The Promoter Targeting Sequence mediates epigenetically heritable transcription memory

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Large gene complexes frequently use “specialized” DNA elements to ensure proper regulation of gene activities. The Promoter Targeting Sequence (PTS) from the Abdominal-B locus of the Drosophila Bithorax complex overcomes an insulator, and facilitates, yet restricts, distant enhancers to a single promoter. We found that this promoter-targeting activity is independent of an enhancer’s tissue or temporal specificity, and can be remembered in all somatic cells in the absence of promoter activation. It requires an insulator for its establishment, but can be maintained by the PTS in the absence of an insulator. More importantly, the promoter-targeting activity can be remembered after the transgene is translocated to new chromosomal locations. These results suggest that promoter targeting is established independent of enhancer activity, and is maintained epigenetically throughout development and subsequent generations.

[Keywords: Abdominal-B; PTS; insulator; promoter targeting; epigenetic inheritance; transcription memory]

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Cell identity, as specified by differential gene expression, relies on heritable epigenetic memory of gene activity through many cell cycles. The most notable examples are the functions of the Drosophila Polycomb Group (PcG) and Trithorax Group (trxG) gene products, which act through dedicated Polycomb/Trithorax Response Elements (PRE/TRE), located at numerous loci in the homeotic gene complex, to maintain the silenced or activated states of the local chromatin throughout development (Kennison 1995; Simon 1995; Hagstrom and Schedl 1997; Pirrotta 1998). However, most epigenetic chromatin marks acquired during development, and in the adult stages, affect only somatic cells and are not transmitted through the germ line. Any epigenetic alterations to the germ-line chromatin are thought to be erased during gametogenesis and fertilization to ensure totipotency in the gametes (Rakyan et al. 2001). However, there is growing evidence that specific marks on the chromatin or the DNA are transmittable through the germ line to the subsequent generations (Rakyan and Whitelaw 2003). The methylation-induced silencing of the agouti and axin-fused loci in mice (Morgan et al. 1999; Rakyan et al. 2003), the swi6-mediated silencing of the mating locus in yeast (Nakayama et al. 2000), and the transcription memory mediated by the Cellular Memory Module (CMM) (Cavalli and Paro 1998; Bantignies et al. 2003) near the Front-abdominal (Fab-7) region of the Drosophila Abdominal-B ( Abd-B) locus, are just a few examples of known activities that can be epigenetically inherited through subsequent generations. These studies raise the possibility that germ-line chromatin is not entirely naive, and that certain regulatory mechanisms might be maintained in the germ-line chromatin and thus be transmittable to the next generation.

The homeotic gene complexes ANT-C and BX-C require elaborate regulatory controls to ensure that each homeotic gene is expressed at the appropriate level in a temporal- and tissue-specific manner. Each of these genes uses a very large and complex control region containing multiple “specialized” regulatory elements, in addition to tissue-specific enhancers. The Abd-B locus is organized into multiple enhancer domains, termed infra-abdominal (iab). Each iab regulates Abd-B expression in a specific abdominal segment (see Fig. 1A) (Duncan 1987; Celniker et al. 1990; Mihaly et al. 1998). Each of these domains is functionally separated from the next by a domain boundary element, such as Front-abdominal (Fab-7) or Fab-8 (Fig. 1A) (Gyurkovics et al. 1990; Galloni et al. 1993; Hagstrom et al. 1996, Zhou et al. 1996, 1999; Mihaly et al. 1997; Barges et al. 2000).

Boundary elements or insulators specifically block the activity of an enhancer when these elements are placed between the enhancer and a promoter. In their endogenous locations, insulators usually function as chromatin boundaries to prevent enhancers from ectopically ac-
tivating the wrong genes, and to restrict chromatin regulatory activities within defined domains by blocking the possible “spreading” of active or silenced chromatin along the DNA [Bell et al. 2001]. This type of activity has been described with respect to histone acetylation in the chicken β-globin LCR [Piroilou et al. 1999], histone methylation in the yeast mating-type locus [Noma et al. 2001, Grewal and Elgin 2002, Hall et al. 2002], and long-range silencing of a Drosophila P[PRE (Sigrist and Pirrotta 1997, Mallin et al. 1998; Bell and Felsenfeld 1999). The best-studied insulators include the vertebrate β-globin insulator [Chung et al. 1993; Bell et al. 1999], the H19/IGF-2 insulator [Bell and Felsenfeld 2000; Hark et al. 2000; Kanduri et al. 2000], the Drosophila ses, scs elements [Kellum and Schell 1992; Zhao et al. 1995; Gaszner et al. 1999], and the suppressor of Hairy wing [suHw] insulator from the gypsy transposon (Geyer and Corces 1992, Dorsett 1993, Gerasimova et al. 2000).

The fact that both Fab-7 and Fab-8 possess potent enhancer blocking activity when assayed in transgenic flies raises the possibility that, within the Abd-B locus, additional mechanisms must be in place to ensure that proper enhancer–promoter interactions are not blocked by this activity. A recent survey of a region 15 kb downstream from the Abd-B promoter [Hendrickson and Sakonju 1995; Hopmann et al. 1995; Sapos et al. 1998] led to the identification of a novel cis-regulatory element, the Promoter Targeting Sequence [PTS; Fig. 1A] [Zhou and Levine 1999]. The minimal 290-bp PTS exhibits an anti-insulator activity, facilitates long-range enhancer–promoter interactions, and usually restricts the enhancer activity to a single promoter when more than one is present in the same transgene [Lin et al. 2003]. These unique properties of the PTS suggest that it may regularly regulate highly specific, long-range enhancer–promoter communications in the Abd-B locus by overcoming the enhancer-blocking activity of the Fab-8 element [Fig. 1A] [Zhou and Levine 1999].

Three types of transgenic strains are usually obtained when transgenes carrying the PTS [and an insulator] are introduced into the fly genome by P-element-mediated transformation. In Type I, the enhancer targets the proximal promoter; in Type II, it targets the distal promoter, and in Type III, no promoter targeting occurs, and the enhancer remains blocked by the insulator [Fig. 1B]. Although the promoter-targeting function varies from strain to strain, this effect is stable in subsequent generations within a specific strain. This phenomenon has led us to hypothesize that promoter targeting is epigenetically heritable. In this study, we report that the PTS cotargets multiple enhancers [i.e., targets two or more enhancers that are divergent in tissue specificity and temporal patterns] to the same promoter. We also found that promoter targeting requires an insulator to be established but could be maintained in all successive generations by the PTS in the absence of the insulator. Finally, we present evidence that promoter targeting is stable after P-element transposition to new chromosomal locations. These results suggest that the PTS maintains an
epigenetic transcriptional memory that can be transmitted to successive generations.

Results

The PTS targets multiple enhancers to the same promoter

In principle, promoter targeting could be affected by several factors such as the local chromatin structure, the identity of the enhancer or the promoter, and the arrangement of different cis elements within the transgene. Among these, the enhancer could play an important role. For example, the PTS could selectively sense the weak, insulator-attenuated enhancer–promoter interaction, and reinforce it on a specific promoter. This model predicts that different enhancers could be targeted independently to different promoters in the same transgenic strain. Alternatively, a targeted promoter could be determined independent of enhancer identity or enhancer activity. This model suggests that the PTS could target all nearby enhancers to the same promoter regardless of their tissue or temporal specificity.

To distinguish between these two models, we challenged the PTS with three different enhancers in the same transgene: an embryonic Neural Ectoderm Enhancer (NEE) from the rhomboid gene (Ip et al. 1992), an embryonic IAB5 enhancer from the Abd-B locus (Busturia and Bienz 1998), and an adult eye-specific enhancer glass from the glass gene (Moses and Rubin 1991) (see Fig. 2). The transgenic vector (W191, Fig. 2I) carrying these enhancers also contains two divergently transcribed genes, white (w) and eve-GFP (Small et al. 1993). In the absence of the insulator, suHw, and the PTS, each of these enhancers is able to activate both w and eve promoters (data not shown), but in the presence of both suHw and the PTS, we obtained three types of transgenic strains as illustrated in Figure 1B. From 31 strains analyzed, different transgenic enhancers target w in four strains and GFP in three other strains. In each of these targeted strains, both NEE and IAB5 target the same promoter, a phenomenon we refer to as cotargeting. An example of a Type I, proximal eve promoter-targeted strain, is shown in Figure 2A–D, and a Type II, distal w promoter-targeted strain is shown in Figure 2E–H. In a separate experiment, we tested hairy stripe I (Hi) (Riddiough and Ish-Horowicz 1991) and IAB5 in transgenic vector W199 (see summary in Fig. 2J). Cotargeting of these two enhancers was also observed. Because NEE, Hi, and IAB5 enhancers use different activators and repressors, these data suggest that promoter targeting is independent of enhancer identity or enhancer-interacting proteins.

To test whether the glass enhancer is also cotargeted with the embryonic enhancers, we surveyed the eye color of adult flies for w-targeted [Type II] versus nontargeted [Type III] or GFP-targeted [Type I] strains. We found that w-targeted strains have consistently red or bright red colored eyes, whereas the remaining strains usually have yellow to orange eyes. For example, the eyes of w-targeted W191 are bright red in all four cases (Fig. 2E), whereas the eyes in nontargeted strains and GFP-targeted strains have yellow or orange, but only in a few cases, red eyes (Fig. 2A,J). These consistently high levels of w activation in adult eye (evidenced by bright red eye color) when embryonic enhancers are targeted to w are seen in all six transgenes tested [summarized in Fig. 2J]. In the absence of glass, the eye color is not affected by which promoter is targeted [data not shown]. When GFP expression is examined in pupa stage (where GFP is not masked by w expression), GFP-targeted strains display strong green fluorescence whereas non-GFP-targeted lines show almost no GFP activity (Fig. 2, cf. C and G). These results suggest that glass is also targeted to the same promoters as the embryonic enhancers.
ers, NEE, HI, and IAB5. Thus, the cotargeting of these enhancers (both embryonic and adult) strongly supports the epigenetic maintenance model.

**The PTS functions only in the presence of an insulator in transgenic embryos**

In addition to overcoming an insulator, the PTS also facilitates the distal enhancer and restricts it to a single promoter, indicating that the PTS does not simply cancel the function of an insulator (Lin et al. 2003). For this reason, we examined whether the PTS exhibits these additional activities in the absence of an insulator. We constructed four types of synthetic P-transposons to test this hypothesis. The first (Table 1A) contains two divergently transcribed reporter genes, w and Transposase (Tp–lacZ, as well as one of the lacZ 3′-located enhancers such as IAB5, IAB8, or NEE. The second type (Table 1B) also contains the insulator, suHw, inserted between the 3′-end of lacZ and one of the enhancers. The third (Table 1C) includes only the PTS in this position, whereas the last type (Table 1D) contains both an insulator (suHw or Fab-8) and the PTS between the 3′-end of lacZ and one of the downstream enhancers. Individual transgenic strains for each of these constructs were analyzed, and the results are summarized in Table 1. In the absence of the insulator or the PTS, each of the enhancers tested could activate both w and lacZ (Table 1A), whereas when an insulator is inserted between the 3′-end of lacZ and the enhancer, transcription of both w and lacZ is blocked (Table 1B). In contrast, inserting only the PTS did not affect enhancer–promoter interactions in any of the tested transgenes (Table 1C). Promoter targeting is observed only when both an insulator and the PTS are inserted in the transgene (Table 1D). The relative order of the insulator and the PTS does not affect promoter targeting (cf. W87 and W88, or W97 and W98 in Table 1D).

A specific example of insulator requirement for promoter targeting is shown in Figure 3: When IAB5 is placed at the 3′-end of lacZ, it activates both the divergently transcribed w and Tp–lacZ fusion genes in the posterior region of the early embryo (see W91 in Fig. 3A). This activity is greatly reduced by inserting the suHw insulator (see W71 in Fig. 3C). In contrast, the addition of

**Table 1. Summary of staining results of different transgenic constructs**

<table>
<thead>
<tr>
<th></th>
<th>transgenic constructs</th>
<th>E-P type</th>
<th>II</th>
<th>II</th>
<th>IV</th>
<th>Promoter targeting</th>
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<td>A</td>
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<td></td>
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<tr>
<td></td>
<td>W91</td>
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<td>0 0 0 0 16 0</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>enhancer plus insulator</td>
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<td></td>
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<tr>
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<td>15 0 0 15 0</td>
<td>0 0 0 0 16 0</td>
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<td></td>
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<tr>
<td>C</td>
<td>enhancer plus PTS</td>
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<tr>
<td></td>
<td>W93</td>
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</tr>
<tr>
<td>D</td>
<td>enhancer plus insulator-PTS</td>
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<td>W67</td>
<td>15 4 0 11 0</td>
<td>4 (27%)</td>
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<td></td>
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<tr>
<td></td>
<td>W68</td>
<td>20 6 1 13 0</td>
<td>7 (35%)</td>
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</table>

The constructs are classified into four groups: (I) enhancer alone inserted at the 3′-end of Tp-lacZ (A); (II) enhancer plus an insulator located between the enhancer and the Tp promoter (B); (III) enhancer plus the PTS (C); and (IV) enhancer plus the PTS and insulator inserted between the enhancer and the Tp promoter (D). Three enhancers, NEE, IAB5, and IAB8 were analyzed and two insulators, Fab-8 and suHw, were tested. With the exception of constructs HN, HNP, and W59, which contain a second HI enhancer as a control, all constructs carry a single enhancer. Enhancer–w and enhancer–lacZ interactions are grouped into four categories based on staining patterns: Type I, no activation of w but strong activation of lacZ shown as w−z+++. Type II, strong activation of w but no activation of lacZ shown as w+++z−. Type III, no activation of either w or lacZ shown as w−z− and Type IV, activation of both w and lacZ with low level of transcription shown as w+z−.

*Indicates that enhancer–lacZ interaction is stronger than that of enhancer–w interaction. Promoter targeting indicates the fact that the lacZ 3′-enhancer selectively activates only one of the divergently transcribed w or Tp promoters (type I or II) and its activity is much stronger than that of the enhancer alone when placed at the same position.
indeed, deleted from the transgene. Note the 400-bp reduction of the PCR fragment size after the deletion of the suHw IAB5 continues to activate lacZ, the same chromosomal position, thereby controlling for position effects. The same genetic background (data not shown), indicating that the suHw insulator was removed from the integrated transgenes. These results strongly suggest that the promoter-targeting function of the PTS can be maintained in the absence of an insulator in subsequent generations. A similar result was obtained when the insulator deletion analysis was done to transgenic strains carrying W98 [Fig. 5A–E] and W97 (data not shown).

Instead of deleting the insulator, we also inactivated the suHw insulator by introducing the transgene W98 into a genetic background mutant for the insulator protein Mod [mdg4] [Dorsett 1993; Cai and Levine 1995; Gerasimova et al. 1995; Scott and Geyer 1995]. Although the insulator function is lost in the control W72 transgene in mod[mdg4] mutant embryos, we did not observe any loss of promoter-targeting function in W98 in the same genetic background (data not shown), indicating the loss of insulator activity did not impede promoter targeting. To test whether the result seen with suHw is a general property of insulators, we also deleted the Fab-8 insulator from promoter-targeted strains carrying the W34 transgene [Table 1, group IV]. None of the examined strains showed any loss of promoter targeting as a result of the insulator deletion [data not shown]. Finally, we followed several of the insulator-deleted promoter targeted strains for ~20 generations, and did not detect any loss of promoter targeting. Taken together,
these results clearly suggest that although an insulator is initially required, it is dispensable once promoter targeting is established, and that the memory of promoter targeting is stable through successive generations.

Promoter-targeting memory could survive transgene transposition to new chromosomal locations

Two possible hypotheses may explain why an insulator is required only initially for promoter targeting, and can be subsequently removed from the chromosomally integrated transgene in promoter-targeted strains. First, an insulator may be necessary to direct the transgene to a chromosomal location where promoter targeting could occur, so that once the transgene is integrated the insulator is no longer needed. Alternatively, when promoter targeting is established, an insulator-independent, epigenetically heritable memory is created to ensure promoter targeting in successive generations. We reasoned that if promoter targeting is epigenetically heritable through the germ line (as opposed to being stable from one cell cycle to the next), it might be possible for this memory to be carried with the transgene when it is moved to new chromosomal locations by introducing transposase into the germ-line lineage. For this reason, we mobilized several PTS transgenic lines, and examined the effects of new chromosomal insertion sites on promoter targeting.

First, as a control, we mobilized nontargeted strains from three similarly constructed transgenes, W14, J18, and W123 [Table 2A]. All three contain the leftward-transcribed w and rightward-transcribed Tp-lacZ gene, the lacZ 3'-located IAB8 enhancer, and the suHw insulator between the enhancer and the promoters, as shown in Table 2. In addition, W14 contains the 290-bp PTS located between the insulator and IAB8 [similar to W97 in Table 1], J18 contains the 625-bp PTS and a 3-kb spacer from the λ genome separating the PTS from the suHw insulator, whereas W123 contains the 625-bp PTS and a 300-bp NEE enhancer located between the w and Tp promoters. Transgenic strains carrying each of these constructs were mated with a strain expressing the Fab-8, and IAB8, inserted at the 3'-end of lacZ. W59 contains the PTS, Fab-8, and NEE in the same position. In addition, W59 contains a reference enhancer, HI, placed at the 5'-end of lacZ [see Table 1, Fig. 4]. W123 contains NEE at the promoter-proximal position and suHw, PTS, and IAB8 at the 3'-end of lacZ [Table 2]. We mobilized three w promoter-targeted strains, W32-1, W59-1, and W123-14. When new insertion strains were examined, all exhibited the same pattern of promoter targeting to the w promoter, no lacZ-targeted strains were recovered [Table 2B]. In situ hybridizations to the w and lacZ transgenes for W59 are shown in Figure 4. In w-targeted strains, the HI and the distal NEE enhancers are mutually inhibitory in the anterior region where both enhancers are active [see Fig. 4A, detailed studies to be described elsewhere]. This activity allows us to test whether the new insertion strains have lost promoter targeting even when the original P-element is still present, because a nontargeted strain would produce strong w activation by HI in the anterior region [Fig. 4B]. As a result, when both the starting w-targeted P-element and a new nontargeted P-insertion exist in the same strain, a composite staining of both transgenes will be observed, and the inhibition of transcription in the anterior region will be masked by the strong HI–w interaction from the nontargeted strain. From a total of 17 new insertions analyzed, no reactivation of HI was observed. A typical new insertion is shown in Figure 4C, whereas a new transposition, W59-Δ10 from the second to the third chromosome, is shown in Figure 4D. In either case, there was no reappearance of robust HI–w interaction similar to that in Figure 4B [indicating a lost of promoter targeting] or NEE–lacZ interaction [indicating targeting to a different promoter], suggesting that there is no loss of promoter targeting or switching of targeted promoter.

We next tested whether the insulator is required for generating heritable promoter targeting at new insertion sites. We transposed promoter-targeted strains carrying W98 and W97 after the insulator had been deleted from the integrated transgenes [summarized in Table 2C]. As shown in Figure 5C, in the original insertion line [W98-19], the IAB8 enhancer was targeted to the Tp promoter in the presence of suHw and the PTS. This is evident as the enhancer strongly activates the Tp promoter as compared with IAB8 alone [W76 in Fig. 5A], and the activity is restricted to the Tp promoter. The deletion of suHw from this strain did not affect the PTS-mediated promoter targeting. The insulator-deleted strain was then mobilized, and a total of nine derivative strains were obtained that lack the original P-element as determined by inverse PCR. When the expression of w and lacZ were analyzed, seven of nine strains exhibited strong IAB8–Tp–lacZ interaction but no IAB8–w interaction, a pattern indistinguishable from the starting promoter-targeted strain [Fig. 5, cf. D and F]. In the remaining two, one had lost promoter targeting, and the other had no apparent promoter activity. We also analyzed a w-tar-
geted, W98-23, for promoter targeting after transposition. A total of six lines were isolated, one of which remained targeted to the \( w \) promoter, but four had lost promoter targeting, resulting in the weak activation of both \( w \) and \( \text{lacZ} \) by IAB8. The remaining strain produced no transcription from either promoter.

W97 was constructed similar to W98 except the position of the insulator and the PTS was switched (Table 1D). As can be seen from the summary in Table 1D, the PTS mediates robust promoter targeting from this position. We first analyzed a \( w \)-targeted strain, W97-29: The deletion of \( suHw \) insulator did not affect promoter targeting to the \( w \) promoter (data not shown). After transgene mobilization, 12 new insertion strains were recovered, eight of which retained promoter targeting, two of which lost promoter targeting, and the remaining two exhibited no detectable transgene expression (summarized in Table 2C). The transposition of a \( \text{lacZ} \)-targeted strain, W97-6, generated five strains, two retained promoter targeting, and one lost promoter targeting.

These results indicate that promoter targeting is remembered to a significant degree during \( P \)-transposition in the absence of an insulator, suggesting the insulator is not required in maintaining promoter targeting during this process. It remains possible, however, that insulator may increase the percentage of inheritance in transposed strains. These results are in contrast to that from obtaining promoter-targeted strains by the standard injection

### Table 2. Summary of enhancer-promoter interactions after \( P \)-element transposition

<table>
<thead>
<tr>
<th>Group</th>
<th>Transgenic strains</th>
<th>E-P interaction in original strains</th>
<th>P-hop</th>
<th>E-P interaction at new insertions</th>
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<tbody>
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<td></td>
<td></td>
<td></td>
<td>5/12</td>
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<td>J18-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4/12</td>
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<tr>
<td>W59-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2/12</td>
</tr>
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</table>

Red oval represents insulator; green rectangle represents the PTS; and N, I8, and H represent NEE, IAB8, and H1 enhancers, respectively. (A) Three representative lines carrying W14, J18, and W123 were transposed to new locations. Each of the three produced promoter-targeted strains. (B) Promoter targeting after \( P \)-element transposition of targeted strains. (C) Summary of promoter-targeted versus non-promoter-targeted strains obtained from transposing in insulator-deleted, promoter-targeted strains [W98-19/W98-23Flp, W97-29/W97-6; see Fig. 4]. An inverse PCR screen was conducted to select strains that have lost the original \( P \)-element.

* indicates that two of the W59-1 lines are transposed from the second to the third chromosome.

** Two of the 33 new insertion strains obtained from W123-14 are transposed from the second to the third chromosome.
Promoter targeting is memorized after P-element transposition. (A) w promoter targeting of the NEE enhancer in W59-1 strain. NEE activates w exclusively, no NEE–lacZ interaction could be detected. Note that the anterior third of the NEE enhancer is repressed, and the reference enhancer HI is also inhibited. (B) Promoter activity of a nontargeted strain (W59-2) showing robust HI enhancer function on both w and Tp promoter, but no transcription activation by NEE. (C) Enhancer–promoter interactions of a strain (W59-1A) obtained by transposing the original W59-1 strain. (D) Transgene transcription of a strain [W901-Δ10] that was transposed from the second to the third chromosome.

Enhancer cotargeting

When given the choice of two promoters in the transgene, the PTS usually targets just one, resulting in exclusive transcription activation of the targeted promoter. What determines which promoter is targeted in a specific transgenic strain is not known, but two models could be proposed regarding the role of the enhancer in promoter selection. First, promoter targeting is determined partially by the enhancer or transcription factors bound to the enhancer. Second, it is epigenetically determined and maintained independent of the identity of the enhancer, and the enhancer-binding proteins. The first model predicts that if multiple enhancers exist in the transgene, the PTS could target different enhancers independently. For example, one enhancer could be targeted to the proximal Tp promoter, whereas the other could be targeted to the distal w promoter in the same transgenic strain. Our results shown in Figure 2 demonstrated that this is not the case. In all transgenic strains examined, different enhancers are cotargeted to the same promoter. These results strongly support the epigenetic inheritance model. Consistent with this model, we observed that both adult eye enhancer glass and embryonic enhancers IAB5 are cotargeted to the same promoter [Fig. 2]. Because the adult glass enhancer and the embryonic enhancer IAB5 are active in different tissues and at different times during the fly life cycle, cotargeting of these two enhancers suggests that in every somatic cell during the life span of the fly, the PTS maintains a memory of the target promoter should an enhancer become active during development.

Mechanisms and implications of insulator requirement in promoter targeting

Several models could be proposed to account for the role of the insulator in PTS function: First, an insulator may be necessary to direct the transgene to “special” nuclear compartments that are compatible with PTS function. The suHw insulator is known to cause the transgene to associate with the nuclear envelope (Gerasimova et al. 2000), whereas the vertebrate β-globin HS4 insulator has been shown to associate with the nucleolus (Yusufzai et al. 2004). In addition, artificially attaching transgenes to nuclear pores creates a chromatin boundary in the transgene (Gerasimova et al. 2000; Ishii et al. 2002). Although it provides an interesting mechanistic explanation, this model could not explain why the insulator is dispensable once promoter targeting is established, because the loss of insulator would dislodge the transgene from the nuclear compartment required for PTS function. The second possibility is that insulator DNA may cause the P-element to integrate into special regions of the chromosome during P-mediated transformation where the PTS can interact with the appropriate local chromatin structure favorable for promoter targeting. Although specific regulatory DNA sequences have been observed that cause preferential insertions into specific chromosomal locations (Bender and Hudson 2000; Kassis 2002), such a
function has not been reported for insulators. As we have demonstrated, an insulator is not necessary to maintain the promoter-targeting memory during transgene mobilization to new locations; thus, it is unlikely that insulator functions by directing the P-element to the right chromosomal location for integration. A third potential mechanism is that an insulator may help recruit proteins that are necessary for PTS activity. This is also unlikely because we observed that promoter targeting only occurs when an insulator is inserted between an enhancer and its promoter (Q. Chen, Q. Lin, L. Lin, and J. Zhou, unpub.). Because the insulator is a constitutive element that presumably recruits the same proteins regardless of its relative locations to the enhancer, or the promoter, the differential results on promoter targeting as a result of its location in the transgene is more likely due to which enhancer it blocks than what proteins it helps recruit for the PTS. We favor the model that the insulator is necessary for promoter targeting because of its domain boundary or enhancer-blocking function. For example, the PTS could recognize a special chromatin structure established by an insulator, such as specific histone or DNA modification, and then establish enhancer–promoter interactions over the insulator.

Two important implications could be gleaned from the role of an insulator in PTS function. First, the Fab insulator elements may play a role in Abd-B that has not been previously realized. These elements are known to function as boundaries that keep individual regulatory domains functionally independent. The current study suggests that they are also necessary for long-range enhancer–promoter interactions in the Abd-B locus, because without an insulator the PTS would not be able to target the distant, Abd-B downstream enhancers such as IAB5 and IAB7 to their promoter, over long distances. Similar roles of insulators in long-range enhancer–promoter interactions have also been suggested by recent studies of the suHw insulator, where paired suHw could facilitate rather than block distant located enhancers [Cai and Shen 2001; Muravyova et al. 2001]. The second implication for the initial requirement of an insulator in promoter targeting is epigenetic inheritance. We observed that an insulator is required for promoter targeting, but only when the transgene is first generated. In established promoter-targeted lines, the insulator could be deleted without any adverse effect on promoter targeting. These results suggest that the PTS could epigenetically maintain promoter targeting through multiple generations [see below].

The epigenetic inheritance of promoter targeting

The most important implication of this study is the heritable nature of PTS function throughout the fly life cycle, which is transmittable into the next generations. This is supported by three sets of experiments presented here: enhancer cotargeting, the initial requirement of an insulator, and P-element transposition. Enhancer cotargeting strongly suggests that promoter targeting is a constitutive chromatin effect that is not determined at the onset of transcription, that it is independent of the function of an enhancer, and that it is epigenetically stable throughout the life cycle of a fly. Because promoter targeting is stable over all successive generations, the memory of the initial targeting event is likely epigenetically maintained by the PTS in the germ line. This idea is further supported by the differential requirement of insulators in the initiation and maintenance of promoter targeting. An insulator must be present to obtain transgenic strains that exhibit promoter targeting, but it could be deleted from the targeted strains, with no loss of promoter targeting. There are two alternative explanations for this result: Either the germ-line cells have the memory of promoter targeting, or the insulator must have helped the transgene to integrate into a chromosomal location where promoter targeting could occur.

More direct evidence for the epigenetic inheritance model comes from our subsequent P-element transposition experiment, which demonstrated that the promoter-targeting effect is remarkably stable even when the transgene is translocated to new locations. In one of our experiments demonstrated in Figure 4, we found that when a w-targeted, W59-1 strain is transposed to various
locations, including two that moved to a different chromosome, enhancers from all new insertions appear to target the same \( w \) promoter, suggesting that a memory is present within the transgene during its transposition to different locations in the germ-line cells. In a separate experiment shown in Figure 5, we generated new insertions from an original promoter-targeted strain, from which the \( suHw \) insulator has been removed by FLP-FRT recombination. Most of the new insertion strains (seven of nine) retain promoter targeting to the same \( Tp \) promoter. Although there are strain variations to the extent of how stable this memory is [Table 2C], these results suggest that an insulator is not required to maintain the promoter-targeting memory during transposition, and that an insulator probably does not function by directing the transgene to chromosome locations favorable for PTS function, as it is not present in the transgene when being transposed. Thus, in the original promoter-targeted strains, the consistent targeting to the same promoter generation after generation is largely due to epigenetic inheritance.

It could still be argued, however, that most P-element transpositions are relatively local where chromatin structures are similar, and hence the same type of promoter targeting can be generated without epigenetic memory. Considering that transpositions of nontargeted strains [Table 2A] are also local hops, yet produce a normal distribution of Type I, II, and III strains [see Fig. 1B], indistinguishable from generating promoter-targeted strains by DNA injection, the local chromatin near the original promoter-targeted transgene is less likely to exert a consistent effect on which promoter should be targeted when the P-element is excised and reinserted nearby. Presently, we could not rule out the possibility that a promoter-targeted transgene may associate with other chromatin structures or nuclear sites where similar function is processed. Consequently, such structure may dictate where the transgene could insert after being transposed. This possibility, however, is hinged on an epigenetic memory in the germ line, because some of the proteins associated with promoter targeting must be present to direct targeting after P-element reinsertion at a new site. We conclude that promoter targeting could be memorized in both somatic and germ-line cells and could be transmitted to successive generations.

Heritable transcription memory has been previously reported in the \textit{Drosophila Abd-B} locus. The 3.7-kb \( Fab-7 \) boundary region also contains a cellular memory module (CMM) \cite{Cavalli1998,Bantignies2003} that can transmit a Polycomb-dependent heritable transcription memory to the offspring, which remains stable for a few generations. Similarly, the PTS-mediated promoter targeting appears to be stable in a majority of new insertions. However, once a newly targeting strain is obtained, it is stable in all successive generations, and can be transmitted through both the male and the female germ lines. It is not known what mechanism the PTS uses to transmit promoter targeting memory to subsequent generations. However, it is clearly different from what is used by the CMM, because PTS function is independent of Polycomb \cite{Zhou2001}. Considering that certain chromatin loop structures could remain intact through spermatogenesis \cite{Klaus2001}, it is likely that the PTS function could be transmitted by a special loop structure [Fig. 1C, part b].

An important prediction of our study is that the inheritance of promoter targeting is not due to the transmission of an enhancer activity in the somatic cell into the germ line. It is a process that has to be determined in the germ-line lineage independent of enhancer functions \cite{Zhou2001} by a combination of the PTS, the promoter, the insulator, and, possibly, the local chromatin structure. The promoter-targeting activity detected in the embryo is not a de novo process, but it is built upon what has been determined in the germ-line cells. This prediction is supported by the FLP-FRT analysis and P-element transposition experiments because only germ-line recombination and transposition events could be recovered by our analysis. We propose that promoter-targeting memory is maintained by the PTS through either a stable chromatin loop or a special chromatin modification. Such structure or modification could be maintained in somatic tissues and germ-line cells in the absence of enhancer activity [Fig. 1C, parts a,b]. When an enhancer becomes active, this structure or modification could automatically guide the enhancer to the targeted promoter [Fig. 1C, part c]. This unique property of the PTS element underscores the possibility that certain aspects of the mechanism controlling long-range enhancer–promoter interactions in the \textit{Drosophila} Hox cluster may be programmed in the germ-line chromatin.

Materials and methods

\textbf{Plasmid constructions}

The construction of the \( P \)-transformant vector HN was described in Zhou and Levine \cite{Zhou1999}. The construct HNP was made by inserting a BamHI–BglII fragment of the 625-bp PTS into the BglII site of HN. Similarly, W59 was made by inserting a 1.7-kb DNA that contains both the PTS and the \( Fab-8 \) insulator. W89 and W91 were made by inserting the 1.6-kb PstI fragment of IAB8, or the 1.0-kb PstI IAB5 DNA into the PstI site of CaSperN vector \cite{Zhou2001}. W71 and W72 were made by inserting a 380-bp fragment of \( suHw \) into the NotI site of W91 and W89, respectively. W93 and W83 were created by inserting the 625-bp PTS into the NotI site of these vectors. To generate the \( P \)-transgene W34, a 700-bp BamHI–BglII fragment containing the 625-bp PTS and part of the Bluescript polylinker, as well as a 1.6-kb BamHI–BglII IAB8 was first cloned into the BglII site of C4PLZ. Then, the 800-bp BglII fragment containing FRT and \( Fab-8 \) was inserted into the recreated BglII site [the 580-bp BamHI fragment of \( Fab-8 \) was first inserted into a BamHI site located between a direct repeat of FRT sites in Bluescript). W97, W98, W87, and W88 were made by inserting an NotI fragment that contains the 625-bp PTS and FRT sites flanking \( suHw \) into W89 and W91, respectively. This NotI fragment was made by first inserting the BamHI–BglII PTS fragment into the BamHI site, followed by adding the FRT-flanked \( suHw \) fragment [made by inserting the BamHI \( suHw \) between the two FRT sites in Bluescript] into the BamHI site.
W123 was made by inserting a 300-bp NEE enhancer between the w and Tp promoters in the transgene shown in Figure 5 of Zhou and Levine (1999). To make W177 and W179, the BamHI–BglII fragment of the lacZ gene from the C4PLZ vector was replaced by the BamHI–BglII fragment of Tp–GFP. A BamHI and BglII fragment was later inserted. The BglII site was then converted into a NotI site, into which an assortment of elements including suHw, PTS, glass, and IAB5 (W177) or IAB8 (W179) was inserted. These DNA fragments were constructed in Blue-Script that contains two NotI sites. To make W182, W191, W198, and W199, the C4PLZ vector was similarly modified as above, but only the eve–GFP gene was inserted. At the 3′-end of GFP, four combinations of different DNA fragments were inserted. For W191, they are suHw, PTS, glass, NEE, and IAB5; for W182, these are Fab-8, PTS, glass, NEE, and IAB5; for W199, these elements are suHw, PTS, glass, HI, and IAB5; and finally for W198, Fab-8, PTS, glass, HI, and IAB5.

P-element transformation and in situ hybridization

P-element transformation vectors containing lacZ and white reporter genes were introduced into the Drosophila germ line by injecting yw"embryos as described previously (Rubin and Spradling 1982). Approximately 20–30 independent transformants were obtained for each of the recombinant P-elements shown. In situ hybridizations were 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 (Department of Genetics and Development, Howard Hughes Medical Institute, Columbia University, New York) and Steve Small (Biological Department, New York University, New York) (Wu et al. 1998). To recombine different DNA elements away from the transgenes, females carrying the transgene [provided that the transgene were located on the second or third chromosome] are mated with males that carry a P-element transformation vector that expresses the Flip recombinase under the control of a sperm-specific tubulin promoter (Wu et al. 1998). The recombinase binds the FRT sites and deletes the intervening DNA. Male flies were collected and mated to yw virgin females to establish stocks that are subsequently analyzed by in situ hybridization.

P-element mobilization

To create new insertion sites for the P-elements, flies carrying the P-elements were mated with flies expressing the Δ2–3 transposase, en mass. Female F1 flies with both the P-element and the transposase were mated with yw males. Male offspring bearing darker eye colors were collected and used to establish individual lines. Each new insertion line was then mapped. P-elements that hopped to different chromosomes were segregated from the original P-elements and kept as stocks.

Genomic DNA preparations and PCR analysis

Genomic DNA preparation and PCR was adopted from BDGP [http://www.fruitfly.org/about/methods/index.html]. Briefly, five anesthetized flies were collected in Eppendorf tubes and frozen at −20°C, then were homogenized in 100 µL of buffer A (100 mM Tris-HCl at pH 7.5, 100 mM EDTA, 100 mM NaCl, 0.5% SDS) with a disposable tissue grinder. After incubation for 20 min at 65°C, 200 µL of LiCl/Kac [1 part 5 M Kac:2.5 parts 6 M LiCl] was added to the tube and incubated for 10 min on ice. The mixture was spun for 15 min, and the supernatant was transferred to a fresh tube, followed by precipitation with 150 µL of isopropanol. The DNA pellet was washed with 70% ethanol, air-dried, and resolved in 50 µL of TE. Two microliters of the DNA sample was used in genomic PCR with primers specific in the PTS and enhancer regions that flank the SuHw insulator. Ten percent of the PCR product was loaded on a 1% agarose gel.

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