50 mM tris–HCl, pH 8; 1 mM EDTA, 1% SDS), the cross-links were reversed, and the precipitated DNA was extracted by the phenol–chloroform method.

The enrichment of specific DNA fragments was analyzed by real-time PCR, using a StepOne Plus Thermal Cycler (Applied Biosystems, United States). The primers used for PCR in ChIP experiments for genome fragments were as follows: tub (5′-gctttcccaaga agctcataca-3′, 5′-ggttcagtgcggtattatccag-3′), rpl32 (5′-gtt cgatccgtaaccgatgt-3′, 5′-ccagtcggatcgatatgctaa-3′), F8_G (5′-tgttggtgagcaagcgaaga-3′, 5′-cgaacattttttacgcgaca tgt-3′), M_G (5′-aaagtcgggtctgcaaataagg-3′, 5′-gcataagc tgcaaaagaaaaacaa-3′), F6_G (5′-agctaaacccgatttgctttg ccg-3′, 5′-ctgcccagtgggagatacaaagat-3′) and A_G (5′-cca acaacaagccaactaactaca-3′, 5′-acgaacaaaaaacgctctcaga ct-3′); for construct fragments: PTS/F8_C and PTS/ F8m_C (5′-ggaaagcaccaacacaagatgtc-3′, 5′-cccccaacatcca gttgattt-3′), M_C and Mm_C (5′-ggaaacgcattacgcacac tta-3′, 5′- ttatagatccccggcgatacc-3′), F6m_C (5′-gcaatcac atttgtcagatactttcg-3′, 5′-gcctcctggccttacaatttact-3′) and A_C (5′-gcctcctggccttacaatttact-3′, 5′-cggatgccttcaca cgtaca-3′).

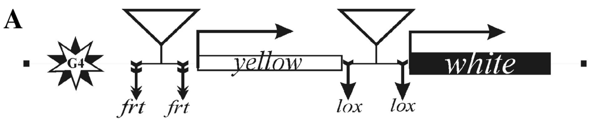
RESULTS

dCTCF-containing regions corresponding to Fab-3, Fab-4 and Fab-6 can support distant interactions in the GAL4/*white* assay

Previously (43), dCTCF sites were identified in the regions that might be putative boundaries between *iab*-4 and *iab*-3 and between *iab*-3 and *iab*-2 domains (Figure 1). Here, these regions are referred to as Fab-4 and Fab-3 elements, respectively. Fab-4 contains only one dCTCF binding site, while Fab-3 contains two such sites. As shown in our previous study (46), dCTCF binding sites can support long-distance interaction and are essential for communication between the Fab-8 boundaries. To find out whether all dCTCF-containing regions are capable of such interaction, we tested Fab-3 (626 bp) and Fab-4 (784 bp) in the GAL4/*white* assay, which is based on the finding that the yeast GAL4 activator bound to sites located upstream of the *yellow* gene fails to stimulate the *white* promoter placed downstream of the *yellow* 3′ end (40). In the test constructs (Figure 2A), 10 GAL4 binding sites (G4) were inserted at −893 relative to the *yellow* transcription start site. As a result, the distance between the *white* gene and G4 was almost 5 kb. To examine the functional interaction between two regulatory elements, one element flanked by frt sites (49) was inserted near G4 and the other, flanked by lox sites (50), was inserted near the *white* promoter. The presence of the frt and lox sites made it possible to delete the DNA fragments tested and to compare stimulation of transcription by GAL4 in transgenic lines before the deletion of the regulatory elements and after it (control).

Initially, we tested whether the interaction between two Fab-3 (Figure 2B) or Fab-4 elements (Figure 2C) could facilitate *white* stimulation by GAL4 across the *yellow* gene. The test DNA fragments were inserted in opposite orientations, since we previously found that other

A



B *white*

	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(F3)Y(F3^R)W					1		6	1		8
+ GAL4		1		2	4	1				8/8
G4(Δ)Y(Δ)W					1		6	1		0/8
+ GAL4					1		6	1		0/8

C

	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(F4)Y(F4^R)W							3	2		5
+ GAL4					1	3	1			5/5
G4(Δ)Y(Δ)W							3	2		0/5
+ GAL4							3	2		0/5

D

	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(F6)Y(F6^R)W								2		2
+ GAL4	1		1							2/2
G4(Δ)Y(Δ)W							2			2/2
+ GAL4							2			0/2

Figure 2. Testing the Fab-3, Fab-4 and Fab-6 elements in the GAL4/*white* assay. (**A**) Reductive scheme of transgenic construct used to examine the interaction between regulatory elements at a distance. The *yellow* and *white* genes are shown as boxes with arrows indicating the direction of their transcription. Downward arrows indicate target sites for Flp recombinase (*frt*) or Cre recombinase (*lox*); the same sites in construct names are denoted by parentheses. GAL4 binding sites (indicated as G4) are at a distance of ~5 kb from the *white* promoter. Triangles indicate positions of elements tested for the interaction. (**B–D**) Experimental evidence that interacting dCTCF-containing regulatory elements facilitate stimulation of *white* by a distantly located GAL4 activator. Superscript 'R' indicates that the corresponding element is inserted in the reverse orientation relative to the *white* gene in the construct. '+ GAL4' indicates that eye phenotypes in transgenic lines were examined after induction of GAL4 expression. The '*white*' column shows the numbers of transgenic lines with different levels of *white* pigmentation in the eyes. 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 y phenotype upon induction of GAL4 or deletion (Δ) of the specified DNA fragment; T is the total number of lines examined for each particular construct. Other designations are as in Figure 1.

boundaries in such a configuration supported *white* stimulation by GAL4 more effectively (orientation-dependent pairing) (40,41,46).

The *white* promoter usually accounts for the basal level of expression, with eye pigmentation ranging from pale yellow to dark yellow. To simplify further presentation of the results, we designated *white* stimulation by GAL4 as strong, moderate or weak when flies from more than half of corresponding transgenic lines acquired eye pigmentation in the ranges of brown to red, orange to dark orange or dark yellow to orange, respectively.

To express the GAL4 protein, we used a transgenic line carrying the GAL4 gene under control of the ubiquitous *tubulin* promoter (40). These experiments showed that the pairs of Fab-3 and Fab-4 elements provided for moderate and weak *white* stimulation by GAL4, respectively (Figure 2 Band C).

To demonstrate that *white* stimulation by GAL4 was supported by either the Fab-3 or Fab-4 pair, we deleted these DNA fragments from transgenic lines. As a result, GAL4 lost the ability to stimulate *white* expression in most of the lines tested. Thus, the interaction between either Fab-3 or Fab-4 elements allowed GAL4 to stimulate transcription of the *white* gene.

We then examined the interaction between the 425-bp Fab-6 boundaries mapped with the aid of transgenic assays (28,29). This DNA fragment contains two dCTCF binding sites and functions as a silencer (Figure 1). It was suggested that the Fab-6 boundary and PRE silencer overlap with each other. In accordance with the silencing activity of Fab-6, we obtained only two transgenic lines carrying two Fab-6 boundaries inserted in opposite orientations (Figure 2D). Both of them showed repression of *yellow* (variegated bristles, data not shown) and *white* (pale yellow eyes). Deletion of the Fab-6 boundaries restored *yellow* (data not shown) and *white* expression. In both transgenic lines (Figure 2D), GAL4 strongly induced *white* expression. When the boundaries were deleted from these transgenic lines, GAL4 lost the ability to stimulate *white* expression.

Thus, all dCTCF-containing elements tested in these experiments proved to support distant interaction between the GAL4 activator and the *white* promoter.

All tested regulatory elements containing dCTCF binding sites are able to interact with each other

Next, we examined whether different dCTCF-containing regulatory elements can interact with each other. In addition to Fab-3, Fab-4 and Fab-6, we tested Mcp and PTS/F8 (Figure 1), the latter consisting of 254 bp from the Fab-8 insulator and 83 bp from the promoter targeting sequence (PTS).

The boundaries were inserted pairwise, in different combinations, near the *white* promoter and GAL4 binding sites (Figure 3). The level of *white* stimulation by GAL4 in the majority of transgenic lines was weak in all variants except for those with the Fab-3/Mcp and Fab-4/Fab-6^{R} pairs, in which it was classified as moderate. These results suggest that all tested boundaries containing dCTCF binding sites interact with each other with different efficiency.

dCTCF is not critical for self-pairing of the Mcp, Fab-6 and PTS/F8 boundaries

The results of our previous study (46) show that two closely spaced binding sites for the dCTCF protein are required for the interaction between the Fab-8 insulators. However, the observation that PTS/F8 interacts with Fab-7, which contains no dCTCF binding sites, suggest that additional protein(s) are involved in the interaction between boundaries. To check this possibility, we mutated binding sites for dCTCF in the Mcp, Fab-6 and PTS/F8 DNA fragments. The results of electrophoretic mobility shift assay confirmed dCTCF binding to the Mcp, Fab-6 and PTS/F8 fragments but not to the Mcp^{m}, Fab-6^{m} and PTS/F8^{m} fragments (Supplementary Figure S1). The binding of dCTCF to Mcp and PTS/F8 in the transgenic

white

	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(F3)Y(F4^{R})W							8	7		15
+ GAL4					5	5	5			13/15
G4(F3)Y(PTS/F8^{R})W						1	4	2		7
+ GAL4				1	2	4				7/7
G4(F3)Y(M^{R})W						3	5	1		9
+ GAL4			1	1	3	2	2			8/9
G4(F3)Y(M)W						3	9	3		15
+ GAL4		1	1	5	5	1	1	1		13/15
G4(F4)Y(F6^{R})W							3	8	4	15
+ GAL4	2	2	1	2	4	4				15/15
G4(F4)Y(PTS/F8^{R})W						2	7	3		12
+ GAL4			1		4	3	4			10/12
G4(M)Y(PTS/F8^{R})W						2	10	7		19
+ GAL4			1	4	2	5	6	1		16/19
G4(M)Y(PTS/F8)W						1	3	2		6
+ GAL4					2	2	2			3/6
G4(F6)Y(M^{R})W						1	4	6		11
+ GAL4			2	2		5	1	1		9/11
G4(F6)Y(PTS/F8^{R})W							1	3		4
+ GAL4				2		1	1			4/4

Figure 3. Testing the functional interaction between regulatory elements containing dCTCF binding sites. For designations, see Figures 1 and 2.

constructs was confirmed by immunoprecipitation with chromatin isolated from pupae (Supplementary Figure S2). At the same time, chromatin immunoprecipitation (ChIP) showed that dCTCF did not bind to Mcp^m, Fab-6^m and PTS/$F8^m$ in transgenic pupae.

Two fragments of the mutated Mcp^m (Figure 4A) or Fab-6^m boundary (Figure 4B) were inserted in opposite orientations relative to each other.

Previously (40), we observed a strong interaction between the Mcp elements inserted in opposite orientations (Figure 4A). In transgenic lines carrying the Mcp^m–Mcp^m pair (Figure 4B), GAL4 induced only a moderate level of *white* expression. Thus, inactivation of dCTCF binding sites partially affected the interaction between Mcp elements. At the same time, the interaction between the mutant Mcp^m elements suggests that additional proteins are involved in the interaction between the Mcp boundaries.

Two copies of the Fab-6^m element still strongly repressed *yellow* and *white* expression, indicating that dCTCF binding sites are not required for the activity of the silencer (data not shown). For this reason, we selected transgenic lines by crossing F_0 males grown from injected embryos with *yw¹¹¹⁸; P[w⁻, tubGAL4]117/TM3,Sb* females carrying the *tubGAL4* gene. It is noteworthy that, in the F_1 generation, *TM3,Sb/+* males carrying the construct with Fab-6 displayed normal *yellow* and *white* expression, but in the next generation these genes were strongly repressed in approximately half of the transgenic lines (data not shown). This observation may be explained by the fact that GAL4 expression in embryos can counteract PRE silencing (51). In all transgenic lines tested, we observed moderate levels of *white* stimulation by GAL4 (Figure 4C). These results suggest that dCTCF is not critical as for PcG-mediated repression as for distant interaction between the Fab-6 boundaries.

We have found previously that PTS/F8 boundaries interact in an orientation dependent manner, with the pairing of the insulators located in opposite orientations providing for a strong level of *white* stimulation by GAL4 (46). Here, we inserted the mutated PTS/$F8^m$ boundaries either in opposite orientations (Figure 4D) or in the same orientation (Figure 4E) and observed a weak level of *white* stimulation by GAL4 in both series of transgenic lines. As a control, we inserted two wild-type PTS/F8 elements in the opposite orientation relative to each other (Figure 4F) like in the construct with the PTS/$F8^m$ elements shown in Figure 4D. As a result, GAL4 induced strong *white* activation in six transgenic lines tested (Figure 4F).

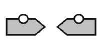
This is evidence that dCTCF is required for the orientation-dependent pairing between the PTS/F8 boundaries.

white

A

	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(M)Y(M^R)W					1	3	1	1		6
+ GAL4		5	1							6/6

B



	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(M^m)Y(M^{mR})W						1	6	8		15
+ GAL4	1		3	4	7					15/15

C

	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4($F6^m$)Y($F6^{mR}$)W					2	2	1	4	6	15
+ GAL4	4		7	2	2					15/15

D

	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(PTS/$F8^{mR}$)Y(PTS/$F8^m$)W						1	8	4		13
+ GAL4				1	4	7	1			13/13

E

	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(PTS/$F8^m$)Y(PTS/$F8^m$)W						2	4	1		7
+ GAL4				2	2	3				7/7

F

	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(PTS/$F8^R$)Y(PTS/F8)W						2	2	2		6
+ GAL4		4	2							6/6

Figure 4. Role of dCTCF in self-pairing of (**A, B**) Mcp, (**C**) Fab-6 and (**D–F**) PTS/F8 boundaries. The results presented in (A) are from ref. 40. For designations, see Figures 1 and 2.

Fab-7 selectively interacts with the Fab-6 and PTS/F8 boundaries

The dCTCF protein does not bind to the Fab-7 boundary (43), but we observed interaction between Fab-7 and PTS/F8 (46). To test whether dCTCF is required for this interaction, we examined the combination of Fab-7 with the PTS/$F8^m$ fragment in which dCTCF binding sites were mutated, with Fab-7 and PTS/$F8^m$ being inserted in opposite orientations (Figure 5A). In eight transgenic lines tested, GAL4 induced a moderate level of *white* expression, which was indicative of interaction between Fab-7 and PTS/$F8^m$. Thus, dCTCF is not required for the functional interaction between Fab-7 and PTS/F8.

Next, we examined interactions of the Fab-7 boundary with Fab-3 (Figure 5B and C), Fab-4 (Figure 5D), Mcp (Figure 5E and F) and Fab-6 (Figure 5G). The boundaries tested in pairwise combinations were inserted either in the same or in opposite orientations relative to each other. The Fab-6/Fab-7 combination accounted for a moderate level of *white* activation by GAL4, which was indicative of interaction between the Fab-6 and Fab-7 boundaries. By contrast, only weak stimulation of *white* expression by GAL4 in minor proportions of transgenic lines was observed when Fab-7 was combined with Fab-3, Fab-4 or Mcp. Therefore, Fab-7 does not interact with these three elements.

Analysis of interactions between the upstream region of the *Abd-B* promoter and the PTS/F8 and Fab-7 boundaries

As we found previously, the 375-bp regulatory element with a dCTCF binding site (A^{CTCF}) located upstream of the *Abd-B* promoter A (Figure 1) can functionally interact with PTS/F8 and Fab-7 boundaries (46). To test the role of the dCTCF binding site in A^{CTCF} in the interaction with the boundaries, we mutated this site (A^{CTCFm}). An electrophoretic mobility shift assay confirmed dCTCF protein binding to A^{CTCF} but not to *white*

A

	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(PTS/F8^{mR})Y(F7)W						1	6	1		8
+ GAL4			2	1	4	1				8/8

B

	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(F3)Y(F7)W						2	7			9
+ GAL4					1	4	4			4/9

C

	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(F3)Y(F7^{R})W					2	6	3			11
+ GAL4					3	7	1			3/11

D

	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(F4)Y(F7^{R})W						1	6	2		9
+ GAL4						2	6	1		2/9

E

	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(F7)Y(M)W						2	7	1		10
+ GAL4					1	3	5	1		3/10

F

	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(F7)Y(M^{R})W					3	11	4			18
+ GAL4				1	5	9	3			6/18

G

	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(F6)Y(F7^{R})W						1	3	2		6
+ GAL4	2		1		2	1				6/6

Figure 5. Testing the interaction of the Fab-7 boundary with (**A**) PTS/F8^{m}, (**B** and **C**) Fab-3, (**D**) Fab-4, (**E** and **F**) Mcp and (**G**) Fab-6. For designations, see Figures 1 and 2.

A^{CTCFm} (Supplementary Figure S1). Unexpectedly, immunoprecipitation with chromatin isolated from pupae of two different transgenic lines confirmed weak dCTCF binding to the endogenous A^{CTCF} region but not to the same region in the transgenic constructs (Supplementary Figure S3).

The A^{CTCF} (Figure 6A) or A^{CTCFm} (Figure 6B) element was inserted near the GAL4 binding sites in direct orientation. In both constructs, Fab-7 was inserted in reverse orientation near the *white* promoter. In the control transgenic lines carrying the A^{CTCF}/Fab-7 pair, a moderate level of *white* activation by GAL4 was observed (Figure 6A). By contrast, the combination of Fab-7 with A^{CTCFm} provided for only weak *white* stimulation by GAL4 (Figure 6B). Thus, the dCTCF binding site in A^{CTCF} is essential but not critical for the functional interaction between A^{CTCF} and Fab-7.

We have shown previously (46) that the A^{CTCF} element inserted in direct orientation near the GAL4 binding sites strongly interacted with PTS/F8 inserted near the *white* promoter in reverse orientation (Figure 6C). Substitution of the A^{CTCF} element by the mutant one led to reduction of *white* stimulation by GAL4 (Figure 6D).

To test the role of dCTCF in this interaction, we compared *white* activation by GAL4 in three transgenic lines carrying the PTS/F8–A^{CTCF} pair in either wild-type or mutant ($CTCF^{y+1}$/+ or $CTCF^{y+6}$/+) background. As a result, we found that a decrease in dCTCF level had no effect on the interaction between the PTS/F8 boundary

white

A

	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(A^{CTCF})Y(F7^{R})W					1	2	9	1		13
+ GAL4			1	2	7	3				13/13

B

	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(A^{CTCFm})Y(F7^{R})W					2		12	3		17
+ GAL4	1				5	6	4	1		13/17

C

	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(A^{CTCF})Y(PTS/F8^{R})W				1		1	3	2		7
+ GAL4	1	2	1	1	1	1				7/7

D

	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(A^{CTCFm})Y(PTS/F8^{R})W						3	13	7		23
+ GAL4		1	2	1	5	7	5	2		15/23

E

	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(A^{CTCF})Y(PTS/F8^{mR})W					1	1	5			7
+ GAL4		1	2	2	2					7/7

F

	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(A^{CTCF})Y(F8^{R})W					1	1	4	3		9
+ GAL4					1	1	4	3		0/9

G

	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(A^{CTCF})Y(F8)W						3	3	2		8
+ GAL4						3	3	2		0/8

H

	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(A^{CTCF})Y(PTS^{R})W						1	5	3		9
+ GAL4						2	4	3		1/9

Figure 6. Analysis of interactions between the A^{CTCF} region of the *Abd-B* promoter and the Fab-7 and PTS/F8 boundaries. Experiments were performed to compare the interactions of (**A**) normal A^{CTCF} and (**B**) mutant A^{CTCFm} with Fab-7. Similar experiments were performed to study the interactions of PTS/F8 with (**C**) A^{CTCF} and (**D**) A^{CTCFm}, as well as (**E**) of PTS/F8^{m} with A^{CTCF}. In addition, A^{CTCF} was tested in combinations with (**F** and **G**) the Fab-8 insulator and (**H**) PTS. The results presented in (C) are from ref. (46). For designations, see Figures 1 and 2.

and the upstream promoter region (data not shown). In addition, the mutated PTS/F8^{m} boundary with the inactivated dCTCF binding sites interacted with A^{CTCF} with the same efficiency as the wild-type PTS/F8 boundary (Figure 6E), suggesting that dCTCF is not required for this interaction. Taken together, these results confirm that an unidentified protein binds to the dCTCF binding site in the upstream *Abd-B* promoter region and this protein contributes to effective distant interactions between A^{CTCF} and PTS/F8.

We also addressed the question as to which part of the PTS/F8 boundary is responsible for the interaction with A^{CTCF}. To test the role of the Fab-8 insulator (609 bp, including two dCTCF binding sites), A^{CTCF} and Fab-8 were placed in the same positions and orientations as in the construct carrying A^{CTCF} and PTS/F8 (Figure 6F). GAL4 failed to stimulate *white* transcription in all of the transgenic lines tested, suggesting the absence of the functional interaction between the Fab-8 insulator and A^{CTCF}.

Similar results were obtained when A^{CTCF} and Fab-8 were inserted in the same orientation (Figure 6G).

In the same way (Figure 6H), we tested the functional interaction between A^{CTCF} and PTS (625 bp). Once again, GAL4 was able to weakly stimulate *white* transcription in only one out of nine transgenic lines tested. These results indicate that both parts of the PTS/F8 boundary are required for its functional interaction with A^{CTCF}.

The upstream region of the *Abd-B* promoter selectively interacts with boundaries

To test whether the A^{CTCF} element is able to interact with other dCTCF-containing regulatory regions, we examined the interaction of A^{CTCF} with Fab-6 (Figure 7A and B), Fab-3 (Figure 7C and D), Fab-4 (Figure 7E) and Mcp (Figure 7F–H).

In experiments with the A^{CTCF}/Fab-6 combination, GAL4 stimulated *white* expression in 23 out of 25 transgenic lines, suggesting that Fab-6 functionally interacted with A^{CTCF} (Figure 7A). Taking into account that the silencing activity of Fab-6 may affect *white* stimulation by GAL4, we can conclude that Fab-6 interacts with the upstream promoter region A. Inactivation of both dCTCF binding sites in the Fab-6 boundary had no effect on its interaction with A^{CTCF} (Figure 7B). These results confirm that dCTCF is not essential for the interaction of the Fab-6 boundary with the upstream region of the *Abd-B* promoter.

By contrast, we observed no *white* stimulation by GAL4 in most transgenic lines carrying either the A^{CTCF}/Fab-3 (Figure 7C and D) or the A^{CTCF}/Fab-4 (Figure 7E) combination of boundaries.

To test the interaction between A^{CTCF} and Mcp, we made three constructs with different combinations of these elements (Figure 7F–7H). No appreciable *white* stimulation by GAL4 was observed in any of the three series of transgenic lines. Thus, the Mcp boundary separating the *Abd-B* and *abd-A* regulatory regions fails to interact with the upstream promoter region of the *Abd-B* gene.

white

A	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(F6^{R})Y(A^{CTCFR})W						2	5	7	11	25
+ GAL4		2	1	2	6	11	3			23/25

B	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(F6^{mR})Y(A^{CTCFR})W							4	4	2	10
+ GAL4	1	1	3	1	2	2				10/10

C	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(A^{CTCF})Y(F3)W						2	5	2		9
+ GAL4						3	4	2		1/9

D	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(A^{CTCFR})Y(F3)W						2	6	10		18
+ GAL4						4	7	7		2/18

E	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(F4)Y(A^{CTCFR})W					1	3	9	4		17
+ GAL4					1	5	7	4		2/17

F	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(M)Y(A^{CTCF})W						1	3	2		6
+ GAL4						1	4	1		1/6

G	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(A^{CTCF})Y(M)W					3	7	6	3		19
+ GAL4					3	8	7	1		3/19

H	R	BrR	Br	dOr	Or	dY	Y	pY	W	N/T
G4(A^{CTCFR})Y(M)W					2	6	7	4		19
+ GAL4					3	5	8	3		3/19

Figure 7. Analysis of interaction between the A^{CTCF} region of the *Abd-B* promoter and (**A**) Fab-6, (**B**) mutant Fab-6^{m}, (**C** and **D**) Fab-3, (**E**) Fab-4 and (**F–H**) Mcp elements. For designations, see Figures 1 and 2.

DISCUSSION

The results of this study show that the putative Fab-3 and Fab-4 boundaries containing dCTCF binding sites are able to support distance interactions. However, we have no evidence whether additional proteins bound to these boundaries are involved in distant interactions such as in the case of the Mcp, Fab-6 and PTS/F8 boundaries.

As follows from our previous data (40,46), the relative orientation of two Mcp or PTS/F8 elements defines the mode of loop formation that either allows or blocks stimulation of the *white* promoter by the GAL4 activator. This phenomenon is explained by the model suggesting that when the insulators are located in opposite orientations, the loop configuration is favorable for communication between regulatory elements located beyond the loop. The loop formed by two insulators located in the same orientation juxtaposes two elements located within and beyond the loop, which leads to partial isolation of the GAL4 binding sites and the *white* promoter placed on the opposite sides of the insulators. Supposedly, this orientation-dependent interaction is accounted for by at least two insulator-bound proteins that are involved in specific protein–protein interactions. In accordance with this model, the results of this study show that dCTCF is required for orientation-dependent pairing of the PTS/F8 boundaries.

As expected, we observed cross interactions between all dCTCF-containing boundaries included in analysis. However, only Fab-6 and PTS/F8 interacted with the Fab-7 boundary (which lacks dCTCF binding sites). It is noteworthy that dCTCF failed to bind to the upstream region of the *Abd-B* promoter A at the pupa stage, which probably accounted for the inability of Fab-3, Fab-4 and Mcp to interact with A^{CTCF}. At the same time, strong interactions were observed between the upstream promoter region and Fab-6, Fab-7, PTS/F8 or the mutant Fab-6^{m} and PTS/F8^{m} boundaries lacking dCTCF binding sites. Thus, as yet unidentified protein(s) other than dCTCF are involved in supporting specific interactions of the Fab-6, Fab-7 and PTS/F8 boundaries with the upstream region of the *Abd-B* promoter.

It appears that one of such proteins was recently identified in the Fab-7 boundary (52).

Inactivation of the dCTCF binding site in the A^{CTCF} region considerably reduced A^{CTCF}/Fab-7 and A^{CTCF}/PTS/F8 interactions, suggesting a role for the dCTCF binding site in these processes. Since no dCTCF binding takes place in the transgenic construct, it is likely that some as yet unidentified protein binds to this site, thereby either supporting enhancer–promoter communication or recruiting other transcription factors to the upstream promoter region.

The above-described results are in accordance with the model that boundaries (Fab-6, Fab-7 and PTS/F8) are required for specific interaction with the region located upstream of the promoter A that facilitates contact between the *iab* enhancer and the *Abd-B* promoter itself (1,43,53,54). As shown recently, the Fab-7 boundary interacts with a region near the *Abd-B* promoter *in vivo* (55). The Fab-7 boundary is capable of almost complete substitution of the Fab-8 boundary, which is indicative of similarity in the mechanism of boundary function (56). On the other hand, the minimal Fab-8 insulator lacking PTS sequencing can only partially substitute for the Fab-7 boundary (56), with the resulting loss of *Abd-B* activation by *iab-6*. These findings are in agreement with our observation that the Fab-7 boundary (but not the Fab-8 insulator or PTS) can interact with the A^{CTCF} region. It appears that proteins required for the interaction with A^{CTCF} effectively bind only to the composite PTS/F8 element. This conclusion is supported by several studies demonstrating that PTS can support long-distance enhancer–promoter interaction in transgenic lines only when combined with a Fab-8- or Su(Hw)-containing insulator (57–60). It may well be that the insulator functions as an auxiliary element helping in opening chromatin and recruiting transcriptional factors to PTS. Thus, our results support the model (60) that the PTS elements in cooperation with corresponding insulators facilitate proper interaction between *iab* enhancers and promoters in the *Abd-B* locus.

Recently, a 255-bp element, named promoter-tethering element (PTE), was found at a distance of 40 bp from the *Abd-B* transcriptional start site (61,62) (Figure 1). This element, located between the *Abd-B* promoter and the A^{CTCF} region used in this study, is capable of selectively recruiting *iab* enhancers to the *Abd-B* promoter in a transgenic assay (62) and supposedly improves the specificity of interaction between the *iab* enhancers and the *Abd-B* promoter (61).

Thus, it seems likely that the boundaries, PTS and PTE cooperate in organizing proper interactions between enhancers and promoters in the *BX-C*. Identification of new proteins (other than dCTCF) bound to the boundaries, PTS and PTE is required for elucidating the mechanism of enhancer–promoter communication in the *BX-C*.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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