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

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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 insulators 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 [scs, scs′, IdefixU3 and Fa^{swb}] 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 (Gerasimova 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 *yacw*^{1118} preblastoderm embryos as described (Rubin and Spradling, 1982; Spradling and Rubin, 1982). The resulting flies were

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crossed with *yacw*^{1118} flies, and the transgenic progeny were identified by their eye or cuticle pigmentation. Chromosomal localization of various transgene inserts was determined by crossing the transformants with the *yacw*^{1118} balancer stock carrying dominant markers: *In(2RL),CyO* for chromosome 2 and *In(3LR)TM3,Sb* for chromosome 3. The transformed lines were tested for transposon integrity and copy number by Southern blot hybridization. Only single-copy transformants were included in the study.

The lines with DNA fragment excisions were obtained by crossing the transposon-bearing flies with the Flp (*w*^{1118}*; S2CyO, hsFLP, ISA/Sco; +*) or Cre (*y*^{1}*w*^{1}*; Cyo, P[w+,cre]/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 Flp 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 *yw*^{1118}*; P[w*$^{-}$*, tubGAL4]117/TM3,Sb* line (Bloomington Stock Center #5138), in which the marker *mini-white* gene was deleted as described (Kyrchanova et al., 2007).

To inactivate Zeste, we used the null *z*^{v77h} mutation (*z*^{v77h} *w*^{67c23}, 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 pCaSpew15(+RI) plasmid was constructed by inserting an additional *Eco*RI site at +3190 of the *mini-white* gene in the pCaSpew15 plasmid. The Wari insulator located on the 3′ side of the *mini-white* gene was deleted from pCaSpew15(+RI) by digestion with *Eco*RI to produce plasmid pCaSpeRΔ700. The 3 kb *Sal*I-*Bam*HI fragment containing the *yellow* gene regulatory region (yr) was cloned into pGEM7 digested with *Xho*I and *Bam*HI (yr-pGEM7). The 5 kb *Bam*HI-*Bgl*II fragment containing the coding region (yc) was inserted in forward orientation into the CΔ plasmid (Savitskaya et al., 2006) digested with *Bam*HI (CΔ-yc). The *Xba*I-*Bam*HI fragment containing yr was then cloned from the yr-pGEM7 vector into CΔ-yc digested with *Xba*I and *Bam*HI (CΔ-y). The CΔ-y(–893) plasmid containing the *Aor*I-*Bgl*II fragment of yc with 893 bp of upstream sequence lacking enhancers was generated by deleting the *Xba*I-*Aor*I fragment (containing wing and body enhancers) from the CΔ-y plasmid.

The 825 bp sequence corresponding to the Wari insulator (PCR amplified with 5′-CGCAAGGAGTAGCCGACATATAT-3′ and 5′-CTTTGGAGTACGAAATGCGTCG-3′ primers) and the 454 bp sequence corresponding to the 1A2 insulator (PCR amplified with 5′-GGAGTACTACTACCAGGC-3′ and 5′-CAAGAACATTTCCGATATG-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^{R}) 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^{R}) plasmid cleaved with *Bam*HI or *Apa*I, 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 *Xho*I or *Bam*HI, respectively.

YG4(1A2), Y(1A2^{R})G4, Y(1A2)G4, YG4(1A2^{R})

The G4-B-lox(1A2) fragment was cloned into the CΔ-y(–893) plasmid cleaved with *Sma*I in either forward [YG4(1A2)] or reverse [Y(1A2^{R})G4] orientation. The lox(1A2)-A-G4 fragment was cloned into CΔ-y(–893) cleaved with *Sma*I in either forward [Y(1A2)G4] or reverse [YG4(1A2^{R})] orientation.

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

The Link-Y plasmid was constructed by cloning the 1828 bp *Hin*cII fragment of the *lacZ* region into CΔ-y cleaved with *Aor*I and *Xba*I. The G4-B-lox(1A2) or lox(1A2)-A-G4 fragments were inserted into Link-Y cleaved with *Sma*I to generate Link-YG4(1A2) or Link-Y(1A2)G4 plasmids, respectively.

YG4(1A2^{m}), YG4(S$^{\times 4}$)

Plasmid vectors containing 454 bp 1A2 insulator with mutated Su(Hw) binding sites (1A2^{m}) and four reiterated Su(Hw) binding sites [S$^{\times 4}$; 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(1A2^{m}) and lox(S$^{\times 4}$). To generate G4-B-lox(1A2^{mR}) and G4-B-lox(S$^{\times 4}$), a fragment containing ten binding sites for yeast GAL4 was cloned into the lox(1A2^{m}) or lox(S$^{\times 4}$) plasmid cleaved with *Bam*HI. Each of the G4-B-lox(1A2^{mR}) and G4-B-lox(S$^{\times 4}$) fragments was cloned into CΔ-y(–893) cleaved with *Sma*I in forward orientation.

ΔPTE-YG4(1A2^{R}), preveYG4(1A2^{R})

The plasmid vectors Δ*Xho*I-yr and Δ*Xho*I-yr-eve were kindly provided by L. Melnikova (Melnikova et al., 2008). The plasmid vector Δ*Xho*I-yr contains the *Xba*I-*Bam*HI *yellow* regulatory region minus deleted upstream sequences (–100 to –69) of the *yellow* gene. In the Δ*Xho*I-yr-eve 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 *Xba*I-*Bam*HI fragment of CΔ-y was replaced by *Xba*I-*Bam*HI fragments from Δ*Xho*I-yr, yr-eve and Δ*Xho*I-yr-eve plasmids to produce Δ*Xho*I-y-CΔ and Δ*Xho*I-eve-y-CΔ. The *Xba*I-*Aor*I 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 *Sma*I, to produce ΔPTE-YG4(1A2^{R}) and preveYG4(1A2^{R}).

WG4(Wari), W(Wari^{R})G4, W(Wari)G4, WG4(Wari^{R})

The G4-X-lox(Wari) fragment was cloned into the pCaSpeRΔ700 plasmid cleaved with *Eco*RI in either forward [WG4(Wari)] or reverse [W(Wari^{R})G4] orientation. The lox(Wari)-B-G4 fragment was cloned into pCaSpeRΔ700 cleaved with *Eco*RI in either forward [W(Wari)G4] or reverse [WG4(Wari^{R})] 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-pCaSpeRΔ700 plasmid. The G4-X-lox(Wari) fragment was cloned into Δas-pCaSpeRΔ700 cleaved with *Eco*RI in forward orientation.

YG4(Wari)

The G4-X-lox(Wari) fragment was cloned into CΔ-y(–893) cleaved with *Sma*I in forward orientation.

WG4(1A2^{R})

The lox(1A2)-A-G4 fragment was cloned into pCaSpeRΔ700 cleaved with *Eco*RI in reverse orientation.

pryWG4(Wari), pryWG4(1A2^{R})

The sequence corresponding to –328 to +169 and containing the *white* gene promoter with upstream sequences was deleted from pCaSpeRΔ700 to produce Δprw-pCaSpeRΔ700. The *yellow* promoter region was PCR amplified with primers 5′-CTGTTCTCAGAACACAACTGTC-3′ and 5′-CACTTAGCTCTAAGCTG-3′. The PCR product was ligated into pBluescript SK+ cleaved with *Eco*RV 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 *Hin*dIII (to leave –494 to +169 bp of the *yellow* promoter) and inserted into Δprw-pCaSpeRΔ700 cleaved with *Xba*I to produce prY-pCaSpeRΔ700. The G4-X-lox(Wari) and the lox(1A2)-A-G4 fragments were cloned into prY-pCaSpeRΔ700 cleaved with *Eco*RI to produce pryWG4(Wari) and pryWG4(1A2^{R}), respectively.

YG4(scs), YG4 (Fab-7), YG4 (MCP), YG4(CTCF$^{\times 4}$)

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′-GATTTCAAGCTGTGTGGCGGGGG-3′ and 5′-CGTGAGCGACCG-AAACTCG-3′), the 350 bp Mcp insulator sequence (PCR amplified with primers 5′-GCTCAGAGTACATAAGCG-3′ and 5′-CCCAATCGTTGT-AAGTGT-3′) and CTCF$^{\times 4}$ were obtained as described (Kyrchanova et al., 2008a; Kyrchanova et al., 2008b). Sequences corresponding to the scs, Fab-7, Mcp insulators and CTCF$^{\times 4}$ fragment were cloned into pBluescript SK+ between *lox* sites to produce lox(scs), lox(Fab-7^{R}), lox(MCP^{R}) and lox(CTCF$^{\times 4R}$) plasmids, respectively. To generate G4-B-lox(scs^{R}), G4-B-lox(Fab-7), G4-B-lox(MCP) and G4-B-lox(CTCF$^{\times 4}$), a fragment containing ten binding sites for yeast GAL4 was cloned into the corresponding plasmids cleaved with *Bam*HI. Then, G4-B-lox(scs^{R}), G4-B-lox(Fab-7), G4-B-lox(MCP) and G4-B-lox(CTCF$^{\times 4}$) fragments were cloned into CΔ-y(–893) plasmid cleaved with *Sma*I 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 RNeasy 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 were used as templates in quantitative real-time PCR (Q-PCR). The results of RT-PCR of *Ras64B* were used to standardize the overall amount of cDNA used in PCR assays. The *yellow*, *white* and *Ras64B* transcripts were detected using the PCR primers listed in Table S1 in the supplementary material.

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 $MgCl_2$, 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% N-lauroylsarcosine, EDTA-free protease inhibitor cocktail) and sonicated to break chromatin into fragments of average length 700 bp. The material was pelleted by centrifugation at 18,000 ***g*** for 5 minutes and the supernatant was transferred to a new tube. The pellet was treated with a second 0.5-ml portion of lysis buffer, and the preparation was centrifuged at 18000 ***g*** for 5 minutes. The two portions of supernatant were pooled, cleared by centrifuging twice at 18000 ***g*** for 10 minutes, and the resultant chromatin extract (1 ml) was used in four ChIP experiments after preincubation with protein A-Sepharose or protein G-Sepharose (see below). One aliquot of chromatin extract after preincubation with Sepharose was kept as a control sample (input).

ChIP experiments involved incubation with rat antibody to CP190, rabbit antibody to Mod(mdg4)-67.2, mouse antibody to RNAP II or rabbit antibody to TBP. Corresponding non-immune IgG were used as nonspecific antibody controls. Antibody-chromatin complexes were collected with either protein A-Sepharose [TBP, Mod(mdg4)-67.2] or protein G-Sepharose (CP190, RNAP II) beads (Sigma). The enrichment of specific DNA fragments was analyzed by real-time PCR using a C1000 thermal cycler (Bio-Rad).

Primers used in ChIP/real-time PCR analysis are listed in Table S2 in the supplementary material.

Antibodies

Antibodies used in the study were: anti-Mod(mdg4)-67.2 against residues 402 to 611, anti-CP190 against residues 386 to 508 (a gift from A. Golovnin), anti-TBP against residues 1 to 55 [a gift from S. G. Georgieva (Vorobyeva et al., 2009)], and the monoclonal antibody 4H8 against the RNAP II 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.

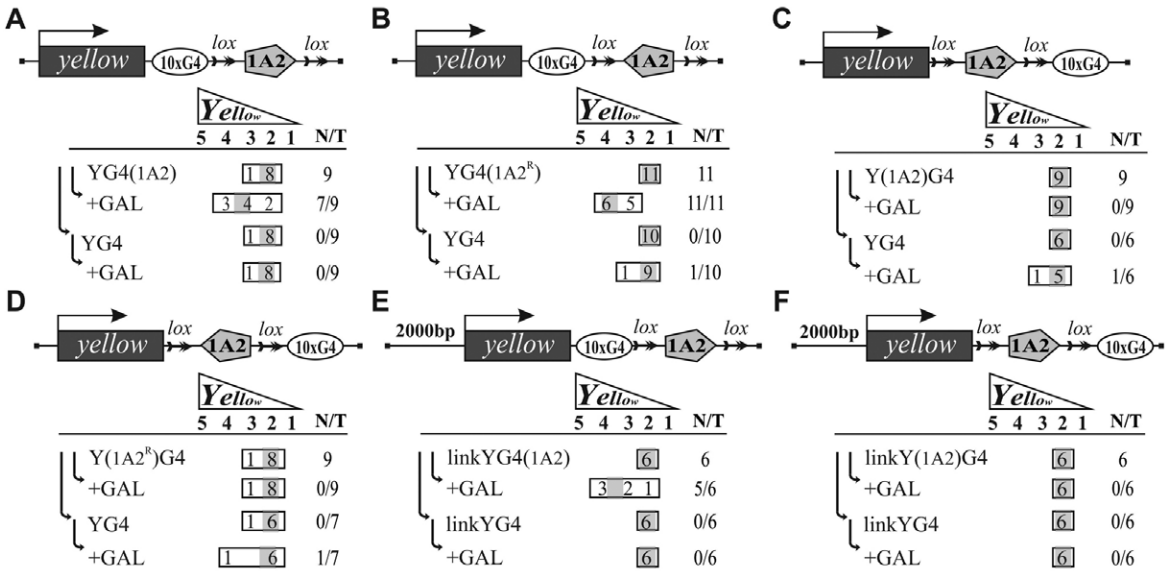

Fig. 1. The 1A2 insulator functionally interacts with the *Drosophila yellow* gene promoter. (**A-F**) Transgene maps (not to scale; key constructs are drawn to scale in Fig. S1 in the supplementary material) showing the *yellow* gene (arrow indicates direction of transcription), the ten binding sites 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 ($1A2^{m}$, 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 ($S^{\times 4}$) 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($1A2^{R}$) 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.

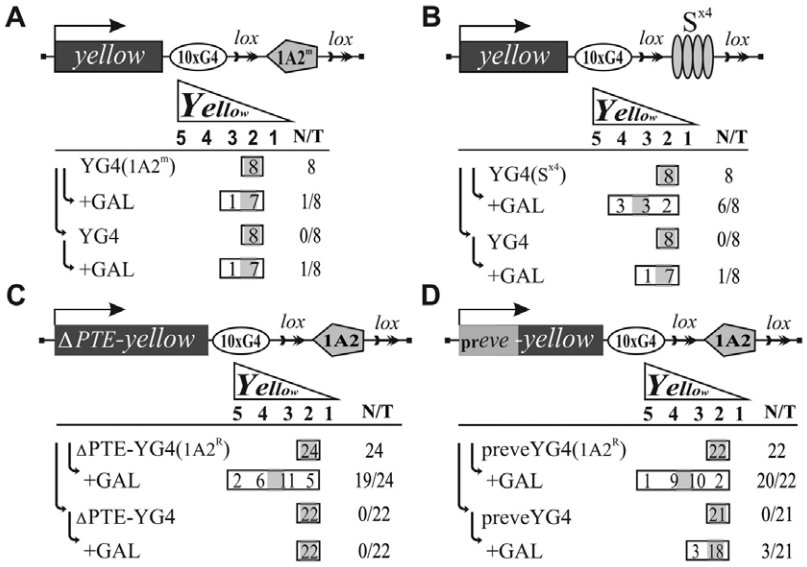

Fig. 2. Role of Su(Hw) and the core promoter region in gene loop formation. (**A-D**) 1A2^{m} is the 1A2 insulator with mutated Su(Hw) binding sites; S$^{\times 4}$, four copies of the third Su(Hw) binding site from the *gypsy* insulator; ΔPTE-*yellow*, deletion of the –100 to –69 region from the *yellow* promoter; preve-*yellow*, replacement of the *yellow* core promoter (–63 to +130) with the *eve* core promoter (–65 to +128). For other designations, see Fig. 1.

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; Soshnev 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 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 *lox* 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

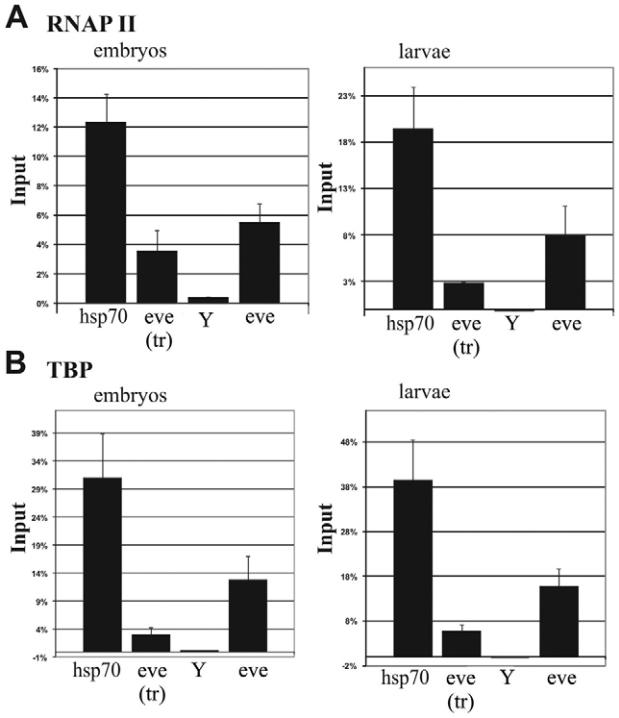

Fig. 3. Binding of RNAP II and TBP to promoters of the *yellow* and *eve* genes at embryonic and larval stages of *Drosophila* development. Results of ChIP with antibodies to (**A**) RNAP II and (**B**) TBP. Diagrams summarize the results of ChIP with specific antibodies followed by real-time PCR (average values of four experiments; error bars indicate s.d.). The ordinate shows the percentage of target sequences in the immunoprecipitated material relative to the input (10% of total cross-linked chromatin), with the genome regions for which DNA enrichment was tested indicated on the abscissa: hsp70, endogenous *Hsp70* promoter; eve (tr), *eve* promoter in the preveYG4(1A2) #12 transgenic line; Y, endogenous *yellow* promoter; eve, endogenous *eve* promoter. Background immunoprecipitation (the average normalized level after chromatin treatment with a nonspecific antibody) was subtracted from normalized specific ChIP signals (obtained with specific antibodies) at each position. The intensity of ChIP signals from the different kinds of material was normalized with respect to the signal on the *Hsp70* promoter.

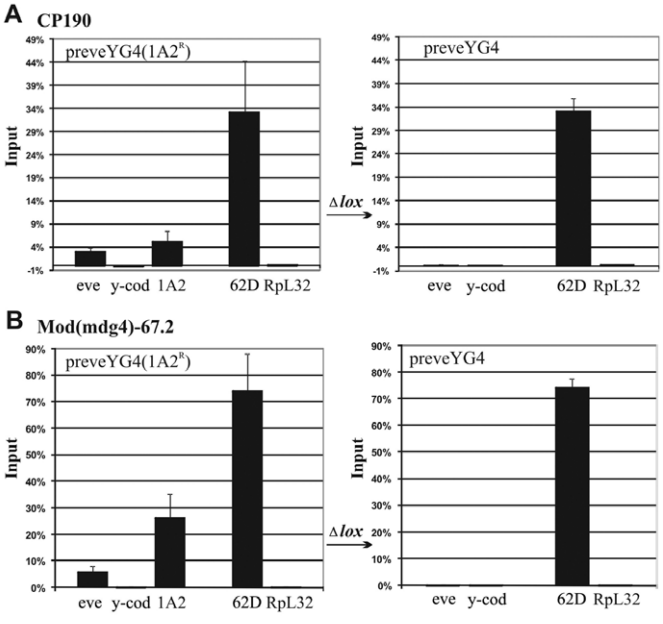

Fig. 4. Demonstration of correlation between the presence of the 1A2 insulator in the transgene and enrichment of insulator proteins at the *eve* promoter by ChIP in *Drosophila* larvae. Diagrams summarize the results of ChIP with antibodies to (**A**) CP190 and (**B**) Mod(mdg4)-67.2 followed by real-time PCR (average values of four experiments; error bars indicate s.d.). ChIP experiments were performed with the material from larvae of the transgenic *yacw*^{1118} line carrying the preveYG4(1A2^{R}) construct in the absence of endogenous 1A2 and the *yellow* gene (homozygote transgenic line #12). The genome regions for which DNA enrichment was tested are indicated on the abscissa: eve, *eve* promoter; y-cod, coding region of the *yellow* gene; 1A2, 1A2 insulator. The region of strongest endogenous Su(Hw) binding (62D), which contains three Su(Hw) binding sites, and the coding region of the *RpL32* gene were used as positive and negative controls, respectively. The data on the right (indicated by the Δ*lox* arrow) were obtained with the derivative transgenic line preveYG4, which carries the construct with a deleted 1A2 insulator. The intensity of ChIP signals from different kinds of material was normalized with respect to the signal on the 62D region. For other designations, see Fig. 3.

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 z^{v77h} 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.

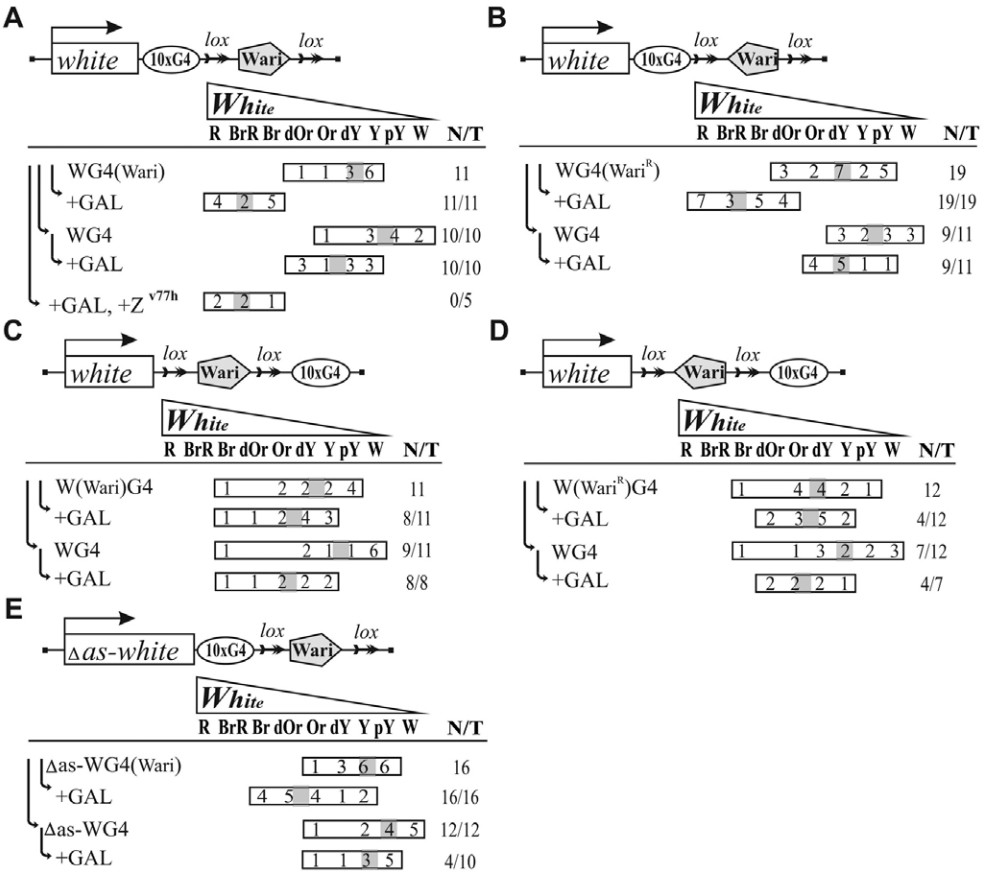

Fig. 5. The Wari insulator functionally interacts with the *white* gene promoter.
(**A-E**) Transgene maps showing the *white* gene (arrow indicates direction of transcription) and Wari insulator (the acute angle indicates its orientation relative to the *white* gene in the genomic position). For *white*, the pigmentation scale ranges from red (R) in wild type, through brownish red (BrR), brown (Br), dark orange (dOr), orange (Or), dark yellow (dY), yellow (Y) and pale yellow (pY) to white (W) in the absence of any expression. '+GAL4, +Z^{v77h}' indicates that eye phenotypes in transgenic lines were examined after induction of GAL4 expression with the null z^{v77h} mutation. For other designations, see Fig. 1.

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 (Gaszner 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 seven GAF (Trl – FlyBase) binding sites required for enhancer blocking (Schweinsberg et al., 2004), and with the Mcp 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 ($CTCF^{\times 4}$), 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 Mcp 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.

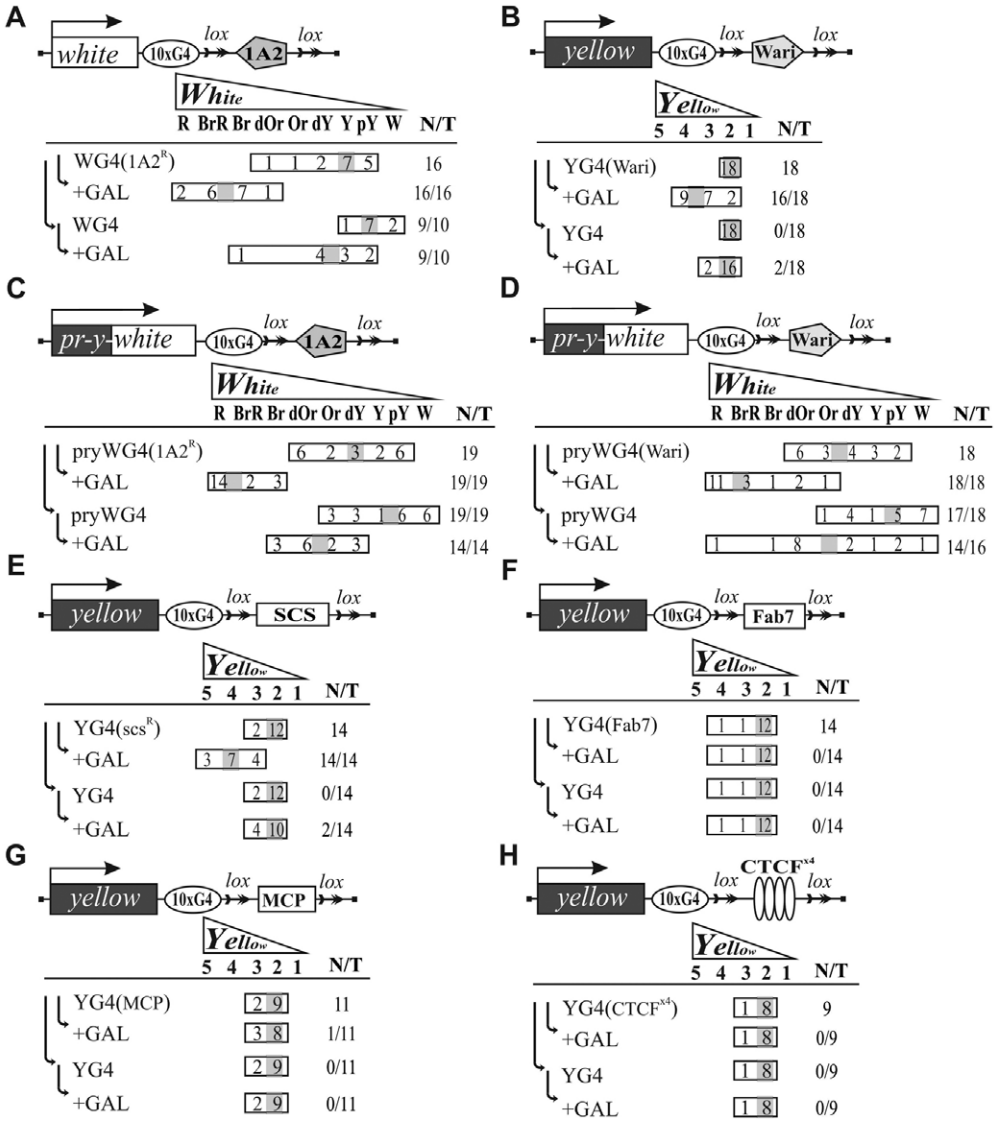

Fig. 6. Testing different *Drosophila* insulators for the ability to facilitate *yellow* stimulation by GAL4. (**A-H**) *pr-y-white* is the *white* gene under the control of the *yellow* gene promoter; scs, the scs insulator; Fab7, the Fab-7 insulator; MCP, the Mcp insulator; $CTCF^{\times 4}$, four CTCF binding sites. For other designations, see Figs 1 and 5.

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

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 1A2 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.

Supplementary material
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Table S1. Primers used for RT-PCR experiments

Primer	Sequence (5′ to 3′)
yellow-RT-forward	TGTGACCCTGATCACCTTGG
yellow-RT-reverse	TCGGGTATTCGGGAAAGCA
white-RT-forward	GCAAATGTCAGCACACGATCAT
white-RT-reverse	GTGGGCTCATCGCAGATCA
Ras64B-RT-forward	GAGGGATTCCTGCTCGTCTTCG
Ras64B-RT-reverse	GTCGCACTTGTTACCCACCATC

Table S2. Primers used for PCR in X-ChIP experiments for DNA fragments from the genome or transgenic constructs

Primer	Sequence (5′ to 3′)
1A2-forward	CTGCAAGCTAGATCCACCTG
1A2-reverse	CTTCGTCTACCGTTGTGC
RpL32-forward	CAGGCCCAAGATCGTGAAGAAGCG
RpL32-reverse	GTTCTCTTGAGAACGCAGGCGACC
62D-forward	TGATACCAGGCGAACAGAAATC
62D-reverse	TTTGGGCTTGGTGAGAACAG
eve-prom-forward	GCTGTCCCGCTCGCACCT
eve-prom-reverse	TGCGTCTTGTGATTCAAAGTTGGC
yell-prom-forward	AAATCATACCAAACCCAGCGAAAGG
yell-prom-reverse	GTGTTCGGGTAATCAGGTGGCTT
y-mid-forward	GTGGCAAAGTGGAACATTTAAAGGC
y-mid-reverse	AGTTTTTCCAATTGAGCCCAGCATT
Hsp70-forward	ACGGCGCACTGTTCTCGTTG
Hsp70-reverse	GCTTGTTCAGCTGCGCTTGTTTG
eve-prom-construct-forward	AAATCATACCAAACCCAGCGAAAGG
eve-prom-construct-reverse	TGCGTCTTGTGATTCAAAGTTGGC