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Characterization of the transvection mediating region of the *Abdominal-B* locus in *Drosophila*

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SUMMARY

Genetic studies have identified an unusual transvection process in the *Abdominal-B (Abd-B)* locus of *Drosophila*. In some cases distal *infraabdominal (iab)* regulatory domains continue to activate the *Abd-B* promoter even when translocated onto different chromosomes. Transvection depends on an approx. 10 kb genomic DNA sequence, termed the *transvection mediating region (tmr*), located immediately downstream of the *Abd-B* transcription unit. Here we report a detailed analysis of this region. Different DNA fragments from the *tmr* were inserted into a variety of *P*-transformation vectors. Analyses of reporter gene expression in transgenic embryos and adults identify at least three cis-regulatory elements, including two enhancers (IAB7 and IAB8) and a new insulator DNA (*Frontabdominal-8, Fab-8*). Evidence is also presented for a *Polycomb* Response Element (*P*RE) linked to the IAB8 enhancer, and an internal promoter in the *iab-8* domain, which transcribes the *iab-7* and *iab-8* cis-regulatory DNA, including the *Fab-8* insulator. We discuss the significance of these findings with regard to *Abd-B* transvection and long-range enhancer-promoter interactions in mammalian *globin* loci.

Key words: Enhancer, Promoter, Insulator, Transvection, *Abd-B*, *tmr*, IAB7, IAB8, *Fab-7*, *Fab-8*, *Drosophila melanogaster*

INTRODUCTION

Enhancers direct stripes, bands and tissue-specific patterns of gene expression in the early *Drosophila* embryo. During the past few years we have become interested in the next tier of gene regulation, namely the regulation of enhancer-promoter interactions within complex genetic loci. Recent studies suggest that insulator DNAs and core promoter elements play an important role in the orderly trafficking of enhancers within complex loci (Foley and Engel, 1992; Li et al., 1994; Ohtsuki et al., 1998; and see reviews by Geyer, 1997; Mihaly et al., 1998a; Udvardy, 1999). Consider a shared enhancer located near three potential target promoters (Fig. 1). In principle, an insulator DNA located between the enhancer and leftward promoter would block the activation of *gene A* without interfering with the expression of *gene B* (Scott and Geyer, 1995; Cai and Levine, 1995). Moreover, recent studies have shown that certain core promoters possess an intrinsic enhancer blocking activity (Ohtsuki and Levine, 1998). The placement of such a promoter at *gene B* would block the activation of *gene C* (see Fig. 1).

To determine whether there are additional cis-regulatory elements that might influence the regulation of enhancer-promoter interactions we have examined the *Drosophila* bithorax complex (BX-C). The BX-C contains more than 300 kb of genomic DNA and genetic studies suggest that the bulk of this DNA corresponds to cis-regulatory information. The BX-C contains only three *Hox* transcription units, *Ultrabithorax (Ubx), abdominal-A (abd-A)* and *Abdominal-B (Abd-B*); each is regulated by 60-80 kb of cis DNA (Lewis et al., 1995; Martin et al., 1995; reviewed by Morata, 1986; Duncan, 1987; Peifer et al., 1987). In the case of the *Abd-B* gene, most of this cis-regulatory DNA is located downstream of the transcription unit.

The 3′ *Abd-B* cis-regulatory region spans approx. 50 kb and contains a series of genetically defined domains, termed *infraabdominal-5 (iab-5), iab-6, iab-7* and *iab-8* (Boulet et al., 1991; Celniker et al., 1990; Sanchez-Herrero, 1991; Fig. 2). Mutations in a given *iab* domain disrupt the morphogenesis of the corresponding abdominal segment. For example, mutations in *iab-5* disrupt abdominal segment 5, or more accurately, parasegment 10, which includes the posterior compartment of the fourth abdominal segment and the anterior compartment of the fifth abdominal segment. Thus far, only three *Abd-B* cis-regulatory elements have been characterized, the IAB5 enhancer, the *Fab-7* insulator and the *iab-7 P*RE (Busturia and Bienz, 1993; Gyurkovics et al., 1990; Galloni et al., 1993; Karch et al., 1994; Zhou et al., 1996; Hagstrom et al., 1996, 1997; Mihaly et al., 1997; summarized in Fig. 2).

It has been suggested that insulator DNAs organize the different *iab* regions into discrete chromatin boundary domains (Galloni et al., 1993; Vazquez et al., 1993; also see Fig. 2). The *Frontabdominal-7* (*Fab-7*) DNA element has been shown to possess an enhancer blocking activity that is similar to those observed for the *scs* and *scs′* chromatin boundary elements of the *hsp70* locus, as well as the Su(Hw) insulator DNA from the *gypsy*

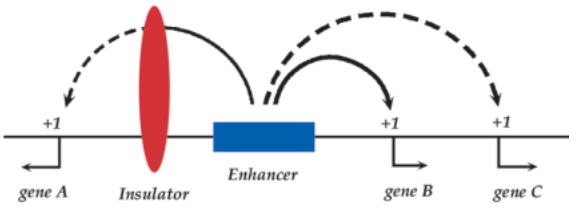

Fig. 1. Summary of enhancer-promoter interactions. The diagram depicts a shared enhancer located near three potential target genes, *A*, *B* and *C*. In principle, an insulator DNA, such as the 340 bp *Su(Hw)* insulator from the *gypsy* retrotransposon, located in the indicated position would block the activation of *gene A*, but would not interfere with enhancer-*gene B* interactions. In addition, the activation of *gene C* can be blocked by promoter competition, whereby the *gene B* promoter is 'stronger' than the *gene A* promoter. Alternatively, the *gene B* promoter region might contain an enhancer blocking activity that would preclude the activation of *gene C*.

retrotransposon (Kellum and Schedl, 1992; Cai and Levine 1995; Scott and Geyer, 1995; Zhou et al., 1996; Hagstrom et al., 1996; Mihaly et al., 1997). A second potential insulator DNA, *Miscadastral pigmentation* (*Mcp*, Lewis, 1978; Karch et al., 1985, 1994), was identified on the basis of chromosomal lesions and DNaseI hypersensitivity, although it is not clear whether it possesses a bona fide enhancer blocking activity in transgenic assays. In the present study we provide evidence for a third insulator DNA in the *Abd-B* 3′ cis regulatory region, *Frontabdominal-8* (*Fab-8*, summarized in Fig. 2).

The presence of one or more insulator DNAs in the *Abd-B* regulatory region poses a potential problem, namely, how do distal enhancers, such as IAB5, overcome the blocking effects of these insulators and interact with the *Abd-B* promoter over long distances (50 kb or more; see Fig. 2)? One hint as to how this might work stems from genetic studies. An unusual transvection phenomenon has been described for *Abd-B*. A genomic DNA sequence of approx. 10 kb, termed the *transvection mediating region (tmr*), permits distal enhancers such as IAB5 to interact with the *Abd-B* promoter even when they are translocated onto different chromosomes (Hendrickson and Sakonju, 1995; Hopmann et al., 1995; Sipos et al., 1998). This transvection process does not depend on *zeste* and is not disrupted by unpairing homologous chromosomes; it is therefore distinct from transvection at other loci such as *Ubx* and *yellow*. The *tmr* is located just downstream of the *Abd-B* transcription unit, and it appears to contain one or more cis-regulatory elements that promote long-range enhancer-promoter interactions.

We have employed a variety of transgenic assays to identify cis-regulatory elements within the *tmr*. Several elements were identified, including two enhancers, IAB7 and IAB8, an insulator DNA (*Fab-8*), a potential *P*RE, and an internal promoter within the *iab-8* domain. We discuss the significance of these findings with regard to *Abd-B* transvection and long-range enhancer-promoter interactions.

MATERIALS AND METHODS

DNA plasmid construction

The 9.5 kb DNA spanning the *tmr* region was cloned as a *Bam*HI-*Hin*dIII fragment from *P1* phage clone #81-16 (DSO7696, L07835; Gary Karpen laboratory, The Salk Institute, La Jolla, CA). The 1.9 kb IAB7 enhancer is a PCR fragment (PII) corresponding to nucleotides (nt) 66,258-68,186 within the DSO7696 sequence of the *Abd-B* gene (Martin et al., 1995; Lewis et al., 1995). Similarly, the 5.3 kb IAB8 enhancer was cloned as a *Bam*HI-*Hin*dIII fragment from nt 59,197-64,580; the 1.7 *Fab-8* DNA corresponds to a PCR fragment from 63,709-65,407. The 0.7 kb IAB7 enhancer is an *Eco*RI-*Sma*I fragment within the 1.9 kb PCR fragment, whereas the

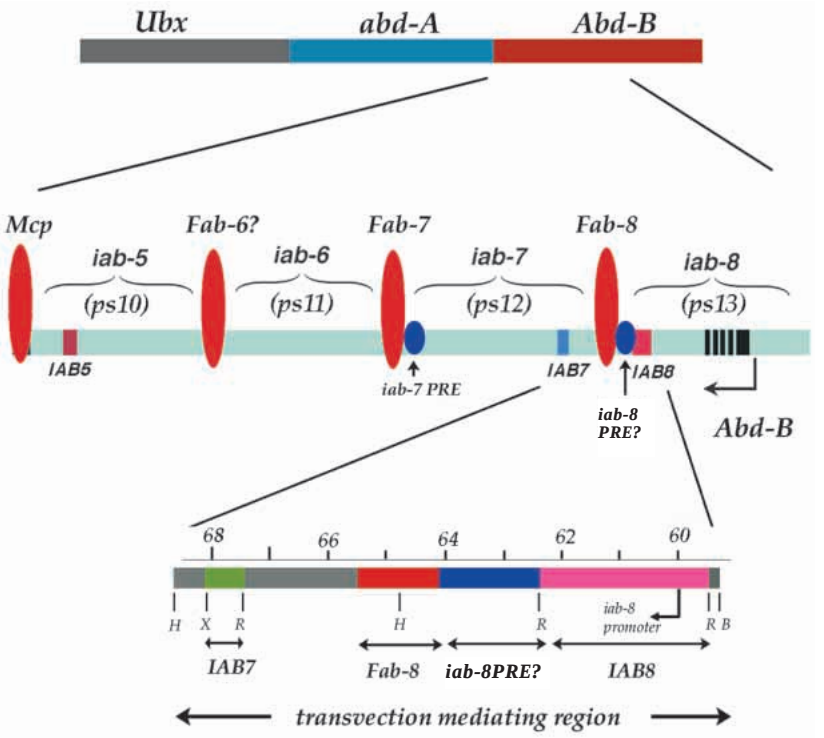

Fig. 2. Summary of the *Abd-B* locus. The bithorax complex is over 300 kb in length and contains three Hox transcription units, *Ubx*, *abd-A* and *Abd-B*. The *Abd-B* locus is over 80 kb in length. The leftward arrow (middle diagram) indicates the transcription start site, and the black rectangles represent exons present in the mature *Abd-B* mRNA. Genetic studies suggest that *iab-8* cis-regulatory elements, which direct expression in parasegment (ps) 13, are located both upstream and downstream of the *Abd-B* transcription unit. The other *iab* regulatory domains that control *Abd-B* expression are also indicated (*iab-5*, *iab-6*, and *iab-7*). These latter domains regulate expression in ps10, ps11 and ps12, respectively. It has been proposed that the 3′ *Abd-B* cis-regulatory DNA contains a series of insulators, which are indicated by the red ovals. Only *Fab-7* has been definitively identified, although there is evidence that *Mcp* also functions as an insulator. This study provides evidence for a *Fab-8* insulator DNA. It is unclear whether *Fab-6* exists. A detailed map of the approx. 10 kb *tmr* is shown at the bottom of the figure. The numbers refer to the DSO7696 sequence of *Abd-B* in kb units. This study provides evidence that the *tmr* also contains two enhancers, IAB7 and IAB8, and an internal promoter in the *iab-8* domain, which is indicated by the leftward arrow near position 60. A potential *iab-8 P*RE is located between position 62 and 64.

2.7 kb IAB8 enhancer was derived from an *Eco*RI fragment within the 5.3 kb DNA.

The *yellow* gene was recovered as a 7.7 kb *Sal*I fragment from a plasmid provided by Gary Struhl (Struhl and Basler, 1993). *Fab-7* and *Fab-8* insulator cassettes were inserted at a *Hin*dIII site 200 bp proximal to the *gypsy* insertion site within the y^2 allele (Geyer et al., 1986). Frt sites are identical to those described by Struhl and Basler (1993). The 5x*glass* enhancer was obtained from the Rubin laboratory (Moses and Rubin, 1991).

The 2xPE-IAB5 *P*-transformation vector used to identify the Fab-8 insulator (Fig. 5) is described in Zhou et al. (1996). Different *tmr* DNAs were cloned into a unique *Bam*HI site located between the 2xPE and IAB5 enhancers.

To generate the construct in Fig. 7E, a 228 bp *Spe*I-*Bam*HI fragment of the *hairy* stripe one enhancer (HI; Zhou et al., 1996) was inserted between the *mini white* and the *transposase*-lacZ promoter of the C4PLZ vector. The 9.5 *tmr* DNA was inserted in the *Bgl*II site at the 3′ end of the *lacZ* gene.

P-transformation, fly strains and in situ hybridization

P-element transformation vectors containing *lacZ* and *white* reporter genes were introduced into the *Drosophila* germline by injecting yw^{67} embryos as described previously (Zhou et al., 1996), with reporter DNA and *PUC hs-π Δ 2-3* at a 10:1 ratio (1 μg/μl) in injection buffer (5 mM KCl, 0.1 mM NaH_2PO_4, pH 6.8, Don Rio laboratory). The Flip recombinase was introduced into transgenic strains by mating *yw P{ry+ hs-Flp};+/+;ry Dr/ry TM3* females with males bearing the indicated transgenes (see Fig. 6).

In situ hybridizations were performed essentially as described (Tautz and Pfeifle, 1989; Jiang et al., 1991), using digoxigenin-UTP-labeled RNA probes. A few modifications to this procedure were introduced. Fixed 2-4 hour embryos were washed in methanol twice, followed by four washes in PBT (PBS plus 0.1% of Tween 80) for 3 minutes each. Embryos were subsequently washed at room temperature with a 1:1 mix of PBT and hybridization buffer (50% formamide, 5× SSC, 100 μg/ml ssDNA, 50 μg/ml heparin and 0.1% Tween 80) for 10 minutes followed by pre-hybridization in the same buffer for at least 1 hour.

Tissue preparations

Adult abdominal cuticles were mounted for light microscopy as described by Duncan (1982). Young adult males (approx. 4 hours) were selected for better visualization of the mosaic pigmentation of eyes and cuticles. Heads were dissected from quick-frozen adults and then immersed in a 1:3 glycerol:ethanol solution. All specimens were mounted on slides with coverslips.

RESULTS

The approx. 10 kb *tmr* is located just downstream of the *Abd-B* transcription unit (Fig. 2). Chromosomal breakpoints within the *tmr* disrupt long-range interactions between *iab-5*, *iab-6* and *iab-7* enhancers and the *Abd-B* promoter (Hopmann et al., 1995). Such disruptions appear to cause a reduction of *Abd-B* function. Cis-regulatory elements were identified within the *tmr* by inserting different DNA fragments into *P*-element transformation vectors containing *lacZ*, *white* and *yellow* reporter genes. The regulatory activities of these DNA fragments were determined by analyzing reporter gene expression in transgenic embryos and adult flies. In situ hybridization was used to analyze reporter gene expression in embryos, while eye color and body color were used to examine reporter gene activity in adults.

Characterization of the IAB7 enhancer

The distal approx. 4 kb region of the *tmr* extends into the *iab-7* domain (Fig. 2; Gyurkovics et al., 1990), which controls the morphogenesis of the tissues that comprise the seventh abdominal segment. Thus far, no enhancers, either embryonic or larval, have been identified in this region. A 1.9 kb PCR fragment, which includes nearly half of the *iab-7* sequences contained within the *tmr*, was inserted into a *P*-transformation

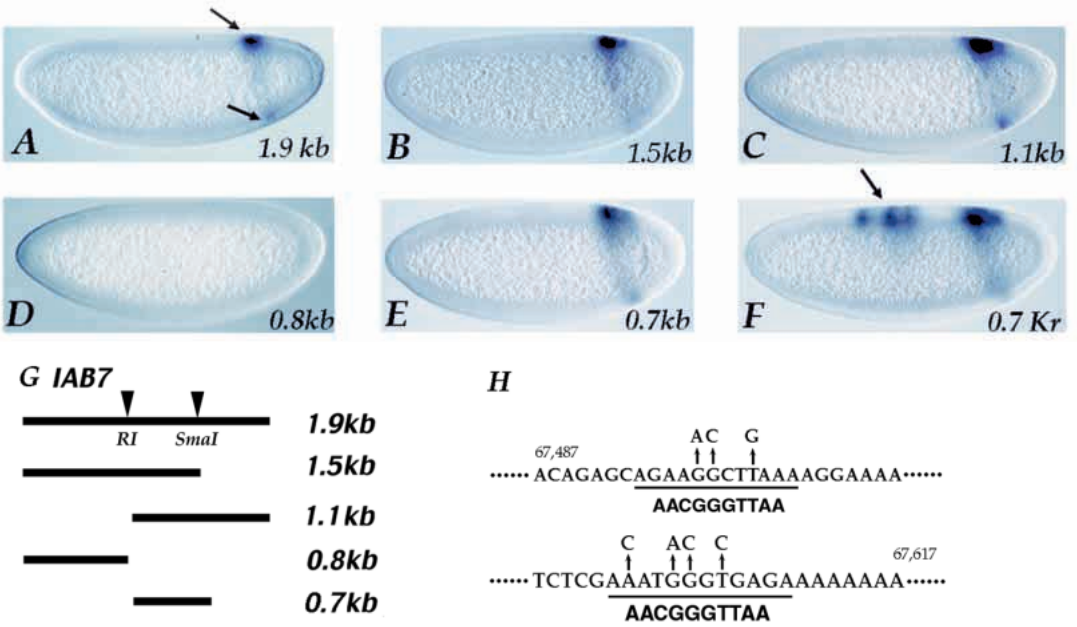

Fig. 3. Characterization of the IAB7 enhancer. The indicated *iab-7* DNA fragments were attached to a *white* reporter gene, and transgenic embryos were hybridized with a digoxigenin-labeled antisense RNA probe. The embryos are oriented with anterior to the left and dorsal up. Most of the embryos are at the midpoint of nuclear cleavage cycle 14. (A) 1.9 kb DNA fragment from the *iab-7* region of the *tmr* directs *white* expression within the limits of a posterior stripe (arrows). Staining is somewhat stronger in dorsal versus ventral regions. (B,C) A similar pattern of *white* expression was obtained with 1.5 kb and 1.1 kb DNA fragments (see summary in G). Again, staining is stronger in dorsal versus ventral regions. The two fragments overlap in a 700 bp region near the middle of the original 1.9 kb fragment. (D) An 800 bp fragment from the distal portion of the 1.9 kb fragment fails to direct *white* expression in transgenic embryos. (E) The 700 bp DNA fragment directs *white* expression in a posterior stripe. (F) Same as E except that the DNA fragment was mutagenized to disrupt two putative Krüppel binding sites (see summary in H). The mutagenized fragment directs an abnormal pattern of *white* expression, whereby staining is detected in central regions along the dorsal surface (arrow). (G) Summary of DNA fragments tested for *iab-7* regulatory activity. (H) Summary of nucleotide substitutions made within the core Krüppel binding sites contained in the minimal 700 bp IAB7 enhancer.

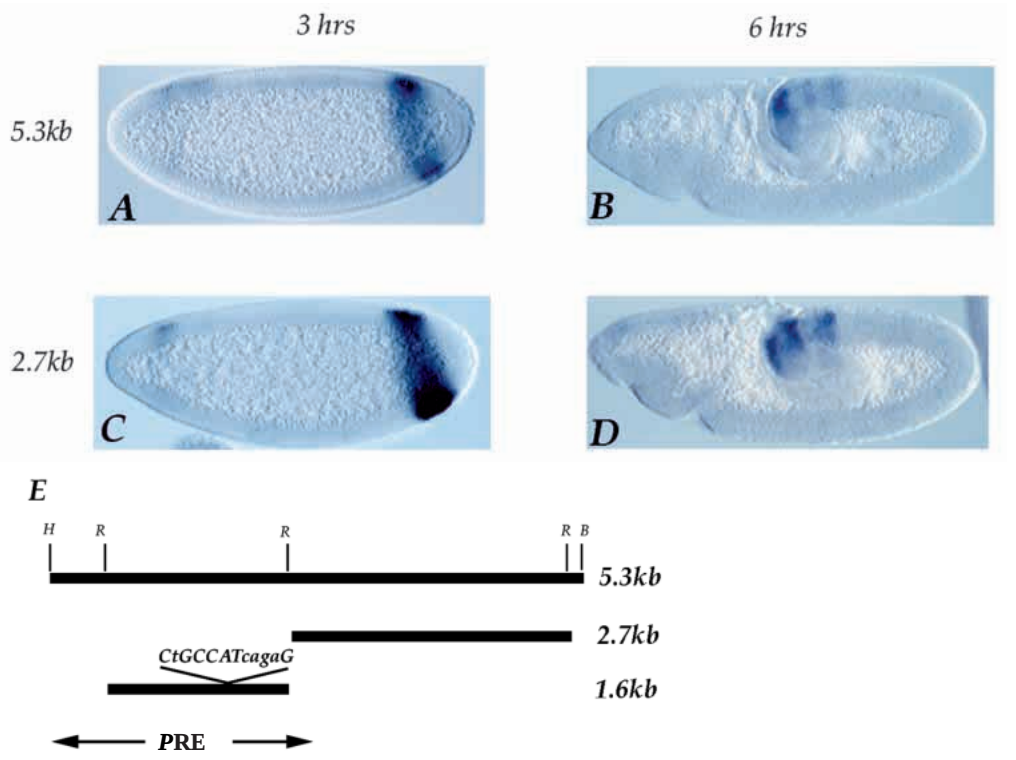

Fig. 4. Identification of an IAB8 enhancer. The indicated DNA fragments (E) from the *iab-8* region of the *tmr* were attached to a *lacZ* reporter gene. Transgenic embryos were hybridizied with a digoxigenin-labeled antisense RNA probe. Embryos are oriented with anterior to the left and dorsal up.
(A) Cellularizing embryo carrying the 5.3 kb DNA fragment. The *lacZ* reporter gene is expressed in a posterior stripe. (B) Same as A except that the embryo has undergone the rapid phase of germ band elongation. *LacZ* expression persists in posterior regions. This pattern persists at later stages of development (data not shown). (C) Cellularized embryo carrying the 2.7 kb DNA fragment. The *lacZ* reporter gene is expressed in a posterior stripe, similar to that observed with the 5.3 kb fragment. (D) Same as C except that the embryo has undergone the rapid phase of germ band elongation. *LacZ* staining persists in posterior regions, but staining is nearly lost in older embryos (data not shown).
(E) Summary of DNA fragments tested for *iab-8* regulatory activity. The 1.6 kb fragment does not activate the *lacZ* reporter gene in transgenic embryos (data not shown). *P*RE, *Polycomb* response element.

vector containing divergently transcribed *white* and *lacZ* reporter genes (see diagrams in Fig. 5). This *iab-7* DNA directs the expression of the *white* reporter gene within the limits of a posterior stripe (Fig. 3A, arrows) that resolves into two stripes in late stage 5 embryos (data not shown). Reporter gene expression was visualized after hybridizing transgenic embryos with a digoxigenin-labeled antisense RNA probe. A similar staining pattern was observed when the *lacZ* reporter gene was monitored via in situ hybridization (data not shown). The early stripes of *white* and *lacZ* expression appear to fall within the limits of parasegments (ps) 12-14 (Martinez-Arias and Lawrence, 1985; Busturia and Bienz, 1993).

Different pieces of the 1.9 kb *iab-7* DNA fragment were analyzed in an effort to identify the minimal IAB7 enhancer (Fig. 3G). A 700 bp fragment retains full activity (Fig. 3E).

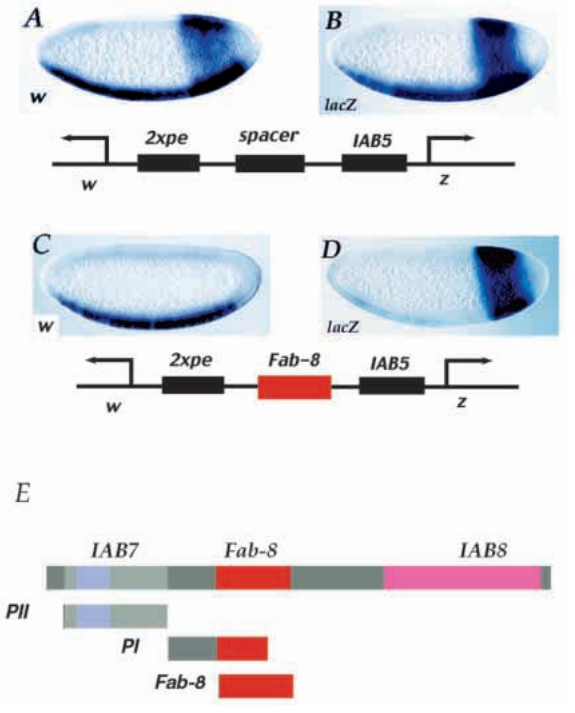

Fig. 5. Identification of an insulator DNA. Transgenic embryos carry the indicated *P*-transposons (see diagrams) and were hybridized with either digoxigenin-labeled *white* (*w*) or *lacZ* antisense RNA probes. Embryos are undergoing cellularization and are oriented with anterior to the left and dorsal up. (A,B) *white* and *lacZ* expression patterns obtained when a 1.6 kb lambda DNA fragment was inserted between the 2xPE and IAB5 enhancers. Both reporter genes exhibit composite patterns of expression, consisting of staining in the ventral mesoderm (2xPE) and abdomen (IAB5). (C,D) *white* and *lacZ* expression patterns obtained when a 1.7 kb '*Fab-8*' DNA fragment was inserted between the 2xPE and IAB5 enhancers. The *white* reporter gene is expressed exclusively in the ventral mesoderm (C), indicating that the IAB5 enhancer is blocked. In contrast, the *lacZ* reporter gene exhibits intense expression in the abdomen, but only weak staining in the ventral mesoderm (D). This pattern suggests that the *Fab-8* DNA fragment attenuates interactions between the distal 2xPE enhancer and *lacZ* promoter. (E) Summary of the *Fab-8* insulator. The location of the insulator is shown relative to the positions of the IAB7 and IAB8 enhancers within the *tmr*. Two other DNA fragments, PII and PI, do not exhibit insulator activity when inserted between the 2xPE and IAB5 enhancers (data not shown).

The 5′ portion of the 1.9 kb fragment lacks regulatory activity (Fig. 3D), and larger fragments containing the minimal 700 bp enhancer direct the same pattern of *white* expression as that observed with the minimal fragment (Fig. 3B,C; compare with E). The IAB7 enhancer directs somewhat stronger expression in dorsal versus ventral regions; a similar asymmetry is observed for the IAB5 enhancer in precellular embryos (e.g. Zhou et al., 1996).

Transcriptional repressors encoded by the gap genes have been shown to be essential for establishing the initial limits of homeotic gene expression (Levine and Harding, 1987). Direct regulatory interactions have been identified for the Krüppel repressor and *iab-2* cis-regulatory elements within the *abd-A* locus (Shimell et al., 1994). Mutations in critical Krüppel binding sites result in an anterior expansion of the normal *abd-A* expression pattern. Genetic studies suggest that Krüppel might also regulate *Abd-B* expression (Harding and Levine, 1988). *Krüppel*$^{-}$ mutants exhibit an ectopic stripe of *Abd-B* expression in central regions of developing embryos, within the presumptive thorax. A computational search of Krüppel recognition sequences throughout the BX-C revealed two closely linked sites within an approx. 130 bp region of the minimal 700 bp IAB7 enhancer (Fig. 3H). Nucleotide substitutions in both core sequences (consensus: AACGGGTTAA) alter the activity of the IAB7 fusion gene in transgenic embryos (Fig. 3F). The *white* reporter gene exhibits ectopic expression in anterior regions (arrow, Fig. 3F) as compared with the normal pattern directed by the wild-type enhancer (Fig. 3E). These results suggest that Krüppel functions as a direct repressor of *Abd-B* expression.

Identification of the IAB8 enhancer

The *tmr* includes approx. 5 kb from the *iab-8* cis-regulatory domain (see summary in Fig. 2, and below). A 5.3 kb *Bam*HI-*Hin*dIII DNA fragment spanning this region was inserted into a *P*-transformation vector containing divergently transcribed *white* and *lacZ* reporter genes (see diagrams in Fig. 5). This DNA directs the expression of both reporter genes within a posterior stripe in transgenic embryos (Fig. 4A). This stripe appears to span parasegments 13 and 14 (Busturia and Bienz, 1993). This expression pattern suggests that the 5.3 kb region belongs to the *iab-8* regulatory domain. Smaller DNA

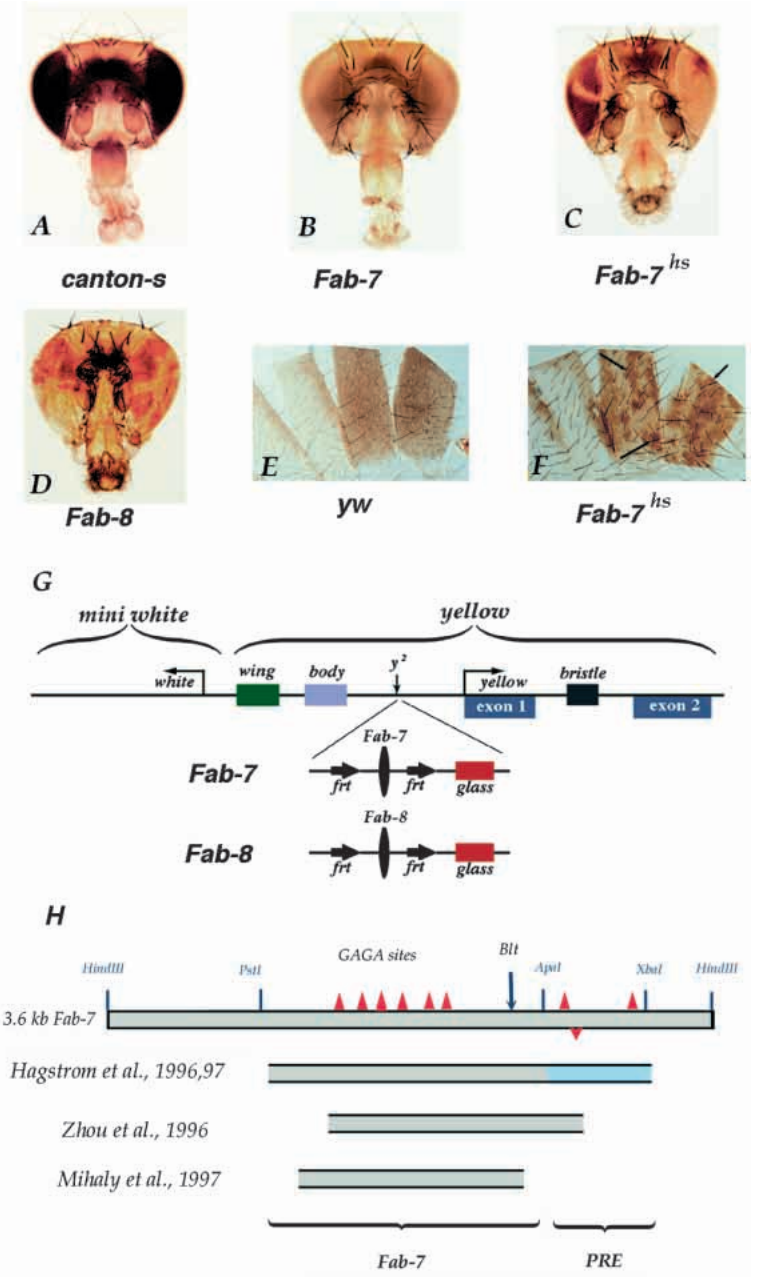

Fig. 6. Insulator activity in adult tissues. The *Fab-7* and *Fab-8* insulators were placed between divergently transcribed *white* and *yellow* marker genes. *yellow* controls pigmentation of the body cuticle, bristles and wing. The insulator DNAs were inserted at a *Hin*dIII site between the distal wing and body enhancers and the *yellow* transcription start site, just next to the insertion site of the *gypsy* retrotransposon in the y^2 mutation (see summary in G). The insulator DNAs were flanked by *frt* sites; five copies of the eye-specific *glass* enhancer were placed in a distal position relative to the *white* reporter gene. (A) Wild-type adult head. The eyes have a bright red pigmentation. (B) Transgenic strain carrying the *Fab-7* insulator DNA positioned between the *white* and *yellow* marker genes. The eyes have a pale yellow, slightly orange pigmentation. (C) Same as B except that the yeast Flip recombinase was randomly expressed via heat shock (see Materials and methods). Mosaic patches of Flip-expressing cells result in the rearrangement of the *frt* cassette so that the *Fab-7* insulator is excised in these cells. The distal *glass* enhancer can now activate *white*, resulting in patches of red pigmented cells in the eyes. (D) Same as C except that the *P*-transposon contains the *Fab-8* insulator DNA. In the absence of the Flip recombinase, the eyes are yellowish. Mosaic expression of the recombinase results in patches of red pigmentation, presumably due to the removal of the *Fab-8* insulator. (E) Portion of the abdominal cuticle from a *yellow*, *white* (*yw*) mutant male. There is a substantial reduction in pigmentation as compared with wild-type flies. (F) Abdominal cuticle from the same transgenic strain as C. The *Fab-7* insulator blocks the interaction of the distal body enhancer with the *yellow* promoter, so that the body cuticle is only slightly pigmented. Mosaic patches of Flip-expressing cells result in clones of pigmented tissue, presumably due to the loss of the *Fab-7* insulator. (G) Summary of the *white*/*yellow* P-transposons. (H) Summary of *Fab-7* insulator DNAs used in previous studies. The experiments shown in C and F involved the use of the *Fab-7* DNA described by Zhou et al. (1996).

fragments were also examined to identify the minimal IAB8 enhancer (Fig. 4E); a distal 1.6 kb fragment failed to direct *lacZ* expression, while the proximal 2.7 kb fragment retains full activity (Fig. 4C).

Both the 5.3 kb, and to a lesser extent, the 2.7 kb DNA fragments continue to direct reporter gene expression in the posterior germ band of stage 10 (Fig. 4B,D) to stage 13 embryos (data not shown). In contrast, the *iab-7* cis-regulatory DNAs direct transient stripes of expression only during early periods of embryogenesis (data not shown). It is therefore conceivable that the *iab-8* region includes a maintenance element, perhaps a *P*RE, which sustains expression during development (reviewed by Hagstrom and Schedl, 1997). Additional evidence for a *P*RE stems from the analysis of eye color in the transgenic strains. Adults carrying the 5.3 kb *iab-8* DNA exhibit strong position effect variegation (PEV) of *mini white* expression (data not shown), which has been shown to be diagnostic for other *P*REs (Chan et al., 1994). The 2.7 kb DNA fragment causes substantially weaker variegation. It is interesting to note that a conserved sequence motif (CRGCCATYDTRG) found in other *P*REs is present in the 5.3 kb, but not the 2.7 kb, *iab-8* DNA (Mihaly et al., 1998b; Brown et al., 1998).

Identification of an insulator DNA

It has been proposed that the different *iab* regulatory regions are organized into separate chromatin loop domains by a series of insulator DNAs (Galloni et al., 1993; Vazquez et al., 1993; see Fig. 2 summary). If so, the most likely location of an insulator within the *tmr* is between the IAB7 and IAB8 enhancers identified in the preceding analyses. Three different DNA fragments spanning this interval, each about 2 kb in length, were tested for insulator activity (summarized in Fig. 5E). The assay involved the use of a *P*-transposon that contains two different enhancers, 2xPE and IAB5, positioned 5′ of divergently transcribed *white* and *lacZ* reporter genes (see diagrams below A,B and C,D in Fig. 5). The 2xPE enhancer corresponds to two tandem copies of the 180 bp proximal enhancer (PE) from the *twist* promoter region, which directs expression in the ventral mesoderm (Jiang et al., 1991; Thisse et al., 1991; Pan et al., 1991). The IAB5 enhancer directs a broad band of expression in the presumptive abdomen (Busturia and Bienz, 1993). When a neutral spacer DNA is inserted between the two enhancers, both *white* and *lacZ* exhibit composite patterns of expression in the mesoderm and abdomen (Fig. 5A,B). Only one of the DNA fragments that was tested in this assay exhibits an insulator activity (Fig. 5C,D). Insertion of this fragment, hereafter called *Fab-8*, in place of the spacer sequence alters both the *white* and *lacZ* staining patterns. The *white* reporter gene is now expressed exclusively in the mesoderm; staining is essentially eliminated in the presumptive abdomen (Fig. 5C). The opposite staining pattern is observed for the *lacZ* reporter gene (Fig. 5D). In this case, expression is detected primarily in the presumptive abdomen, while staining in the mesoderm is markedly reduced. These results suggest that the *Fab-8* DNA functions as an insulator, and selectively blocks the interaction of a distal enhancer with a target promoter. It blocks interactions between IAB5 and *white*, as well as 2xPE and *lacZ*. *Fab-8* is located approximately 2.5 kb 3′ of the IAB7 enhancer and 2 kb 5′ of IAB8 within the *tmr* (see Fig. 2, summary).

Fab-8 and the previously identified *Fab-7* insulator were tested for their ability to block enhancer-promoter interactions in heterologous tissues (Fig. 6). The *P*-transformation vector used for these experiments contains the *white* and *yellow* genes; the latter reporter gene is normally expressed in the body cuticle, wings and bristles of adult flies (summarized in Fig. 6G). The *yellow* promoter region contains separate enhancers that regulate expression in the wings and body cuticle (Geyer and Corces, 1987). Previous studies have

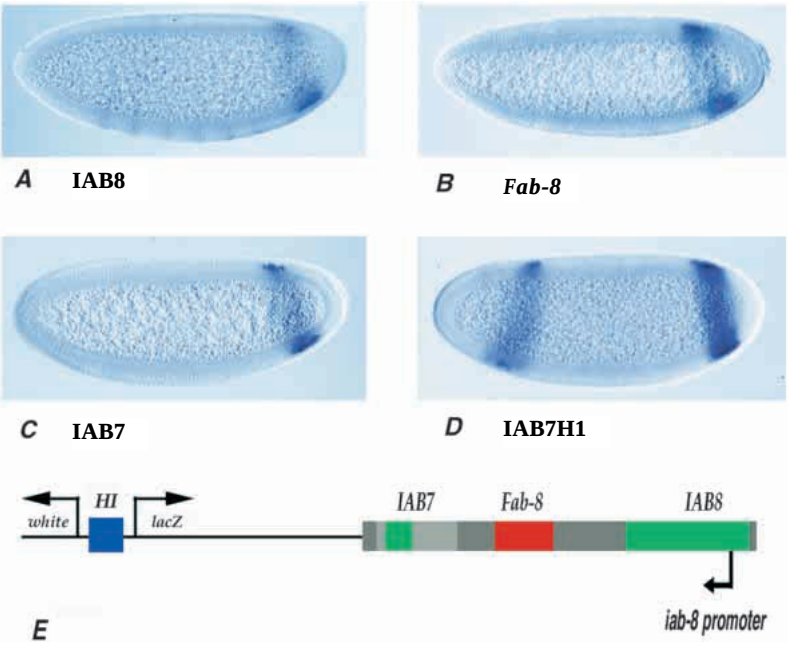

Fig. 7. The *iab-8* domain contains an internal promoter. Cellularized embryos were hybridized with different *Abd-B* cis DNAs and oriented with anterior to the left and dorsal up. (A) Staining pattern obtained after hybridization with the 2.7 kb IAB8 DNA fragment. Transcripts are detected in a posterior stripe. Weaker stripes are also observed in more anterior regions. These latter stripes appear to be in register with the pair-rule *ftz* pattern. The *Abd-B* transcription unit is also expressed in a transient pair-rule pattern in early embryos. (B) Staining pattern obtained after hybridization with the 1.7 kb *Fab-8* insulator DNA. *Fab-8* transcripts are detected in a posterior stripe. This result suggests that the insulator DNA is transcribed. It is not clear whether this transcription influences the enhancer-blocking activity of the insulator. (C) Staining pattern obtained after hybridization with the 700 bp IAB7 enhancer DNA. IAB7 transcripts are detected in a posterior stripe, which is similar to those observed with the IAB8 and *Fab-8* probes. This pattern is distinct from that obtained when the IAB7 enhancer is attached to a reporter gene. (D) IAB7 staining pattern obtained in a transgenic embryo carrying both the approx. 10 kb *tmr* (summarized in E) and the heterologous H1 enhancer from the *hairy* promoter region. The H1 enhancer leads to the ectopic activation of IAB7 transcripts in anterior regions.
(E) Summary of the transgene used in D. The *tmr* was cloned at the 3′ end of the *lacZ* gene. In situ hybridization assays with different *iab-8* DNA fragments suggest that the internal promoter is located in the indicated position, just 3′ of the *Abd-B* transcription unit (data not shown).

identified the y^2 mutation as an insertion of the *gypsy* retrotransposon (Geyer et al., 1986). The *gypsy* insulator specifically blocks the activities of the distal wing and body enhancers, but not the intronic bristle enhancer (see review by Geyer, 1997).

P-transposons were prepared that contain either a *Fab-7* or *Fab-8* cassette (Fig. 6G). Each cassette contains five copies of an eye-specific enhancer from the *glass* gene, and an insulator DNA (*Fab-7* or *Fab-8*) flanked by binding sites (*frt*) for the yeast Flip recombinase. Transgenic flies carrying either insulator cassette possess only yellow to light orange eyes (Fig. 6B, compare with A), presumably due to a block in *glass-white* interactions. These transgenic strains were mated with flies containing the Flip recombinase under the control of the *hsp70* promoter. Heat-induction of the recombinase in first to second instar larvae results in red patches, due to the mosaic removal of the insulator cassette (Fig. 6C,D). These results suggest that the block in *glass-white* interactions (Fig. 6B) is due to the *Fab-7* and *Fab-8* insulators, and not the result of position effects resulting from differing sites of transgene integration. When the insulators are removed by the Flip recombinase, the *glass* enhancer leads to robust expression of the *white* gene. The insulator cassettes also block the activation of the *yellow* gene by the wing and body enhancers but not the intronic bristle enhancer (data not shown). Heat-induced expression of the Flip recombinase results in patches of pigmented body cuticle (Fig. 6F; compare with E), due to the mosaic activation of *yellow* by the distal body enhancer.

The *Fab-7* insulator DNA that was used in the preceding analysis is summarized in Fig. 6H. It is distinct from *Fab-7* sequences examined in other studies, although all three *Fab-7* DNAs contain a cluster of six linked GAGA sites. GAGA binds to a zinc finger protein, Trithorax-like (Trl), and *Trl*-GAGA interactions have been shown to be important for the enhancer blocking activity of the *eve* promoter (Farkas et al., 1994; Bhat et al., 1996; Ohtsuki and Levine, 1998). It is conceivable that GAGA is also important for the insulator activity of *Fab-7*.

Identification of an internal promoter

Previous studies provide evidence that the *Abd-B* 3′ cis-regulatory DNA is transcribed (Sanchez-Herrero and Akam, 1989; Casares and Sanchez-Herrero, 1995). To investigate this issue, different DNA fragments from the *tmr* were used for in situ hybridization assays (Fig. 7). All three cis-regulatory elements, IAB7, *Fab-8* and IAB8, detect specific RNAs (Fig. 7A-C). Staining is observed in a posterior stripe, similar to the pattern directed by the IAB8 enhancer when attached to a reporter gene (see Fig. 4). The use of small DNA fragments spanning the *iab-8* regulatory region suggests that there may be one major internal promoter, located just downstream of the *Abd-B* transcription unit (data not shown; Fig. 7E). This promoter can respond to heterologous enhancers (Fig. 7D). A *P*-transposon was prepared that contains the 9.5 kb *tmr* and the heterologous 200 bp *hairy* stripe 1 (H1) enhancer (Fig. 7E). Transgenic embryos were then hybridized with the IAB7 enhancer as a probe. Normally, the enhancer is expressed in a posterior stripe (Fig. 7C). However, the enhancer is expressed in both a posterior stripe and an anterior band in transgenic embryos (Fig. 7D). The latter pattern is presumably due to activation of the *iab-8* promoter by the H1 enhancer.

DISCUSSION

Previous studies have shown that the *tmr* is important for an unusual transvection process, whereby distal *Abd-B* enhancers continue to activate the *Abd-B* promoter even when translocated onto different chromosomes (Hendrickson and Sakonju, 1995; Hopmann et al., 1995; Sipos et al., 1998). In an effort to determine the basis for this phenomenon, we have attempted to identify all of the cis-regulatory elements within the *tmr*. Several disparate elements were identified, including two embryonic enhancers, IAB7 and IAB8, an insulator DNA (*Fab-8*), an internal promoter, and possibly a *P*RE. Thus, it would appear that the *tmr* contains a densely packed assortment of cis-regulatory elements. Previous studies identified only three elements in the entire 50 kb 3′ regulatory region, the IAB5 enhancer, the *Fab-7* insulator and *iab-7 P*RE (Busturia and Bienz, 1993; Gyurkovics et al., 1990; Galloni et al., 1993; Karch et al., 1994; Zhou et al., 1996; Hagstrom et al., 1996, 1997; Mihaly et al., 1997).

The Abd-B regulatory region contains multiple insulators

The identification of the *Fab-8* insulator is consistent with the previous proposal that each *Abd-B iab* domain may be flanked by insulators (Galloni et al., 1993; Vazquez et al., 1993, see Fig. 2, summary). For example, it now appears that the *iab-7* region is flanked by *Fab-7* and *Fab-8*. Future studies will determine whether *Mcp* functions as a bona fide insulator, and whether there is an insulator, *Fab-6*, located between the *iab-5* and *iab-6* regulatory regions.

It has been proposed that insulator DNAs organize the 3′ *Abd-B* cis regulatory regions into a series of separate chromatin loop domains (Galloni et al., 1993; Vazquez et al., 1993). For example, it is possible that proteins bound to the *Fab-7* and *Fab-8* insulators interact and organize *iab-7* cis-elements within a discrete chromatin loop. Such loops might help account for the *Abd-B* transvection process. Chromatin loops might interact with proteins that recognize the *Abd-B* promoter region, thereby facilitating long-range interactions of distal enhancers with the *Abd-B* promoter.

The *iab-8* internal promoter

Previous studies have shown that cis-regulatory regions of the BX-C are transcribed (Sanchez-Herrero and Akam, 1989; Casares and Sanchez-Herrero, 1995). In the case of the *Abd-B* 3′ cis DNA, it would appear that there is a major internal promoter located just downstream of the *Abd-B* transcription unit (summarized in Fig. 2). The identification of an *iab-8* promoter is reminiscent of the situation seen in mammalian *immunoglobulin* genes, whereby unrearranged, germline genes contain internal promoters that are thought to maintain neighboring intronic enhancers in an open conformation for interactions with appropriate transcriptional regulatory proteins (e.g. Xu and Stavnezer, 1992). It is conceivable that the *iab-8* promoter plays a similar role in *Abd-B* regulation. The maintenance of an open chromatin conformation might help account for *Abd-B* transvection. Perhaps proteins bound to the 3′ region serve as ‘signposts’ for the distal *Abd-B* enhancers.

The *iab-8* promoter might also play a more specific role in ensuring peak expression of *Abd-B* in parasegment 13 (and

perhaps ps 14). Transcription of the 3′ region might inactivate the *Fab-7* and *Fab-8* insulators within parasegment 13 so that all distal regulatory elements can activate the *Abd-B* promoter in this posterior region of the embryo. It is also conceivable that the *iab-8* promoter, as well as other internal promoters in the 3′ region, might direct long-range enhancer-promoter interactions. Perhaps such promoters help 'reel-in' distal enhancers to the *Abd-B* transcription unit.

There are parallels between the organization of the *Abd-B* locus in *Drosophila* and mammalian *globin* loci. Both contain insulator DNAs and exhibit extensive transcription of the cis-regulatory DNA (Chung et al., 1993; Ashe et al., 1997). It has been shown that exogenous DNAs from the *globin* locus, either the *globin* transcription units or the neighboring cis DNAs, physically associate with the endogenous *globin* locus in transfected tissue culture cells. This physical interaction of exogenous DNA with the endogenous gene leads to the activation of the internal promoters in the endogenous locus. There may be a similar long-range physical interaction between distal cis-regulatory elements and the *Abd-B* locus in the transvection phenomenon. Future studies will determine whether the cis-elements identified in this study are sufficient to account for transvection, or whether the *tmr* contains additional elements that facilitate long-range enhancer-promoter interactions.

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