Insulators form gene loops by interacting with promoters in *Drosophila*

Maksim Erokhin, Anna Davydova, Olga Kyrchanova, Alexander Parshikov, Pavel Georgiev* and Darya Chetverina*

SUMMARY
Chromatin insulators are regulatory elements involved in the modulation of enhancer-promoter communication. The 1A2 and Wari insulators are located immediately downstream of the *Drosophila* yellow and white genes, respectively. Using an assay based on the yeast GAL4 activator, we have found that both insulators are able to interact with their target promoters in transgenic lines, forming gene loops. The existence of an insulator-promoter loop is confirmed by the fact that insulator proteins could be detected on the promoter only in the presence of an insulator in the transgene. The upstream promoter regions, which are required for long-distance stimulation by enhancers, are not essential for promoter-insulator interactions. Both insulators support basal activity of the yellow and white promoters in eyes. Thus, the ability of insulators to interact with promoters might play an important role in the regulation of basal gene transcription.

KEY WORDS: Chromatin insulator, Gene loop, Insulator-promoter interaction, CTCF, Su(Hw), *Drosophila*

INTRODUCTION
Insulators regulate gene activity in a variety of organisms. The defining feature of insulators as a class of regulatory elements is their ability to block enhancer-promoter interactions only when positioned between them (for reviews, see Sun and Elgin, 1999; Kuhn and Geyer, 2003; Brasset and Vaury, 2005; Zhao and Dean, 2005; Wallace and Felsenfeld, 2007; Maksimenko et al., 2006; Valenzuela and Kamakaka, 2006; Maeda and Karch, 2007; Phillips and Corces, 2009; Core and Lis, 2009).

Two mutually non-exclusive but rather complementary mechanisms can account for the ability of insulators to block enhancers and support long-distance interactions. Experiments with transgenic lines suggest that the interaction between insulators can result in the formation of chromatin loops that either block or facilitate long-distance enhancer-promoter communication depending on the nature of the interacting promoters as well as on the distances between all the elements involved (enhancers, insulators and promoters) and their relative ‘strength’ (Muravyova et al., 2001; Cai and Shen, 2001; Conte et al., 2002; Kuhn et al., 2003; Gruzdeva et al., 2005; Savitskaya et al., 2006; Kyrchanova et al., 2008a). Alternatively, insulator action can be explained by the ability of insulators to form direct contacts with either an enhancer (the decoy model) or a promoter, thereby inactivating them. For example, the insulator protein CTCF binds to the unmethylated maternal allele of the imprinting control region (ICR) in the Igf2/H19 imprinting domain and blocks enhancer-promoter communication by directly interacting with Igf2 promoters (Li et al., 2008). Insulators of the *Drosophila* Abd-B gene can establish contact with a region upstream of the promoter that is required for proper enhancer-promoter communication (Cléard et al., 2006; Kyrchanova et al., 2008b). Several *Drosophila* insulators [sces, scs’, IdexiU3 and Fa[wdb]] have been shown to contain promoters (Vazquez and Schedl, 2000; Conte et al., 2002; Kuhn et al., 2004), which, according to the decoy model (Geyer, 1997), may tether enhancers in nonproductive interactions. The stalled promoters of the *bithorax* complex display insulator activity in embryos (Chopra et al., 2009). Many insulator proteins, such as CTCF, CP190, Mod(mdg4)-67.2 [Mod(mdg4) – FlyBase] and BEAF (BEAF-32), are frequently found bound to the promoters (Smith et al., 2009; Bartkuhn et al., 2009; Jiang et al., 2009; Bushey et al., 2009; Nègre et al., 2010).

Previously, two well-studied tissue-specific *Drosophila* genes, *yellow* (Golovnin et al., 2003; Parnell et al., 2003) and *white* (Chetverina et al., 2008), were shown to contain insulators immediately downstream of their coding regions. The *yellow* gene is responsible for dark pigmentation of the larval and adult cuticle and its derivatives, whereas the *white* locus determines eye pigmentation. The 1A2 insulator located on the 3’ side of the *yellow* gene contains two binding sites for the Su(Hw) protein. Additional proteins, Mod(mdg4)-67.2, CP190 and E(y)2 (Gerassimova et al., 1995; Pai et al., 2004; Kurshakova et al., 2007), interact with Su(Hw) and are required for the activity of Su(Hw)-dependent insulators. None of the known DNA-binding insulator proteins binds to the Wari insulator located on the 3’ side of the *white* gene (Chetverina et al., 2008). However, we observed stage-specific binding of CP190 and E(y)2 to the Wari insulator, which was indicative of its relationship to Su(Hw) insulators (Erokhin et al., 2010).

Here, we present evidence that the 1A2 and Wari insulators interact with their target promoters and that this facilitates the formation of a gene loop between the promoter and terminator regions.

MATERIALS AND METHODS
*Drosophila* strains, germline transformation and genetic crosses
Flies were maintained at 25°C on standard yeast medium. The construct, together with a P element containing defective inverted repeats (P25.7wc) that was used as a transposase source (Karess and Rubin, 1984), were injected into *yaucwI3* preblastoderm embryos as described (Rubin and Spradling, 1982; Spradling and Rubin, 1982). The resulting flies were
crossed with yacw1118 flies, and the transgenic progenesis were identified by their eye or cuticle pigmentation. Chromosomal localization of various transgene inserts was determined by crossing the transfectants with the yacw1118 balancer stock carrying dominant markers: In(2Rl),CyO for chromosome 2 and In(3LR)TM3,SB for chromosome 3. The transformed lines were tested for transposon integrity and copy number by Southern blot hybridization. Only single-copy transfectants were included in the study.

The lines with DNA fragment excisions were obtained by crossing the transposon-bearing flies with the Fip (w1118, S2CyO, hsFpl, Isa/Sco; +) or Cre (y1w; CyO, P[w+]/Sco; +) recombinase-expressing lines (Golic and Lindquist, 1989; Siegal and Hartl, 2000). The Cre recombinase induces 100% excisions in the next generation. A high level of Fip recombinase (almost 90% efficiency) was produced by heat shock treatment (2 hours, daily) during the first 3 days after hatching. All excisions were confirmed by PCR analysis.

To induce GAL4 expression, we used the modified yw1118; P[w', tubGAL4]117/TM3,SB line (Bloomington Stock Center #5138), in which the marker mini-white gene was deleted as described (Kyrehanova et al., 2007).

To inactivate Zeste, we used the null allele mutation (z177k w67c2), Bloomington Stock Center #1385, which contains a 314 bp deletion that removes RNA leader sequences and the AUG initiation codon of zeste (Pirrotta et al., 1987).

To estimate the levels of yellow and white expression, we visually determined the degree of pigmentation in the abdominal cuticle and wing blades (yellow) and in the eyes (white) of 3- to 5-day-old males developing at 25°C, with reference to standard color scales. The pigmentation scores were independently determined by two investigators.

On the five-grade scale for yellow, grade 5 pigmentation was that of wild type; grades 4 and 3 corresponded to partial stimulation of the yellow gene by GAL4, grade 2 corresponded to the basal level of yellow expression in the absence of GAL4, and grade 1 corresponded to complete loss of yellow expression. Identical data for the wing and body pigmentation were obtained in all experiments.

On the nine-grade scale for white, red (R) eyes corresponded to the wild type and white (W) eyes to the total loss of white expression; intermediate pigmentation levels, in order of decreasing gene expression, were brownish red (BrR), brown (Br), dark orange (dOr), orange (Or), dark yellow (dY), yellow (Y), and pale yellow (pY).

### Plasmid construction

The constructs were based on the CaSpeR vector (Pirrotta, 1988). The pCaSpep15(+R1) plasmid was constructed by inserting an additional EcoRI site at +3190 of the mini-white gene in the pCaSpep15 plasmid. The Wari insulator located on the 3' side of the mini-white gene was deleted from pCaSpep15(+R1) by digestion with EcoRI to produce plasmid pCaSpepΔ700. The 3 kb Sal-BamHI fragment containing the yellow gene regulatory region (yr) was cloned into pGEM7 digested with XhoI and BamHI (yr-PGEM7). The 5 kb BamHI-BglII fragment containing the coding region (yc) was inserted in forward orientation into the CaSpeR plasmid (Savitskaya et al., 2006) digested with BamHI (Ca-yc). The Xbal-BamHI fragment containing yr was then cloned from the yr-PGEM7 vector into Ca-yc digested with XhoI and BamHI (Ca-yc). The Ca-yc-yr fragment containing the Aorl-BglII fragment of yc with 893 bp of upstream sequence lacking enhancers was generated by deleting the Xbal-Aorl fragment (containing wing and body enhancers) from the Ca-yc-yr plasmid.

The 825 bp sequence corresponding to the Wari insulator (PCR amplified with 5'-GGGAATCTCCGATCTACTCGC-3' and 5'-CTTTGGAGGATTGTATCCGATAT-3' primers) and the 454 bp sequence corresponding to the 1A2 insulator (PCR amplified with 5'-GAGATCTGTCGGTGATCC-3' and 5'-GAGGAAGTCCAGATG-3' primers) were obtained as described (Chetverina et al., 2008; Golovnin et al., 2003). These sequences were cloned into pBluescript SK+ between lox sites to produce lox(Wari) and lox(1A2) plasmids, respectively. To generate G4-B-lox(1A2) or lox(1A2)-A-G4, a fragment containing ten binding sites for the yeast GAL4 protein (two copies of 5' binding sites for yeast GAL4 from the pUAST vector) was cloned into the lox(1A2) plasmid cleaved with BanHI or ApaI, respectively. To generate G4-X-lox(Wari) or lox(Wari)-B-G4, a fragment containing ten binding sites for yeast GAL4 was cloned into the lox(Wari) plasmid cleaved with XhoI or BamHI, respectively.

YG4(1A2), Y(1A2)G4, Y(1A2)G4, YG4(1A2)

The G4-B-lox(1A2) fragment was cloned into the Ca-yc-yr fragment containing the Smal in either forward [YG4(1A2)] or reverse [Y(1A2)G4] orientation. The lox(1A2)-A-G4 fragment was cloned into Ca-yc-yr fragment cleaved with Smal in either forward [Y(1A2)G4] or reverse [YG4(1A2)] orientation.

Link-YG4(1A2), Link-Y(1A2)G4

The Link-Y plasmid was constructed by cloning the 1828 bp HincII fragment of the lacZ region into Ca-yc-yr fragment containing Aorl and Xbal. The G4-B-lox(1A2) or lox(1A2)-A-G4 fragments were inserted into Link-Y cleaved with Smal to generate Link-YG4(1A2) or Link-Y(1A2)G4 plasmids, respectively.

YG4(1A2), YG4(1A2)

Plasmid vectors containing 454 bp I-yr insulator with mutated Su(Hw) binding sites (1A2m) and four reiterated Su(Hw) binding sites [S'S' made by tetramerization of the third Su(Hw) binding site] were kindly provided by A. Golovnin (Golovnin et al., 2003; Golovnin et al., 2005). The corresponding fragments were cloned into pBluescript SK+ between lox sites to generate lox(1A2m) and lox(S'S') cleaved with G4-B-lox(1A2m) and G4-B-lox(S'S') fragments, a fragment containing ten binding sites for yeast GAL4 was cloned into the lox(1A2m) or lox(S'S') plasmid cleaved with BamHI. Each of the G4-B-lox(1A2m) and G4-B-lox(S'S') fragments was cloned into Ca-yc-yr fragment cleaved with Smal in forward orientation.

**∆PTE-YG4(1A2), preveYG4(1A2)**

The plasmid vectors ∆Xhol-yr and ∆Xhol-yr-ye were kindly provided by L. Melnikova (Melnikova et al., 2008). The plasmid vector ∆Xhol-yr contains the Xbal-BamHI yellow regulatory region minus deleted upstream sequences (~100 to ~69) of the yellow gene. In the ∆Xhol-yr-ye plasmid, the sequence corresponding to ~63 to +130 was replaced with a 193 bp sequence (~65 to +128) from the eve gene promoter region. The Xbal-BamHI fragment of ∆Xhol-yr was replaced by Xbal-BamHI fragments from ∆Xhol-yr, eve-ye and ∆Xhol-yr-ye plasmids to produce ΔXhol-yr-C∆ and ΔXhol-yr-C∆. The Xbal-Aorl fragment containing yellow gene enhancers was deleted from both plasmids. The lox(1A2)-A-G4 fragment was cloned into these plasmids in reverse orientation, downstream of the yellow gene cleaved with Smal, to produce ∆PTE-YG4(1A2) and preveYG4(1A2).

WG4(Wari), W(Wari)G4, W(Wari)G4, W(4G(Wari))

The G4-X-lox(Wari) fragment was cloned into the pCaSpeRΔ700 plasmid cleaved with EcoRI in either forward [WG4(Wari)] or reverse [W(Wari)G4] orientation. The lox(Wari)-B-G4 fragment was cloned into pCaSpeRΔ700 cleaved with EcoRI in either forward [W(Wari)G4] or reverse [WG4(Wari)] orientation.

**Δas-WG4(Wari)**

The plasmid vector Δas-CaSpeR, which contains a deletion of the white promoter region from ~113 to ~20, was kindly provided by M. Kostyuchenko (Kostyuchenko et al., 2009). The full-length promoter in pCaSpeRΔ700 was replaced by a mutated promoter to produce Δas-CaSpeRΔ700 plasmid. The G4-X-lox(Wari) fragment was cloned into Δas-CaSpeRΔ700 cleaved with EcoRI in forward orientation.

**YG4(Wari)**

The G4-X-lox(Wari) fragment was cloned into Ca-yc-yr fragment cleaved with Smal in forward orientation.

WG4(1A2)

The lox(1A2)-A-G4 fragment was cloned into pCaSpeRΔ700 cleaved with EcoRI in reverse orientation.
pryWG4(Wari), pryWG4(1A2R)
The sequence corresponding to +328 to +169 and containing the white gene promoter with upstream sequences was deleted from pCaSpeR7A700 to produce Δprw-pCaSpeR7A700. The yellow promoter region was PCR amplified with primers 5'-CTGGTCTCAGAACACACTGTC-3' and 5'-CACTTAGCTCTAAGCTG-3'. The PCR product was ligated into pBluescript SK+ cleaved with EcoRV to produce prY-pSK and sequenced to confirm that no unwanted changes had been introduced into the yellow promoter sequence. The prY-pSK plasmid was cleaved with HindIII to leave +494 to +169 bp of the yellow promoter) and inserted into Δprw-pCaSpeR7A700 cleaved with XbaI to produce prY-pCaSpeR7A700. The G4-Lox-lox(Wari) and the lox(1A2)-A-G4 fragments were cloned into prY-pCaSpeR7A700 cleaved with EcoRI to produce pryWG4(Wari) and pryWG4(1A2R), respectively.

Y4G(sc), Y4 (Fab-7), Y4 (MCP), Y4(CTCF-4)
The 990 bp scs insulator sequence corresponding to +510-1503 bp in the GenBank sequence (accession no. X63731), the 858 bp sequence corresponding to the Fab-7 insulator (PCR amplified with primers 5'-GATTCTCAACGTGTGGCGGGGGG-3' and 5'-CGTGAAGCGCCGAAAATCG-3'), the 350 bp Mcp insulator sequence (PCR amplified with primers 5'-GCTGAGATTGCTAACAACG-3' and 5'-CCCCAATCTGTTGTAATGTT-3') and CTFCF-6 were obtained as described (Kyrchanova et al., 2008a; Kyrchanova et al., 2008b). Sequences corresponding to the scs, Fab-7, Mcp insulators and CTFCF-6 fragment were cloned into pBluescript SK+ between lox sites to produce lox(scs), lox(Fab-7), lox(MCP) and lox(CTCF-4) plasmids, respectively. To generate G4-B-lox(sc5), G4-B-lox(Fab-7), G4-B-lox(MCP) and G4-B-lox(CTCF-4), a fragment containing ten binding sites for yeast GAL4 was cloned into the corresponding plasmids cleaved with BamHI. Then, G4-B-lox(sc5), G4-B-lox(Fab-7), G4-B-lox(MCP) and G4-B-lox(CTCF-4) fragments were cloned into pA-γ(-893) plasmid cleaved with Smal in forward orientation.

RT-PCR
RNA was isolated from ~50 μl of 0- to 24-hour embryos with TRI reagent (Ambion) according to the manufacturer’s instructions. Purified RNA pools were digested with DNase I (RNase-free; BioLabs) and repurified using the RNaseasy Mini Kit (Qiagen). For reverse transcription, 3 μg of the generated RNA was incubated with ArrayScript reverse transcriptase (Ambion) in the presence of dNTPs, oligo(dT) (Fermentas) and RNase inhibitor (Ambion) in the supplied reaction buffer at 42°C for 1.5 hours, according to the manufacturer’s instructions. The reverse transcriptase was inactivated by heating at 95°C for 5 minutes. To control DNA digestion by DNase I, additional negative control experiments were performed without reverse transcriptase in the reaction mixture. The generated cDNA pools containing ten binding sites for yeast GAL4 were cloned into the corresponding plasmids cleaved with BamHI. Then, G4-B-lox(sc5), G4-B-lox(Fab-7), G4-B-lox(MCP) and G4-B-lox(CTCF-4) fragments were cloned into pA-γ(-893) plasmid cleaved with Smal in forward orientation.

Chromatin immunoprecipitation (ChIP)
For each experiment, 150-200 mg of the initial material (embryos, larvae or pupae) was collected. The material was homogenized in 5 ml of buffer A1 (15 mM Hepes pH 7.6, 60 mM KCl, 15 mM NaCl, 4 mM MgCl2, 0.5% Triton X-100, 0.5 mM DTT, 10 mM sodium butyrate) supplemented with EDTA-free protease inhibitor cocktail (Roche, Switzerland) and formaldehyde as a cross-linking agent (final concentration 1.8%). The reaction was stopped by adding glycine (final concentration 225 mM). The homogenate was cleared by passing through a 100-μm nylon cell strainer (BD Falcon) and pelleted by centrifugation at 4000 g at 4°C for 5 minutes. After washing in three 3-ml portions of buffer A1 at 4°C (5 minutes each) and 3 ml of lysis buffer without SDS, the pellet was treated with 0.5 ml of complete lysis buffer (15 mM Hepes pH 7.6, 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.5 mM DTT, 0.1% sodium deoxycholate, 10 mM sodium butyrate, 0.1% SDS, 0.5% Nonidet P-40) for 15 minutes on ice. The sample (input).

Antibodies
Antibodies used in the study were: anti-Mod(mdg4)-67.2 against residues 402 to 611, anti-CPI90 against residues 386 to 508 (a gift from A. Golovin), anti-TBP against residues 1 to 55 [a gift from S. G. Georgieva (Vorobyeva et al., 2009)], and the monoclonal antibody 4H8 against the RNAPII CTD repeat sequence YSPTSPS (ab5408, Abcam).

RESULTS

The 1A2 insulator functionally interacts with the yellow gene promoter
To analyze interactions between distantly located regulatory elements of tissue-specific genes in Drosophila, we used an assay that is based on the inability of the yeast GAL4 activator to stimulate a promoter that is located a relatively long distance (~5 kb) from the corresponding gene (Kyrchanova et al., 2007; Kyrchanova et al., 2008b).

To test the interaction between the 1A2 insulator and the yellow promoter, we used a yellow gene with just 900 bp of sequence upstream of the promoter. Ten sites for the yeast GAL4 activator protein were placed on the 3’ side of the yellow gene, and the 1A2 insulator flanked by lox sites was inserted downstream of these GAL4 binding sites in forward (Fig. 1A) or reverse (Fig. 1B) orientation relative to the yellow gene in its genomic position. If the insulator interacted with the promoter, GAL4 would be placed in close proximity to the promoter, which would allow activation of the promoter. To express the GAL4 protein, a transgenic line carrying the GAL4 gene under the control of the ubiquitous tubulin promoter was used.

In all transgenic lines, flies had yellow pigmentation of the wing and body cuticle owing to the absence of tissue-specific enhancers in the constructs. Induction of GAL4 expression considerably increased pigmentation of flies in most of the lines carrying either of the constructs. When the 1A2 insulator was deleted from the transgenic lines, GAL4 lost the ability to stimulate transcription (Fig. 1A,B). Thus, the GAL4 activator cannot stimulate yellow expression when located on the 3’ side of the yellow gene, and the 1A2 insulator facilitates the interaction of GAL4 with the transcriptional machinery at the promoter. Similar 1A2-dependent activation was observed at the embryo stage in one transgenic line tested (see Fig. S2 in the supplementary material).

The orientation of the 1A2 insulator had no significant effect on yellow activation by GAL4 (Fig. 1A,B). We next examined whether the relative orientation of 1A2 and GAL4 binding sites is important for yellow stimulation by GAL4. The 1A2 insulator was inserted upstream of the GAL4 binding sites in either forward (Fig. 1C)
The 1A2 insulator functionally interacts with the Drosophila yellow gene promoter. (A–F) Transgene maps (not to scale; key constructs are drawn to scale in Fig. S1 in the supplementary material) showing the yellow gene (arrow indicates direction of transcription), the ten binding sites for GAL4 activator (10×G4), and the 1A2 insulator (the acute angle indicates its orientation relative to the yellow gene in the genomic position), arrows flanking the insulator indicate lox sites for Cre recombinase. Below the schemes are the expression data for each parental construct shown in the scheme and for those derived from it by in vivo excision of elements flanked by lox sites. ‘+GAL4’ indicates that eye phenotypes in transgenic lines were examined after induction of GAL4 expression. The horizontal color scales are headed by tapering gene names. On the five-grade scale for yellow, grade 5 pigmentation is that of wild type; grades 4 and 3 correspond to partial stimulation of the yellow gene by GAL4; grade 2 corresponds to the basal level of yellow expression in the absence of GAL4; and grade 1 corresponds to complete loss of its expression. Each figure within a frame shows the number of transgenic lines with the corresponding pigmentation grade, with the frame itself showing the range of pigmentation; T is the total number of lines examined for each particular construct or for derivative constructs; N is the number of lines in which the phenotype (i.e. expression level) changed as compared with the parental construct upon induction of GAL4 or deletion of the specified DNA fragment. The shaded cursor in each frame indicates the ‘mean color’ on the scale above; thus, cursor positions and shifts in different rows can be compared directly, but the cursors themselves are not associated with the numbers that they may cover.

Fig. 1. The 1A2 insulator functionally interacts with the Drosophila yellow gene promoter. (A–F) Transgene maps (not to scale; key constructs are drawn to scale in Fig. S1 in the supplementary material) showing the yellow gene (arrow indicates direction of transcription), the ten binding sites for GAL4 activator (10×G4), and the 1A2 insulator (the acute angle indicates its orientation relative to the yellow gene in the genomic position), arrows flanking the insulator indicate lox sites for Cre recombinase. Below the schemes are the expression data for each parental construct shown in the scheme and for those derived from it by in vivo excision of elements flanked by lox sites. ‘+GAL4’ indicates that eye phenotypes in transgenic lines were examined after induction of GAL4 expression. The horizontal color scales are headed by tapering gene names. On the five-grade scale for yellow, grade 5 pigmentation is that of wild type; grades 4 and 3 correspond to partial stimulation of the yellow gene by GAL4; grade 2 corresponds to the basal level of yellow expression in the absence of GAL4; and grade 1 corresponds to complete loss of its expression. Each figure within a frame shows the number of transgenic lines with the corresponding pigmentation grade, with the frame itself showing the range of pigmentation; T is the total number of lines examined for each particular construct or for derivative constructs; N is the number of lines in which the phenotype (i.e. expression level) changed as compared with the parental construct upon induction of GAL4 or deletion of the specified DNA fragment. The shaded cursor in each frame indicates the ‘mean color’ on the scale above; thus, cursor positions and shifts in different rows can be compared directly, but the cursors themselves are not associated with the numbers that they may cover.

1C or reverse (Fig. 1D) orientation. In both series of transgenic lines, we observed no yellow stimulation by GAL4, which indicated that the relative position of 1A2 and GAL4 binding sites is critical for the ability of GAL4 to activate the promoter (Fig. 1C,D).

These results suggest that proteins bound to the 1A2 insulator and the promoter region can interact with each other, and that this in turn facilitates long-distance stimulation of yellow transcription by GAL4. To check the possibility that 1A2 interacts with an unidentified insulator element located near to the site of transgene insertion rather than with the yellow promoter, we inserted a 2 kb spacer upstream of the yellow gene. The results (Fig. 1E,F) were similar to those obtained in the transgenic lines without the spacer (Fig. 1A,C), indicating that yellow activation by GAL4 is unlikely to result from the interaction of 1A2 with another insulator.

The 1A2 insulator contains two binding sites for Su(Hw) (Golovnin et al., 2003; Parnell et al., 2003). However, additional (as yet unidentified) proteins are important for 1A2 activity (Soshnev et al., 2008). To analyze the role of Su(Hw) in the promoter-binding activity of 1A2, we mutated both Su(Hw) binding sites (1A2m, Fig. 2A) and observed no yellow stimulation by GAL4. Hence, we concluded that the Su(Hw) binding sites are required for the functional interaction of 1A2 with the yellow promoter. To test whether Su(Hw) binding sites can support yellow activation by GAL4 independently of other sequences in the 1A2 insulator, a fragment containing four copies of the third Su(Hw) binding site (S24) from the gypsy insulator was tested in the assay (Fig. 2B). The synthetic Su(Hw) binding region functioned similarly to the 1A2 insulator in facilitating yellow stimulation by GAL4. Thus, the insulator complex formed at the Su(Hw) binding sites can interact with the yellow promoter.

The yellow sequence from −100 to −69 (the promoter targeting element, PTE) is essential for the ability of enhancers to stimulate the promoter from a distance (Melnikova et al., 2008). To test this region for a role in the interaction with the 1A2 insulator, we deleted the PTE from the yellow gene (Fig. 2C). In the corresponding transgenic lines, 1A2 facilitated yellow stimulation by GAL4. Therefore, the PTE is not necessary for the promoter-insulator interaction.

We then tested whether the core promoter element determines the specificity of interaction with the 1A2 insulator. The yellow core promoter region contains TATA, initiator (Inr) and atypical downstream promoter (DPE) elements. We replaced the yellow core promoter sequence (−63 to +130) with a 193 bp sequence [−65 to +128 (Morris et al., 2004)] from the eve gene promoter region [the preveYG4(1A2m) construct]. Like the yellow promoter, the eve promoter contains a TATA box and Inr (Kutach and Kadonaga, 2000). In transgenic lines, we observed strong yellow activation by GAL4 in the presence of the 1A2 insulator (Fig. 2D). Therefore, this insulator can interact with different TATA core promoters.
CP190 and Mod(mdg4)-67.2 are detected on the eve promoter only in the presence of 1A2 at the 3’ end of the yellow gene

The Su(Hw), CP190 and Mod(mdg4)-67.2 proteins are responsible for the activity of the 1A2 insulator (Golovnin et al., 2003; Sosnnev et al., 2008). In the case of 1A2 insulator-promoter interaction, it can be expected that these insulator proteins will be detected on the yellow promoter in a chromatin immunoprecipitation (ChIP) assay. According to available data, however, the Su(Hw), Mod(mdg4)-67.2 and CP190 proteins bind to the 1A2 insulator but are not detected on the yellow promoter in Drosophila embryos (Nègre et al., 2010). We also failed to detect Mod(mdg4)-67.2 and CP190 on the yellow promoter in Drosophila wild-type embryos, second instar larvae and late pupae (see Fig. S3 in the supplementary material) and also in second instar larvae and late pupae of the homozygous transgenic line carrying the yellow gene with the downstream 1A2 insulator (see Fig. S4 in the supplementary material). In addition, we performed ChIP experiments for the yellow and white transgenes at the embryo stage (0-24 hours) and for yellow at the late pupa stage with and without GAL4 activator in heterozygous transgenic constructs carrying the corresponding gene with the downstream 1A2 insulator, but also failed to detect the insulator proteins CP190 and Mod(mdg4)-67.2 on the promoter of either gene (see Fig. S4 in the supplementary material).

The absence of insulator protein enrichment on the yellow promoter could be explained by the expression of yellow only in certain tissues where the presence of insulator proteins at the promoter cannot be detected by ChIP using chromatin isolated from whole flies. Indeed, we observed only slight or no enrichment of the yellow promoter with RNA polymerase (RNAP) II and TATA binding protein (TBP) (Fig. 3). By contrast, the eve promoter was active in most embryonic and larval cells, which was confirmed by efficient RNAP II and TBP binding to the promoter both in the genome and in the transgenic construct (Fig. 3).

Consequently, we analyzed CP190 and Mod(mdg4)-67.2 binding to the eve promoter by ChIP in two transgenic lines carrying a chimeric eve-yellow gene and the downstream 1A2 yellow insulator (Fig. 4; see Fig. S5 in the supplementary material). As expected, CP190 and Mod(mdg4)-67.2 bound to the eve promoter in the transgenic constructs in embryos (0-16 hours) and second instar larvae.

Moreover, the insulator proteins were detected on the eve promoter only in the presence of the 1A2 insulator. These results confirm the 1A2-eve promoter interaction identified in the GAL4 transcriptional assay. Thus, insulator proteins interact with the promoter in an insulator-dependent manner, and insulator-promoter interactions appear to take place only when the gene is in the transcriptionally active state.

The Wari insulator functionally interacts with the white gene promoter

To determine whether interactions between promoters and insulators are a common phenomenon, we tested the interaction between the white promoter and the Wari insulator in our assay. The Wari insulator flanked by lax sites was inserted downstream of the white gene and GAL4 binding sites in either forward or reverse orientation relative to the white gene in its genomic position (Fig. 5A,B).

In transgenic lines carrying either of the constructs, GAL4 strongly activated white in the presence of Wari at the adult (Fig. 5A,B) and embryo (see Fig. S2 in the supplementary material) stages, with deletion of the insulator resulting in significant reduction of white stimulation by GAL4 (Fig. 5A). Thus, the Wari insulator functionally interacts with the white promoter. As in the case of the yellow promoter-1A2 insulator pair, stimulation of the white gene by GAL4 was significantly reduced when Wari was inserted in either orientation between the gene and the GAL4 binding sites (Fig. 5C,D).

In most transgenic lines, flies had eye pigmentation in the pale yellow to orange range corresponding to the basal level of white expression. After deletion of the Wari insulator, eye pigmentation in most cases was reduced, varying from white to dark yellow, suggesting a role for Wari in supporting basal promoter activity.

The white promoter region referred to as the ‘anchor site’ is known to be essential for white stimulation by the eye enhancer (Qian et al., 1992). To test whether this promoter region is required for the interaction with the Wari insulator, the sequence corresponding to the anchor site (–113 to –17 relative to the white transcription start site) was deleted from the construct (Fig. 5E). Flies in corresponding transgenic lines showed a decrease in eye
pigmentation compared with those from transgenic lines carrying the unmutated white promoter region. Eye pigmentation was further reduced after deletion of Wari, which provided additional evidence for the role of this insulator in supporting basal promoter activity. In the presence of Wari, however, GAL4 effectively stimulated white expression, indicating that the insulator complex interacts with the promoter lacking the anchor site.

In addition to the anchor site, distant enhancer-promoter communication depends on Zeste protein binding to the enhancer and promoter of the white gene (Qian et al., 1992; Kostyuchenko et al., 2009). Inactivation of Zeste by crossing transgenic lines containing the unmutated white promoter and expressing GAL4 with the null zv77h mutation had no effect on the insulator-promoter interaction in our assay (Fig. 5A). Thus, as in the case of the yellow promoter-1A2 insulator interaction, it appears that the core elements of the white promoter are essential for the interaction with the Wari insulator.

The 1A2 and Wari insulators are interchangeable in the interaction with the yellow and white promoters

The eve and yellow promoters that were tested in pairs with the 1A2 insulator belong to the group of TATA-containing promoters, whereas the white promoter contains only Inr and DPE elements (Kutach and Kadonaga, 2000). To test whether the ability to interact with insulators depends on the type of core promoter, the 1A2 and Wari insulators were inserted downstream of the white and yellow genes, respectively (Fig. 6A, B). GAL4 binding sites were placed between the genes and insulators. In both cases, GAL4 effectively stimulated gene expression only in the presence of the insulator. Thus, 1A2 can functionally interact with the white promoter and Wari with the yellow promoter. Interestingly, deletion of 1A2 considerably reduced eye pigmentation in most transgenic lines (Fig. 6A), indicating that this insulator also supports the basal activity of the white promoter.
Next, we inserted the 1A2 (Fig. 6C) or Wari (Fig. 6D) insulator downstream of the white gene as regulated by the yellow promoter, with GAL4 binding sites placed between the white gene and the insulator. As in the previous constructs, GAL4 strongly activated white expression with the yellow promoter (Fig. 6C,D), but only in the presence of either insulator. Deletion of either insulator considerably reduced eye pigmentation in most of the transgenic lines tested. Thus, both insulators proved to potentiate the yellow promoter in the eye. This provides evidence that the Wari and 1A2 insulators can interact with promoters containing different combinations of core elements and that the insulators improve the basal activity of both promoters in the eye.

**Only certain insulators can facilitate stimulation of the yellow promoter by GAL4**

The above results suggest that at least two different insulators can interact with the yellow promoter. The question arises as to whether the promoter-insulator interactions are selective. We examined several well-described Drosophila insulators in our assay with the yellow gene. The complex scs insulator contains two oppositely directed promoters and a binding site for the Zw5 (Dwg – FlyBase) protein that is essential for enhancer blocking (Gasztner et al., 1999). This insulator was inserted downstream of GAL4 binding sites in reverse orientation relative to its genomic position (Fig. 6E). In the resulting transgenic lines, we observed strong yellow stimulation by GAL4 in the presence of the scs insulator.

Next, we performed experiments with the Fab-7 insulator, which contains a binding site for the CTCF insulator protein (Holohan et al., 2007). These insulators failed to support yellow stimulation by GAL4 (Fig. 6F,G). Additionally, we tested the effect of a sequence comprising four CTCF binding sites (CTCF/H11003), which also failed to facilitate yellow activation by GAL4 (Fig. 6H). These results indicate that only some insulators can facilitate yellow stimulation by GAL4. Thus, it appears likely that insulator-promoter interactions are selective. However, it is also possible that the Fab-7 and Mep insulators interact with the yellow promoter but fail to facilitate yellow stimulation by GAL4.

**DISCUSSION**

In this study, we have shown that the 1A2 and Wari insulators, which are located on the 3’ side of the yellow and white genes, respectively, can interact with their target promoters. Thus, insulators can support a gene loop that brings together a promoter and a terminator. Moreover, the results obtained by ChIP assay suggest that insulator-promoter interactions are transcription dependent. To date, transcription-dependent gene looping has been demonstrated in yeast (O’Sullivan et al., 2004; Ansari and Hampsey, 2005; Singh and Hampsey, 2007; Tan-Wong et al., 2009), human (Tan-Wong et al., 2008) and HIV provirus (Perkins et al., 2008). In yeast, loop formation was reported to be organized by TFIIB and the Ssu72 and Pta1 components of the 3’-end processing machinery (Singh and Hampsey, 2007; Ansari and Hampsey, 2005). It is possible that this mechanism is conserved between eukaryotes and that the interaction between an insulator and a promoter is required to facilitate the formation of a gene loop and/or its stabilization.
It has been suggested that gene loop formation might be a common feature of gene activation that serves to promote efficient transcriptional elongation and transcription reinitiation by facilitating RNAP II recycling from the terminator to the promoter, reinforcing the coupling of transcription with mRNA export and enhancing terminator function (Singh and Hampsey, 2007; Ansari and Hampsey, 2005). Here, we have found that the interaction of insulators with promoters is required for the basal activity of the white and yellow promoters in the eye. In addition to the possible role of a gene loop in the enhancement of RNAP II recycling and mRNA export, insulators might serve to bring to the promoter the remodeling and histone modification complexes that improve the binding and stabilization of the TFIID complex. Recently, Chopra et al. (Chopra et al., 2009) have found that the enhancer-blocking activity of several promoters and insulators depends on general transcription factors that inhibit RNAP II elongation. These authors suggest that insulators interact with components of the RNAP II complex at stalled promoters and that the resulting chromatin loops can prevent the inappropriate activation of stalled genes by enhancers associated with the neighboring locus. Here, we have found that the upstream promoter regions required for interactions with enhancers are not necessary for insulator-promoter interactions, which provides evidence that insulator proteins can interact with general transcription factors or proteins involved in the organization of promoter architecture. Certain types of insulators [the Su(Hw)-dependent 1A2, the Zw5-dependent scs, and Wari] can effectively interact with the yellow promoter, whereas others appear not to (the GAF-dependent Fab-7 and CTCF-dependent Mcp). GAF and CTCF are frequently found bound to promoter regions (Smith et al., 2009; Bartkuhn et al., 2009; Bushey et al., 2009; Nègre et al., 2010), which indicates that insulators that utilize these proteins are also involved in long-distance interactions with some promoters. For example, it is speculated that the Fab-7 insulator can interact with stalled promoters, such as the Abd-B promoter (Chopra et al., 2009; Core and Lis, 2009).

Here, we have shown that the GAL4 activator is unable to stimulate the promoter when GAL4 binding sites are placed downstream of the insulator. It appears likely that the loop is also formed between the insulator and promoter in this case, but that GAL4 is rendered outside the loop and blocked by the insulator. Thus, a chromatin loop formed by the promoter and insulator can

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**Fig. 6. Testing different Drosophila insulators for the ability to facilitate yellow stimulation by GAL4.** (A-H) pr-y-white is the white gene under the control of the yellow gene promoter; scs, the scs insulator; Fab7, the Fab-7 insulator; MCP, the Mcp insulator; CTCF, four CTCF binding sites. For other designations, see Figs 1 and 5.
prevent undesirable interactions with downstream regulatory elements. This provides evidence that the promoter-binding capacity of at least some insulators might contribute to their enhancer-blocking activity.

The genome-wide analysis of binding sites for insulator proteins has shown that they are present at the 3' and 5' UTRs of many Drosophila genes (Nègre et al., 2010). The IA2 and Wari insulators at the 3' end of the yellow and white genes were identified only as a result of the extensive use of these genes in insulator assays. Thus, it appears that insulators are likely to be located at the 3' UTRs of many genes. Further experiments are required to resolve this issue and to elucidate the mechanisms and functional role of insulator-promoter interactions in transcriptional regulation.

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Competing interests statement
The authors declare no competing financial interests.

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