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The promoter targeting sequence facilitates and restricts a distant enhancer to a single promoter in the *Drosophila* embryo

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SUMMARY

Transcriptional enhancers in large gene complexes activate promoters over huge distances, yet little is known about the mechanism of these long-range interactions. We report that the promoter targeting sequence (PTS) from the *Abdominal-B* locus of the *Drosophila* bithorax complex facilitates the activity of a distantly located enhancer in transgenic embryos and that it restricts the enhancer to a single promoter. These functions are heritable in all successive generations. We also show that the PTS functions only when itself and an insulator are located between the enhancer and the promoter. These findings suggest that the PTS may facilitate long-range enhancer-promoter interactions in the endogenous *Abdominal-B* locus. We propose that the PTS establishes a stable chromatin structure between an enhancer and a promoter, which facilitates yet restricts an enhancer to a single promoter.

Key words: *Abdominal-B*, Insulator, PTS, *Fab-8*, *suHw*, IAB5

INTRODUCTION

Large developmentally regulated gene complexes usually contain enhancers that activate their promoters from great distances. However, many enhancer-interacting transcription factors can only stimulate transcription over very short ranges when assayed in yeast (Dorsett, 1999). It is conceivable that more complex organisms adopt additional mechanisms to facilitate long distance enhancer-promoter communications. One such mechanism is suggested by transacting factors Chip and Nipped-B, which by themselves do not activate transcription but facilitate the action of a remote enhancer in the *Drosophila cut* locus (Rollins et al., 1999; Torigoi et al., 2000). A different type of mechanism is provided by specialized *cis*-regulatory elements. The locus control regions (LCRs) found in the *β-globin* locus, the *immunoglobulin* locus, and several other large loci define a class of gene and tissue-specific long range acting elements (Bulger and Groudine, 1999; Fernandez et al., 1998; Grosveld et al., 1993; Li et al., 2001). These elements usually contain cell type-specific transcription regulator interacting sites and confer to transgenes high levels of insertion site-independent gene expression. However, a generic *cis*-regulatory element that does not possess enhancer activity but can facilitate long-range enhancer-promoter communications has not been found.

Recent genetic studies in the homeotic selector gene *Abdominal-B* from the *Drosophila* bithorax complex (BX-C) have identified a *cis*-regulatory region, termed *transvection mediating region* (*tmr*), that may facilitate long-range enhancer-promoter interactions (Hendrickson and Sakonju, 1995; Hopmann et al., 1995; Sipos et al., 1998). *Abd-B* contains an extended 3′ regulatory region that is functionally subdivided into distinct enhancer domains called *infra-abdominal* (*iab*)*-5*, *iab-6*, *iab-7* and *iab-8* by insulators, or boundary elements such as *Frontabdominal* (*Fab*)*-7* and *Fab-8* (Barges et al., 2000; Hagstrom et al., 1996; Karch et al., 1985; Mihaly et al., 1998; Mihaly et al., 1997; Zhou et al., 1999; Zhou et al., 1996) (Fig. 1A,B). Enhancer elements from the 3′ regulatory region can activate the *Abd-B* promoter over the intervening insulators and long distances. These enhancers continue to activate *Abd-B* when the 3′ regulatory region is translocated to different chromosomal locations, or even to a different chromosome (from the third to the Y chromosome) (Hendrickson and Sakonju, 1995; Hopmann et al., 1995; Sipos et al., 1998). This strong regulatory interaction depends on the 9.5 kb *tmr* (Hopmann et al., 1995). These observations suggest that an enhancer-facilitating mechanism exists in *Abd-B*, possibly within the *tmr*. Consequently, a novel *cis* element, the promoter targeting sequence (PTS) (Zhou and Levine, 1999) has been identified from the *tmr*. The PTS has a distinctive anti-insulator activity, allowing an enhancer to activate its promoter over the intervening insulator in transgenic embryos. In the presence of an insulator, the PTS also appears to have a promoter targeting activity, allowing an enhancer to activate only one promoter when two are present in the transgene. We report that the PTS facilitates long-range enhancer-promoter interactions in transgenic embryos, and that it mediates the promoter targeting function by restricting enhancer activities to a single promoter. We also show that the PTS functions only when itself and an insulator are located between an enhancer and a promoter.

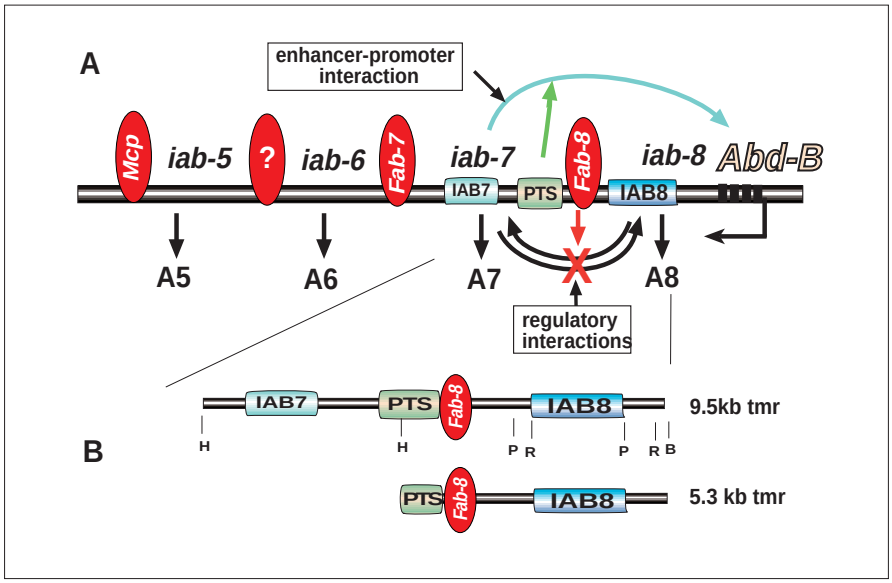

Fig. 1. *Cis* interactions in *Abd-B*. (A) *Abd-B* has an extended 5′ regulatory region that can be subdivided into four regulatory domains, termed *infra-abdominal* (*iab*)*-5*, *iab-6*, *iab-7* and *iab-8*. Each of these controls the development of a corresponding abdominal parasegment (PS). For example, *iab-5* regulates *Abd-B* function in PS10 (roughly the fifth abdominal segment, or A5), *iab-6* controls A6, and so on. Neighboring *iab* genes are separated by domain boundary elements such as *Fab-7* and *Fab-8*, which function to prevent cross-regulatory interference between neighboring *iab* genes. These elements are also potent insulators that can block enhancer-promoter interactions in transgenic constructs. The PTS element has an anti-insulator activity that allows an enhancer to activate a promoter despite an intervening insulator. In the *Abd-B* locus, PTS may mediate enhancer-promoter interactions by allowing enhancers to overcome the intervening *Fab* elements and activates *Abd-B* promoter. (B) Detailed map of the 9.5 and 5.3 kb *tmr* elements. B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; and R, *Eco*RI.

MATERIALS AND METHODS

Plasmid constructions

To generate *P*-transgene shown in Fig. 2A,B, the 1.6 kb IAB8 enhancer was inserted either at a *Bam*HI site upstream of or a *Pst*I site downstream of the *Transponsase* (*Tp*)-*lacZ* gene of the C4PLZ vector (Zhou et al., 1996). Construct #17 and #28 were generated by inserting the 5.3 kb *Bam*HI-*Hin*dIII fragment of the *tmr* region at the *Bgl*II site, 3′ of *lacZ*. The forward orientation (IAB8 proximal to *lacZ* gave rise to #17, whereas the reverse orientation (IAB8 distal to *lacZ*) generated #28. Transgene W14 was made similar to the construct in Fig. 5A of Zhou and Levine (Zhou and Levine, 1999), except a 290 bp 5′ DNA sequence was used instead of the 625 bp PTS. To build construct W32, a 2 kb *Hin*dIII and *Pst*I fragment (Fig. 1B) from the *tmr* containing 290 bp PTS, 590 bp *Fab-8* and additional 1.2 kb 5′ DNA was cloned into the *Bam*HI site between two FRT sites previously inserted into the *Bam*HI site of a *pBluescript* SK+ that lacks the *Bgl*II site. In parallel, a 2.7 kb *Eco*RI fragment (Fig. 1B) from the *tmr* that contains the 1.6 kb IAB8 enhancer was inserted into the *Pst*I site of the C4PLZ vector. The FRT flanked *Pst*I-*Hin*dIII fragment was then excised as a *Bgl*II fragment and inserted into the *Bgl*II site of the IAB8-containing C4PLZ vector. To make W78 and W79, the 1 kb IAB5 enhancer is first inserted into the *eve-lacZ* 3′ located *Pst*I site of –42 *eve* Casper (Zhou et al., 1996). The *eve* promoter was then replaced by *Tp* promoter. A *Xba*I site located between *Pst*I and the 3′ of *lacZ* was converted into a *Not*I site creating *TpCasperNIAB5* (*TpCasperN* that lacks IAB5 was also made, see later). In the meantime, the 340 bp *Spe*I-*Bam*HI *suHw* insulator and the *Bgl*II fragment of FRT flanked 625 bp PTS were sequentially inserted into *Spe*I-*Bam*HI and *Bam*HI sites of a modified pBluescript that contains an additional *Not*I site converted from the *Kpn*I site. The *Not*I fragment containing FRT sites, PTS, and *suHw* was inserted into the *Not*I site of *TpCasperNIAB5*. W81 and W82 were generated similarly, except IAB5 was included in the *Not*I fragment and inserted into the *TpCasperN* vector.

P-element transformation 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 (Rubin and Spradling, 1982). Between 30 and 60 independent transformants were obtained for each of the recombinant P-element shown. In situ hybridization was performed essentially as described in previous reports (Tautz and Pfeifle, 1989; Zhou et al., 1999).

Fly strains and crosses

Transgenic flies expressing the Flip recombinase were kindly provided by Gary Struhl and Steve Small (Wu et al., 1998). To recombine different FRT-flanked DNA element away from the transgene, females carrying the transgene were mated with males that express the Flp recombinase under the control of a sperm-specific *tubulin* promoter (Wu et al., 1998). In F_1 males, the recombinase binds the FRT sites and deletes the intervening DNA. These male flies were collected and mated to *yw* virgin females to establish stocks that are subsequently analyzed by RNA in situ hybridization.

Quantitative analysis of the activity of PTS

Embryos from different transgenic strains were collected and stained with anti *white* or *lacZ* RNA probes in parallel. Enhancer strength was quantified by measuring the differential absorption of transmitted light (ΔEV) between spot *a* (unstained region) and *b* (stained region) in the embryos in Fig. 5A using a digital spot light meter (Sekonic L608). The reading reflects the relative intensity ($2^{\Delta EV}$–1) of the staining that is linear with staining reaction time during a 1 hour incubation (see Fig. 5B). It is assumed that alkaline phosphatase (AP) activity represents enhancer strength, which can be expressed as: enhancer strength=constant × ($2^{\Delta EV}$–1)/time. To compare enhancer strength, heterozygous embryos from different samples are fixed and stained in parallel for 45 minutes. The ΔEV values of approximately 30 embryos are measured. Enhancer strengths are plotted as bar graphs in Fig. 5C,D.

RESULTS

The 5.3 kb *tmr* exhibits a directional enhancer activity

Previous studies have showed that the *Fab-8* insulator could block enhancers (including IAB8) from activating a promoter

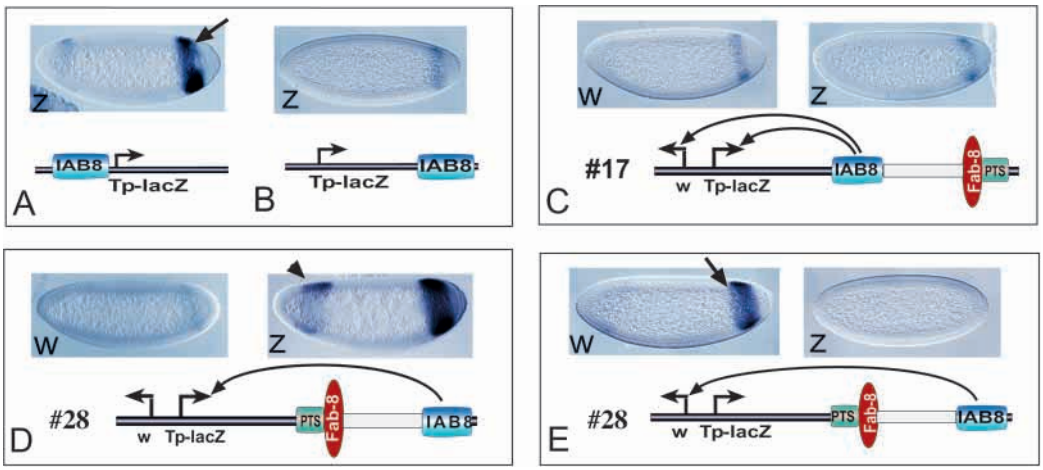

Fig. 2. The 5.3 kb *transvection mediating region* (*tmr*) exhibits orientation-dependent promoter targeting activity. Whole-mount RNA in situ hybridization was performed on 2- to 4-hour-old embryos using anti *white* or *lacZ* RNA probes. All embryos are oriented anterior towards the left and dorsal side upwards. Arrows in the posterior regions of the embryos indicate IAB8 activity. (A) IAB8 activates *Tp-Z* when located 5′ proximal to the *Tp* (*Transposase*) promoter. (B) IAB8 activates *Tp-lacZ* from a 3′ position, 4.5 kb away from the *Tp* promoter. (C) The 5.3 kb *Bam*HI-*Hin*dIII *tmr* fragment was inserted just 3′ end of the *lacZ* gene in a C4PLZ vector in the forwards (5′-3′) orientation. It consists of 290 bp 5′ of the 625 bp PTS, the entire 580 bp *Fab-8* insulator, the IAB8 enhancer, a *Polycomb* response element (PRE) located between *Fab-8* and IAB8 (Barges et al., 2000; Zhou et al., 1999), and about 500 bp of additional genomic sequence 3′ to the 1.6 kb IAB8. In this orientation, IAB8 is proximally located and activates both the divergently transcribed *white* (*w*) and the *Tp-lacZ* fusion gene. (D) In the reverse (3′→5′) orientation, IAB8 can direct strong transcriptional activation of either *w* or *lacZ*. In two out of 12 lines examined, IAB8 activates only *lacZ*. Many of these intensely stained embryos also exhibit anterior staining (arrowhead). This is probably caused by the lack of repressor binding sites in the transgenic regulatory regions that normally repress *Abd-B* enhancer activity in the anterior region of the embryo. (E) In five of these strains, IAB8 activates only *w*. In the remaining lines, the intervening *Fab-8* insulator (data not shown) presumably blocks the IAB8 enhancer.

when it is interposed between the enhancer and its promoter (Zhou et al., 1999; Zhou and Levine, 1999; Barges et al., 2000). A recent study has also demonstrated that the 625 bp PTS element is able to overcome the enhancer blocking effect of the *Fab-8* insulator (Zhou and Levine, 1999). We tested whether the minimal 290 bp 5′ DNA from the 625 bp PTS could overcome the *Fab-8* insulator, and, in addition, facilitate the IAB8 enhancer activity. We studied a 5.3 kb *Bam*HI-*Hin*dIII fragment from the *tmr* that contains the minimal 290 bp of the PTS, the 580 bp *Fab-8* insulator and the 1.6 kb IAB8 enhancer (Zhou et al., 1999; Zhou and Levine, 1999) (see Fig. 1B).

The IAB8 enhancer directs a narrow band of transcription in the posterior region of the embryo (see arrow in Fig. 2A). Similar to other early *Drosophila* enhancers that have been tested (Ohtsuki et al., 1998; Zhou et al., 1996), its activity attenuates as its distance from the promoter increases (compare Fig. 2A with 2B). This 5.3 kb *tmr* was placed in both the forward (5′→3′ when IAB8 is between the promoters and *Fab-8*/PTS, see construct #17 in Fig. 2) and the reverse orientation (construct #28 in Fig. 2). To monitor transcriptional activity of the IAB8 enhancer, embryos from individual transgenic strains were collected and subjected to whole-mount RNA in situ hybridization for the *white* (*w*) or *lacZ* genes.

In the forward orientation, IAB8 weakly stimulated both the divergently transcribed *w* and *Tp-lacZ* genes (Fig. 2C; Table 1). Its activity on the *Tp* promoter is very similar to that of the 1.6 kb IAB8 alone at the same location (Fig. 2B), suggesting that the distally located PTS and *Fab-8* do not affect the communication between IAB8 and the transgenic promoters. In the reverse orientation, however, IAB8 activates the transgenic promoters despite the intervening *Fab-8* insulator. In any particular strain, IAB8 exhibits the selective activation of either the *Tp-lacZ* (Fig. 2D) or the *w* (Fig. 2E) gene. This effect is seen in about 50% of the transgenic lines (Table 1). In the remaining strains, IAB8 does not activate any of the transgenic promoters due to the enhancer blocking effect of the *Fab-8* insulator (data not shown). These results are similar to that of the previous study (Zhou and Levine, 1999), suggesting that the 290 bp PTS exhibits the anti-insulator and promoter targeting activities. This is confirmed by transgene W14 (Table 1), where PTS could overcome a heterologous *suHw* insulator and target IAB8 to the *w* or *Tp* promoters. It should be noted that in the forward orientation (Fig. 2C), IAB8 is located 5.5 kb away from the *w* promoter and 4.9 kb away from the *lacZ* promoter, whereas in the reverse orientation (Fig. 2D,E), it is 8.2 kb away from *w* and 7.5 kb away from *lacZ*. Rather than reducing the activity, the greater distance between IAB8 and the promoters caused by inserting *Fab-8* and PTS resulted in an increase of IAB8 enhancer activity. This is seen by comparing the activities of IAB8 on *Tp-lacZ* in Fig. 2B versus 2D, or *w* in Fig. 2C versus 2E. In either case, the enhancer activity is much stronger when both PTS and *Fab-8* are located between IAB8 and the promoters. These results indicate that PTS may facilitate the long distance interactions between IAB8 and the *w* or the *Tp* promoter.

The PTS, in combination with an insulator, facilitates a distant enhancer and restricts the transcription activation to only one of the two available promoters

To confirm the enhancer facilitating activity and to eliminate position effects due to differential chromosomal insertion sites of the transgene, we used the Flp-FRT system, which causes the removal of FRT-flanked DNA sequences from the transgene after introducing the Flp recombinase by genetic cross (Golic and Lindquist, 1989). This technique permits the analysis of the transgene in the same chromosomal context before and after the test DNA is removed. We flanked a 2.0 kb *Pst*I-*Hin*dIII fragment from the *tmr* region (see Fig. 1B) that contains both the minimal 290 bp PTS and the 580 bp *Fab-8* insulator with the FRT sites and placed this group of elements between the

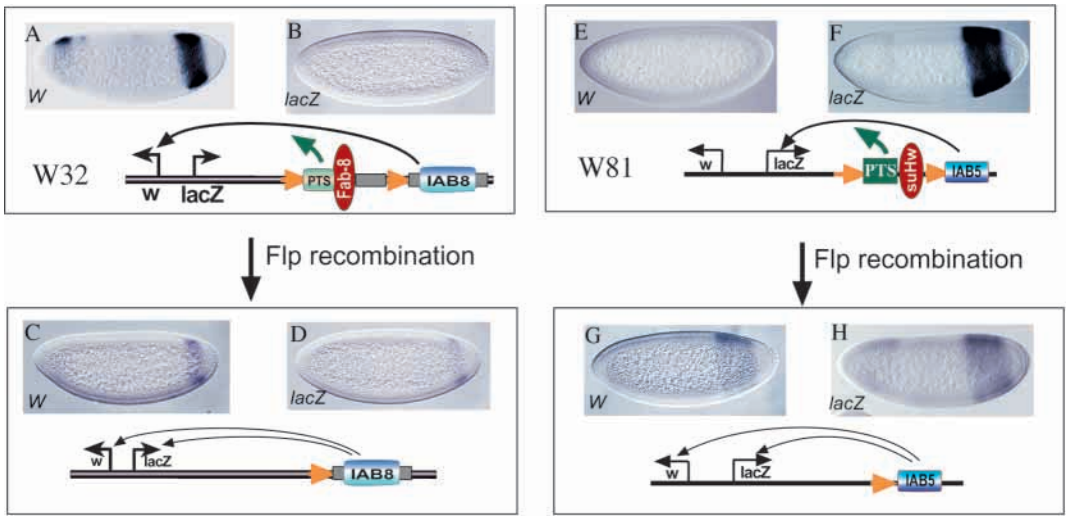

Fig. 3. PTS facilitates and restricts enhancer activity to a single promoter. Constructs W32 and W81 were tested. The former contains an FRT flanked PTS and *Fab-8*, and the IAB8 enhancer, where as the latter contains FRT flanked PTS and *suHw*, plus a 1.0 kb IAB5 enhancer. Large orange arrowheads indicate FRT sites. (A,B) A representative line exhibiting a selective IAB8-*w* interaction is shown. (C,D) The same strain after removal of the *Fab-8* insulator and the PTS by Flp-mediated recombination (Golic and Lindquist, 1989). There is a severe reduction of IAB8-*w* interaction with the accompanying activation of *lacZ*. (E,F) Most transgenic strains carrying the W81 display an IAB5-*lacZ* interaction as shown here. (G,H) The same strain after Flp-FRT analysis. Note the reduction of IAB5-*lacZ* interaction and the concomitant appearance of IAB5-*w* interaction.

Table 1. Analysis of the functions of the PTS in transgenic embryos

Construct		Number of lines	W+++Z–	W–Z+++	W+Z+	W–Z–
17	w lacZ IAB8 Fab-8 PTS	11	0	0	10	1
28	w lacZ PTS Fab-8 IAB8	12	5	2	0	5
W14	w lacZ suHw PTS IAB8	26	3	6	0	17
W32	w lacZ PTS Fab-8 IAB8	14	4	2	0	6
W81	w lacZ PTS suHw IAB5	38	2	12	0	24
W78	w lacZ PTS suHw IAB5	23	4	2	0	17
W79	w lacZ suHw PTS IAB5	36	1	8	0	27
W82	w lacZ IAB5 PTS suHw	42	0	0	42	0

W+++Z–, transgenic strains showing intense *w* activation but no *lacZ* expression; W–Z+++, transgenic lines exhibiting no *w* transcription but very strong *lacZ* transcription; W+Z+, strains displaying weak to robust transcription of both genes; W–Z–, no transcription activity from either promoters.

3′ end of *lacZ* and 2.7 kb *Eco*RI fragment from the same region (Fig. 1B) that contains the 1.6 kb IAB8 enhancer (W32 in Fig. 3). When transgenic embryos were analyzed, results similar to those without FRT sites were obtained. In about half of the transgenic strains IAB8 selectively activates one of the two divergently transcribed *w* and the *Tp-lacZ* genes (Table 1). In the remaining strains, IAB8 does not activate any of the transgenic promoters. An example of a PTS-mediated IAB8-*w* interaction is shown in Fig. 3A,B. Here, IAB8 strongly activates *w* but not the closely positioned *Tp* promoter (compare Fig. 3A with 3B). However, after the removal of PTS and *Fab-8* by Flp-mediated recombination, the intense and selective IAB8-*w* interaction disappeared. Instead, IAB8 activates both *w* and *Tp-lacZ*, but with greatly reduced activity, despite the fact that the enhancer is now 2.0 kb closer to the promoters (Fig. 3C,D). Similar result was obtained when we place the *Fab-8* and PTS elements between the 3′ end of *lacZ* and the heterologous Neural Ectoderm Enhancer (NEE) from the *rhomboid* gene (Ip et al., 1992) (data not shown). These results strongly suggest that the PTS, in combination of the *Fab-8* insulator, facilitates enhancer-promoter interaction, and that it restricts the enhancer activity to a single promoter.

To confirm that the enhancer-facilitating and single promoter activating effects were due to the PTS but not a possible synergy between DNA sequences located within the *tmr*, we tested the PTS in the absence of *Fab-8* and other *tmr* sequences.

The PTS, the heterologous *suHw* insulator (Cai and Levine, 1995; Dorsett, 1993; Geyer and Corces, 1992), and the IAB5 enhancer (Busturia and Bienz, 1993) were placed at the 3′ of *lacZ* in the order given (see construct W81 under Fig. 3E,F). PTS and *suHw* were flanked by a direct repeat of FRT sites so that both elements could be removed by recombination. In about one third of all transgenic strains, IAB5 selectively activated only one of the two (*w* or *Tp*) promoters (see Fig. 3E,F for selective IAB5-*lacZ* interaction), suggesting that the PTS mediates promoter targeting in these transgenic lines (Table 1). The activities of IAB5 on the targeted promoters are consistently strong, with slight variations among different strains (Fig. 5D). In the remaining lines, IAB5 does not activate either of the promoters. Presumably, PTS does not function in these lines, and the IAB5 enhancer is blocked by the intervening *suHw* insulator. The enhancer-facilitating activity was confirmed by Flp-FRT analysis (Fig. 3G,H). Similar to Fig. 3A-D, the simultaneous removal of both PTS and *suHw* dramatically reduced IAB5-*lacZ* interaction (over 10-fold reduction, see Fig. 5D). By contrast, IAB5-*w* interaction, which was undetectable before the recombination, could now be detected (compare *w* activity in Fig. 3G with 3E).

To confirm that the PTS could indeed help IAB5 overcome the *suHw* insulator, we also constructed transgenes W78 and W79 that are similar to W81, but contain an FRT-flanked PTS (Table 1). In the former, PTS was placed between the 3′ end of *lacZ* and the *suHw* insulator, while in the latter, PTS was interposed between *suHw* and IAB5. Transgenic embryos carrying either of these constructs exhibit a selective activation of *w* or *lacZ* in any given strain (see Fig. 4A,B for selective activation of *lacZ* by IAB5 in transgene W78). Removal of the PTS by Flp-mediated recombination caused the loss of IAB5 activated transcription, as IAB5 became blocked by the *suHw* insulator (compare Fig. 4B with 4D). These experiments indicate that the PTS exhibits the anti-insulator and promoter targeting activities when it is either upstream or downstream of an insulator.

In summary, these results clearly demonstrate that the enhancer facilitating function depends on the PTS element, not other unknown elements located within the *tmr*. This result also indicates that the promoter targeting activity is not a result of random positional effect, or preferential insertion of the transgene into specific chromosomal locations that may silence one of the promoters present in the transgenic vector. It is due to a PTS-dependent, active restriction of the enhancer activity to only one of the two available promoters (Fig. 3G,H). The enhancer-facilitating and single promoter activating effects are genetically stable in that they are memorized in up to 60 generations without a loss or change of promoter targeting.

Quantitative analysis of the enhancer facilitating and single promoter activating effects

We also conducted semi-quantitative analyses of PTS-mediated enhancer facilitating and single promoter activating activities by quantifying RNA in situ hybridization. The staining intensity (as measured by optical absorption of stained *Drosophila* embryos, Fig. 5A) shows linear relationship with staining time 60 minutes after the addition of substrates for alkaline phosphatase (AP) (Fig. 5B). Assuming that the AP activity directly reflects enhancer strength and that the AP activity remains constant during an 1 hour incubation, we can compare the activity of the same enhancer in different transgenes if the embryos are fixed and stained in parallel. Using this method, we found that IAB8 is two- to fivefold stronger when it is located less than 100 bp from the *Tp* promoter than when located 5.5 kb downstream of *Tp-lacZ* (Fig. 2A,B, and Fig. 5C). We then compared IAB8 and IAB5 activities with or without facilitation by the PTS. We found that PTS facilitates IAB8 about eightfold and IAB5 ten- to 17-fold, respectively (Fig. 5C,D). In transgenic lines (W81) showing specific IAB5-*lacZ* interaction, no *w* expression could be detected, but after the Flp-mediated removal of PTS and *suHw*, *w* became activated by IAB5 (Fig. 5D). In two of the four lines (W81-2 and W81-4) *w* expression was detectable within 45 minutes, whereas in the remaining two (W81-1 and W81-3), *w* expression could be detected only after extended incubation (~2 hours, data not shown). The strength of IAB5-*w* or IAB5-*lacZ* interactions after the removal of *suHw*/PTS is similar to that of IAB5 alone originally cloned at the same location (data not shown). These results provided quantitative evidence for the enhancer-facilitating and single promoter-activating activities of the PTS.

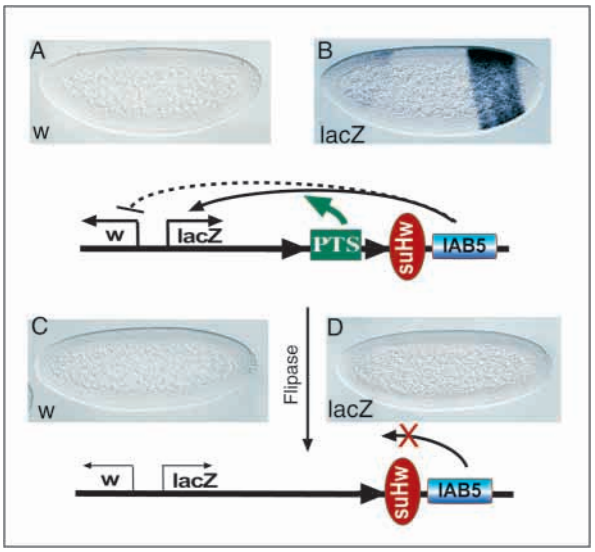

Fig. 4. Anti-insulator and promoter targeting by the PTS element. (A,B) Transgenic embryos carrying construct W78. The IAB5 enhancer selectively activates the *lacZ* but not the *w*. (C,D) The same transgenic strain after the removal of the PTS by Flip-mediated recombination. Notice that the IAB5-*lacZ* interaction is blocked by the *suHw* insulator. Arrowheads indicate FRT sites.

The PTS and an insulator must be located between an enhancer and a promoter

The results shown in Fig. 2 also suggest that PTS must be interposed between an enhancer and a promoter. To test whether this is true, the PTS and *suHw* DNA were placed distal to the IAB5 enhancer, downstream of *lacZ* (W82 in Table 1). From over 40 transgenic strains isolated and examined, none displayed enhancer facilitating and the distinctive single promoter-activating effects (Table 1). In these lines, IAB5 activated both the *w* and *Tp* promoters, with activities similar to that of IAB5 alone in the same location (data not shown). This result, together with the data from Fig. 2 suggests that PTS activity is location dependent, in that it only functions

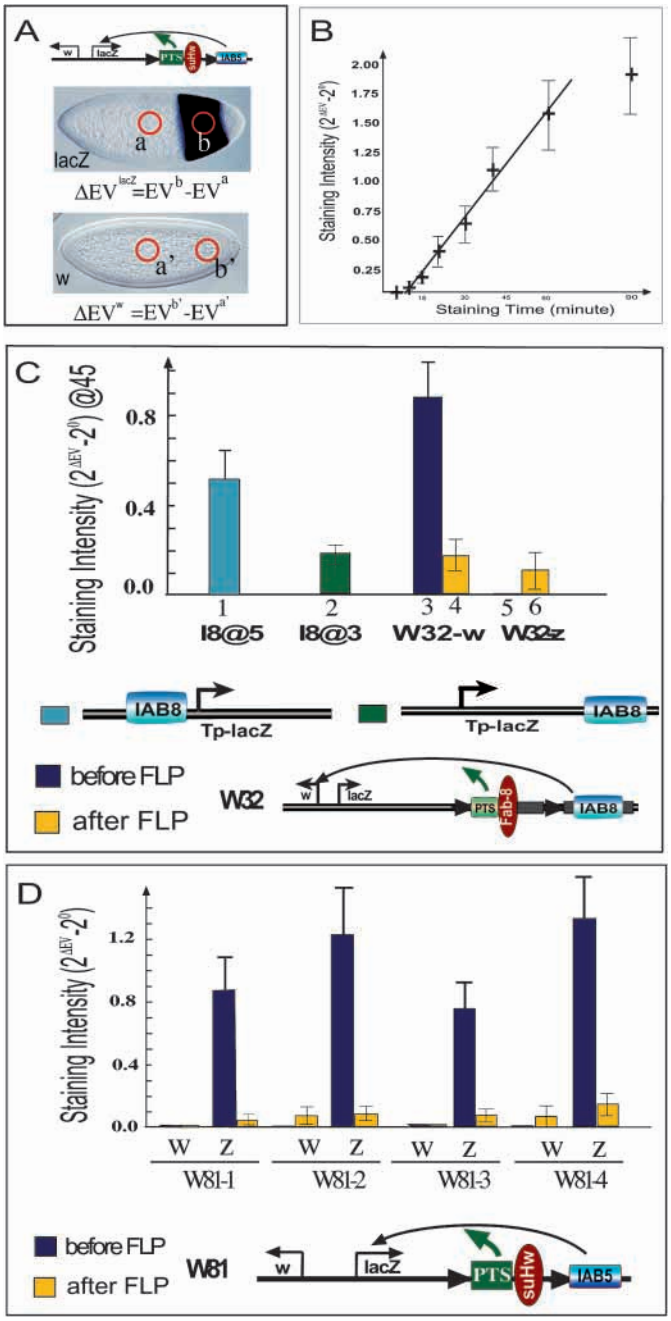

Fig. 5. Quantitative analysis of the activity of PTS. (A) Representative embryos showing IAB5-*lacZ* promoter targeting. Red circles indicate the areas measured for relative staining intensity. (B) The graph displays staining intensity as a function of time. We detected almost no staining in the first 7 minutes after adding the substrate NBT/BCIP. This may be due to the time needed for the substrate to diffuse into the embryos. Between 10 and 60 minutes, staining intensity ($2^{\Delta EV}$–1) was linear with staining time. (C) To compare IAB8 enhancer strength in different P-elements, heterozygous embryos were fixed and stained in parallel for 45 minutes. Approximately 30 embryos were measured. Lanes 1 and 2 show the staining intensities for the IAB8 enhancer on the *lacZ* promoter when it is located about 100 bp (lane 1, gray), or 5.5 kb (lane 2, green) away from the *lacZ* promoter. Five strains for each of these transgenes were analyzed. Lane 3 (blue) and 4 (yellow) indicate IAB8-*w* interaction (W32-*w*) before and after the removal of PTS and *Fab-8* in W32. Lane 5 and 6 shows IAB8-*lacZ* interaction (W32-*Z*) in the same strain before and after the removal of PTS and *Fab-8*. (D) Analysis of enhancer activities before (blue) and after (yellow) the removal of PTS and *suHw* from W81. Four different transgenic strains (W81-1, W81-2, W81-3 and W81-4) were shown.

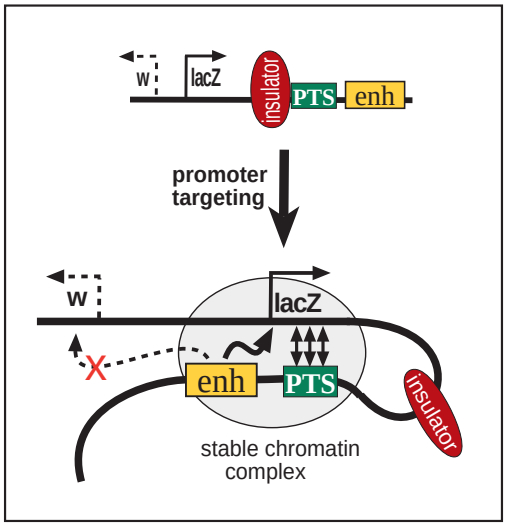

Fig. 6. Model for PTS function. We propose that PTS establishes a stable association between DNA sequences near the enhancer and promoter that results in a constant physical proximity between the two. This type of association could not be blocked by an insulator, and would facilitate weak, long-range enhancer-promoter interactions, while at the same time preventing the enhancer from interacting with other promoters.

when itself (and an insulator) is located between the enhancer and the promoter.

DISCUSSION

In summary, we have shown that PTS facilitates long-range enhancer-promoter interactions in transgenic embryos. The enhancer facilitating activity depends on the anti-insulator and promoter targeting functions in that it only facilitates an enhancer when it is targeted to a promoter (compare Fig. 2C with 2D,E). We have also provided evidence that the promoter targeting function is due to restricting the access of an enhancer to a single promoter, and not due to, for example, positional effects that might inactivate the other promoter present in the transgene. As shown in Fig. 3, the IAB5 or the IAB8 enhancer alone is capable of activating both the *w* and *Tp* promoters after the PTS/insulator fragment is removed by recombination. It is possible that the anti-insulator, promoter targeting, and enhancer-facilitating activities are inseparable and are possibly different aspects of the same activity. For example, enhancer-facilitating effect could be at least in part resulted from restricting the enhancer to a single promoter, which would prevent the enhancer from activating other promoters and consequently increase the probability of activating the 'target' promoter.

In the *Abd-B* locus, the enhancer-facilitating property of the PTS could help distal enhancers such as IAB7 overcome the long distances and direct robust transcription activation of *Abd-B*. This notion is consistent with the genetic functions of the *tmr* and the loss-of-function phenotype of PTS mutants (Castrillon et al., 1993; Gyurkovics et al., 1990; Zhou and Levine, 1999). The single promoter-activating function could ensure that enhancers in the BX-C activate the cognate *Abd-B* promoter only. In this study, we have also shown that promoter targeting only occurred when PTS/insulator was placed between an enhancer and a promoter. This location-dependent characteristic of PTS suggests that strategic placement of PTS within *Abd-B* can facilitate specific enhancers. For example, in the *Abd-B* locus, PTS and *Fab-8* are located between IAB7 (but not IAB8) and *Abd-B* promoter (Fig. 1A). In this arrangement, PTS may facilitate only the distal IAB7 but not the proximal IAB8 to the *Abd-B* promoter. In fact, this appears to be the case, as deletion of PTS causes a loss-of-function transformation of the seventh but not the eighth abdominal segment (Castrillon et al., 1993; Gyurkovics et al., 1990; Zhou and Levine, 1999). On this note, it is possible that multiple PTS-like elements exist in the BX-C to mediate long-distance regulatory interactions.

It can be seen from Fig. 3 that the PTS-facilitated single enhancer-promoter interaction is stronger than the sum of enhancer-*w* and enhancer-*Tp* interactions when the IAB5 or IAB8 enhancers are placed alone at the 3′ of *lacZ*. These results suggest that the PTS-mediated enhancer facilitation is not just the consequence of restricting an enhancer to a single promoter, it must also actively promote long distance enhancer-promoter communications. It is possible that the PTS functions by establishing an insulator-insensitive, stable chromatin structure between the enhancer and a promoter, e.g. forming a ‘stable loop’ and bringing the enhancer closer to the promoter (Fig. 6). Similar ‘loop’ hypothesis has been proposed based on genetic analysis of the enhancer-promoter interactions in the *Abd-B* locus (Galloni et al., 1993). This model can not only explain the anti-insulator activity but can also account for the enhancer-facilitating and the single promoter activating activities. Such a stable association would ensure that enhancer-interacting activators are constantly present at the promoter, which would result in efficient promoter activation and, at the same time, prevent the enhancer from activating other promoters.

Our study also suggests that PTS functions as a generic element in transgenic embryos as it can target and facilitate a heterologous neuroectoderm enhancer NEE (Ip et al., 1992). It is possible that in other large genetic loci such as the odorant receptor gene complex (Mombaerts, 1999) and the neural cadherin-like adhesion gene complex (Wu and Maniatis, 1999), where only one among several dozen promoters is activated in any given cell, PTS-like elements may contribute to the promoter-selective transcriptional activation.

In transgenic embryos, the PTS does not appear to exhibit promoter-specific activity as it can target either the *w* or the *Tp* promoter present in the transgene. It is not known what determines which of the two promoters to select. One possibility is that the decision is made by the interaction between the PTS and local chromatin structure. Alternatively, the selection could be a stochastic process. In the endogenous BX-C, however, the PTS must target the *Abd-B* promoter. Additional mechanism(s), therefore, must be in place to ensure enhancer-promoter specificity in the *Abd-B* locus.

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