The role of Polycomb-group response elements in regulation of *engrailed* transcription in *Drosophila*

Sarah K. DeVido, Deborah Kwon, J. Lesley Brown and Judith A. Kassis*

Polycomb group proteins are required for long-term repression of many genes in *Drosophila* and all metazoans. In *Drosophila*, DNA fragments called Polycomb-group response elements (PREs) have been identified that mediate the action of Polycomb-group proteins. Previous studies have shown that a 2 kb fragment located from –2.4 kb to –395 bp upstream of the *Drosophila engrailed* promoter contains a multipartite PRE that can mediate mini-*white* silencing and act as a PRE in an *Ubx*-reporter construct. Here, we study the role of this 2 kb fragment in the regulation of the *engrailed* gene itself. Our results show that within this 2 kb fragment, there are two subfragments that can act as PREs in embryos. In addition to their role in gene silencing, these two adjacent PRE fragments can facilitate the activation of the *engrailed* promoter by distant enhancers. The repressive action of the *engrailed* PRE can also act over a distance. A 181 bp subfragment can act as a PRE and also mediate positive effects in an enhancer-detector construct. Finally, a deletion of 530 bp of the 2 kb PRE fragment within the endogenous *engrailed* gene causes a loss-of-function phenotype, showing the importance of the positive regulatory effects of this PRE-containing fragment. Our data are consistent with the model that *engrailed* PREs bring chromatin together, allowing both positive and negative regulatory interactions between distantly located DNA fragments.

**KEY WORDS:** Polycomb group genes, Polycomb-group response elements (PREs), Gene silencing, *Drosophila*

**INTRODUCTION**

Early in *Drosophila* development, spatially restricted DNA-binding transcription factors directly control the patterned expression of many genes. These direct regulators soon disappear while the patterned expression of their targets is maintained. The Trithorax group (trxG) and Polycomb-group genes (PcG) are responsible for maintaining expression in the ‘ON’ and ‘OFF’ states, respectively. Some of the PcG proteins are present in two protein complexes, Polycomb repressive complex 1 (PRC1) and PRC2 which repress transcription through modifications of histones and inhibition of chromatin remodeling (for a review, see Ringrose and Paro, 2004). A third complex that contains the PcG DNA-binding protein Pleiohomeotic (Pho) and a modified histone-binding protein, Sfmbt, has recently been identified (Klymenko et al., 2006). trxG genes encode proteins found in chromatin remodeling and co-activator complexes (see Ringrose and Paro, 2004). Two members of the trxG family, *trx* and *ash-I*, seem to play special roles to counteract the silencing action of PcG proteins (Poux et al., 2002; Klymenko and Müller, 2004). Both PcG and trxG genes have been implicated in stem cell and cancer development in mammals (reviewed by Sparmann and van Lohuizen, 2006).

In *Drosophila*, PcG proteins work through DNA elements called PcG response elements (PREs). Endogenous PREs extend over several kilobases and can be divided into subfragments with similar activities (Kassis, 1994; Horard et al., 2000). Minimal PRE fragments have been identified using two types of assays in transgenic *Drosophila*. In mini-*white* silencing, the PRE represses the expression of the linked marker gene mini-*white* in transgenic *Drosophila*. As the degree of repression is often enhanced in homozygotes, this effect has been called ‘pairing-sensitive silencing’ and the fragments that mediate it ‘pairing-sensitive elements’ or PSEs. The second assay, which more accurately reflects the true biological function, tests the ability of the PRE to maintain correct patterned expression of a reporter construct (for a review, see Kassis, 2002). Several protein-binding sites are known to be important for PRE function (reviewed by Müller and Kassis, 2006; Ringrose and Paro, 2007), and computer programs exist to predict PREs based on clustering of some of these sites (Ringrose et al., 2003; Fiedler and Rehmsmeier, 2006), but it is still not possible to predict accurately what constitutes a PRE (reviewed by Ringrose and Paro, 2007).

Trithorax-group proteins act through trxG response elements or TRES. TRES are not as well defined and, at least for the bithorax complex and the *hedgehog* gene, seem to overlap with or closely adjoin PREs (Cavalli and Paro, 1998; Tillib et al., 1999; Maurange and Paro, 2002). It is not yet known how the same DNA fragment can act as both a PRE and a TRE especially in light of recent experiments that show that the *bcd* PRE is bound by PcG proteins in both the on and off transcriptional state (Papp and Müller, 2006). Several studies provide evidence that transcription through a PRE inactivates it (Hogg and Karch, 2002; Rank et al., 2002; Bender and Fitzgerald, 2002), and some suggest that transcription through a PRE converts it into a TRE (Schmitt et al., 2005). Although this is an attractive model, another recent study suggests that transcription through the *bcd* PRE actually contributes to transcriptional silencing (Petruk et al., 2006). Thus, the exact relationship between TRES and PREs is not yet understood.

We have been studying a PRE from the *Drosophila engrailed* (*en*) gene. *en* encodes a homeodomain-containing protein important for segmentation in the embryo and formation of posterior compartments in adults. *en* is regulated by PcG- and Trx-group genes in both embryos and larvae (Moaized and O’Farrell, 1992; McKeon et al., 1994; Breen et al., 1995). We have been studying a multipartite PRE near the *en* transcription start site (from –2.4 kb to

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Accepted 2 December 2007
–395 bp) that is bound by PcG proteins in tissue culture cells, embryos and adults (Strutt and Paro, 1997; Nédre et al., 2006; Schwartz et al., 2006; Comet et al., 2006). We previously studied its mini-white silencing activity and ability to act as a PRE in an Ubx-bad-reporter construct (Kassis et al., 1991; Kassis, 1994; Americo et al., 2002). Here, we examine the role of this DNA at the en gene itself. We find that, in addition to its role as a PRE in maintaining en-like expression from a reporter gene in embryos, this DNA is able to play an activating role in transgenes. This activation function occurs relatively late in development, beginning at about 6 hours, when the maintenance phase normally begins. Two adjoining subfragments of this multipartite PRE are both able to mediate positive and negative regulatory effects with many different tissue-specific enhancers. We also generated a 530 bp deletion of DNA from the endogenous en gene. This deletion generates a weak loss-of-function en phenotype, suggesting that this DNA plays a redundant role as a PRE, but is required for en activation. Our data are consistent with a model whereby en PREs facilitate interactions between distantly located regulatory elements to maintain either transcriptional activation or silencing.

MATERIALS AND METHODS

Construction of plasmids

P[en1] was constructed by cutting construct H (Kassis, 1990) with SphI. This resulted in a singly cut vector at a natural SphI site located at –395 upstream of the transcription start site of en. The next contiguous upstream 8 kb SphI fragment was cut out of the en lambda phage clone E8 (Kuner et al., 1985) and cloned into SphI-cut construct H. The orientation of the fragment was verified by PCR and sequencing. The en DNA present in P[en1] is from Canton S and contains some sequence polymorphisms from the published genomic sequence (data not shown). For P[ΔPSE2], the 8 kb SphI fragment was cut with ClaI, generating a 7.5 kb SphI-ClaI fragment. A PCR fragment containing the 181 bp deletion was created using the primers GTCTGGACAAATCAGATTCGA (which contained the upstream natural ClaI site), and GCCGCAATGCTTTCCAGACACTTTCA, which added a synthetic SphI site at –576 bp upstream of the en transcription start site. The 7.5 kb SphI-ClaI fragment, ClaI-SphI cut PCR fragment and SphI cut construct H were ligated. The resulting clone was sequenced to confirm the 181 bp deletion. For P[Δboth] were ligated. The resulting clone was sequenced to confirm the maintenance of en gene. This deletion generates a weak loss-of-function en phenotype, suggesting that this DNA plays a redundant role as a PRE, but is required for en activation. Our data are consistent with a model whereby en PREs facilitate interactions between distantly located regulatory elements to maintain either transcriptional activation or silencing.

Transgenic lines

P[en1], P[ΔPSE2] and P[Δboth] were generated by injections into homozygous Df(1)w67C2, y embryos in our laboratory using standard procedures. Some P[en1], P[ΔPSE2] and P[Δboth] lines were also obtained by transposon mobilization using a P[Δ2-3] line (Robertson et al., 1988). P[en2] and P[en3] were obtained from injections into w1118 by Genetic Services (Sudbury, MA). The chromosomal insertion sites of transgenes were localized by inverse PCR. The presence of a single transgene insertion site was confirmed for P[en3]-tou, P[en3]-en and P[en3]-9C by Southern blotting (data not shown). Derivatives of P[en2] and P[en3] were generated by treating with hsFLP and hsCre recombinase separately, and the 181 bp deletion was verified by PCR.

Generations of deletions in the en gene in situ

We used an insertion of P[en1-ry] at –412 in the en gene (Kassis et al., 1992) as the recipient and an insertion of P[ΔPSE2] into tou (P[ΔPSE2]-tou) as the donor to try to generate a gene conversion event to precisely delete PSE2. Males of the genotype P[en1-ry]-en/Sp P[ΔPSE2]-tou; ry506/ry506 P[ry2 Δ2-3]99B were crossed to Sco/Cyo; ry506/ry506 virgins. Sp+: ry+ that were either CyO or Sco (ry) derivatives of P[en1-ry]-en chromosome were crossed to Sco/Cyo; ry506/ry506 virgins or males. Flies of the genotype P[en1-ry]-en/Cyo were analyzed by PCR for the presence of the P-ends and for the deletion of PSE2. These flies were also crossed to Df(2R)enX31 to check whether they complemented this en inv. deficiency chromosome. Three-hundred and three ry+ flies were screened. No gene conversion events were recovered, but we did obtain four deletions of DNA flanking the P-element, two with small deletions in the direction of the en promoter, one large deletion and the one described here.

RESULTS

We are studying a DNA fragment that extends from –2.4 kb to –395 bp upstream of the en major transcription start site (Fig. 1A). This fragment of DNA has no enhancer activity on its own (Kassis, 1990), but possesses two other activities. First, it acts as a pairing-sensitive silencer (Kassis et al., 1991), and, second, it can mediate P-element homing, that is, it causes P-constructs that contain it to insert in the genome near the endogenous en gene at a significant frequency (Kassis et al., 1992). We previously identified two subfragments that act as strong pairing-sensitive silencers of mini-white (from –1944 to –1503 and from –576 to –395 bp (PSE2, Fig. 1A) (Kassis, 1994). This fragment of DNA is within a 3.2 kb region that is bound strongly in embryos and adults by the PcG proteins Polyhomeotic (Ph) and Pc as shown by chromatin immunoprecipitation experiments on a genomic tiling microarray (Nédre et al., 2006; Comet et al., 2006). Here, we have divided this 2 kb fragment into two pieces: PSE1 (extending from –2.4 kb to –576 bp) and PSE2 (extending from –576 to –395 bp). PSE1 and PSE2 contain consensus binding sites for all the proteins thought to play a role in PRE activity, including Pho, GAGA Factor/Psp, Dsp1, Sp1 and Zeste (Fig. 1A). We were particularly interested in the activity of PSE2 as we have studied the binding sites required for the PRE and PRC activity of this fragment (Americo et al., 2002; Brown et al., 2005).

As PSE1 and PSE2 do not contain any en enhancers, we examined their activity in the context of a larger en reporter construct. Eight kilobases of upstream en sequences are sufficient to drive a reporter gene in en-like stripes in embryos but not imaginal discs (Hama et al., 1990) (S.K.D., D.K., J.L.B. and J.A.K., unpublished). P[en1] contains 8 kb of upstream en sequences, the en promoter, and 188 bases of the untranslated leader (from –8 kb to –188 bp) fused to Adh-lacZ (Fig. 1B). We obtained six lines for this construct, and, as expected, all expressed lacZ in en-like stripes throughout embryonic development (an example is shown in Fig.
In order to assess whether P[en1] contains PRE activity, we crossed it into a polyhomeotic (ph) mutant background. Both En and β-gal are expressed throughout the embryo in a ph mutant, instead of in stripes as seen in the wild-type embryo (Fig. 1C). This suggests that this reporter construct contains a PRE for en expression.

To test the role of PSE1 and PSE2 in regulating en expression, we first made a derivative of P[en1] with a deletion of PSE2 (P[ΔPSE2], Fig. 1B), and another that deleted both PSEs (P[Δboth]).

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We obtained four lines with this construct. For two lines, β-gal was accurately expressed in stripes throughout development (Fig. 2B,F). One of these two lines was unusual in that it was inserted in E(Pc), the gene next to invected (inv) and this will be discussed below. The third P[en2] line had weak misexpression similar to that seen in Fig. 2G, and the fourth line had strong misexpression, similar to that seen in Fig. 2H. We examined the effect of loss of PSE1, PSE2 and both at all chromosomal locations. Loss of either PSE1 or PSE2 increased misexpression (in all but P[en2]-E(Pc), see below), with the most misexpression seen in P[en2]Δboth at three out of four insertion sites (misexpression in the fourth line was already massive in the ΔPSE1 and ΔPSE2 derivatives). In Fig. 2 we show an example of one line, from an insertion on the X chromosome, where β-gal was accurately expressed in the starting P[en2] line. Removal of PSE2 led to weak misexpression between the stripes (Fig. 2G). Removal of PSE1 led to considerably more lacZ misexpression (Fig. 2H), with the most misexpression seen when both PSE1 and 2 were removed (Fig. 2I). β-Gal expression was correctly initiated from all lines (Fig. 2B,E) and misexpression did not begin until about 7 hours of development, consistent with the role of these fragments as PREs. These data show that PSE2 can act as a PRE with en enhancers. The repressive role of PSE2 is best seen by comparing the amount of misexpression seen in P[en2]ΔPSE1 (Fig. 2H), which contains PSE2, with the much more extensive misexpression seen in P[en2]Δboth (Fig. 2I). However, these data also show that deletion of PSE2 alone led to very little misexpression, showing that PSE1 can largely substitute for the role of PSE2 in the intact reporter construct.

At one chromosomal location, lacZ was only weakly misexpressed when both PSEs were deleted (Fig. 3). In this case, P[en2] was inserted in the 5' region of the E(Pc) gene, ~70 kb away from the endogenous en PRE, and 20 kb upstream of the inv transcription start site. The inv gene is expressed in the same pattern as en, shares sequence homology in the homeodomain region of the protein, and is functionally redundant with en (Gustavson et al., 1996). We suggest that when P[en2] is inserted into E(Pc), the en endogenous PRE, located 70 kb away, is able to compensate for the loss of this fragment within the construct. Alternatively, the regulatory sequences within the construct are able to interact with a presumed PRE located at the 5' end of the inv gene, about 20 kb away from the insertional site of P[en2]-E(Pc). Pc, Psc and Ph have all been shown by chromatin-immunoprecipitation experiments to bind to sequences just upstream and including the transcription start site of inv (Strutt and Paro, 1997; Nègre et al., 2006; Comet et al., 2006).

Taken together, these data indicate that PSE1 and 2 act together to repress en transcription between the stripes in embryos. As PcG proteins are known to bind to these fragments and the misexpression seen when deleting these fragments occurs at a time when PcG proteins act to repress en between the stripes (Moazed and O’Farrell, 1992), these fragments act as bona fide PREs for en expression.

**PSE1 and PSE2 can also act as positive elements**

Construct P[en3] acts as an enhancer trap; that is, it contains no information for patterning on its own and expression of lacZ is governed by enhancers and silencers flanking its genomic insertion site (Fig. 4A) (Kassis, 1990). Because the fragment of en DNA in P[en3] causes P-elements to home to the en locus (Kassis et al., 1992), we recovered two lines with en DNA inserted into the chromosomal region of en, one 6 kb upstream of the endogenous en transcription start site (line P[en3]-en) and one 66 kb upstream, in the tou transcription unit (line P[en3]-tou; Fig. 4). As expected, β-gal is expressed in exactly the same way as endogenous en when P[en3]-en is inserted into the lacZ gene expression (Fig. 4D). Although there was no β-gal detected early, lacZ was expressed in en-like stripes beginning at about 6 hours of development, around the time when en would begin to be regulated by the PcG and trxG genes. Interestingly, when the stripes are turned on in P[en3]-tou, it is done so in a stochastic way: initially single β-gal-expressing cells were observed, but by about 6.5 hours of development, every cell in a stripe is expressing β-gal (Fig. 4D). β-Gal is also detected in the posterior compartment of the P[en3]-tou imaginal discs, although at a lower level than in line P[en3]-en.

What happens when PSE1 or PSE2 are deleted from P[en3]-en and P[en3]-tou? Deletion of both PSE1 and PSE2 from P[en3]-en did not change the expression of lacZ (Fig. 5). Thus, these elements are not important for expression of lacZ when P[en3] is inserted ~6 kb, just 4 kb upstream of the endogenous PSE1 and PSE2 fragments. By contrast, lacZ expression from P[en3]-tou was somewhat altered by the deletion of PSE2, and dramatically by the loss of PSE1 (Fig. 5). In embryos, deletion of PSE1, and to a lesser extent, PSE2 lead to a loss in the intensity of the stripes, especially in the head and thoracic segments. Loss of both PSE1 and PSE2 led to both a decrease in the intensity of the stripes and a rise in the background staining, suggesting both a loss of activation and repression. Deletion of PSE1 and PSE2 from P[en3]-tou also had...
effects in larval tissues. A wing disc is shown in Fig. 5, and similar effects were seen in all other discs (data not shown). Expression of β-gal in larval tissues was entirely dependent on the presence of PSE1 or PSE2. Loss of PSE1 had the most dramatic effect. P[en3]-tou wing discs had very little β-gal activity but this small amount of activity is due to the activating effects of PSE2, as there is no activity in P[en3]-both wing discs. Deletion of PSE1 from P[en3]-tou caused most of the β-gal activity in wing discs to be lost. Similar results were seen in other discs (data not shown). Deletion of PSE1 and PSE2 from P[en3]-en had no effect on the expression of β-gal at any stage examined.

PSE1 and PSE2 can act as positive regulatory elements

We wanted to know whether PSE1 and PSE2 could interact with other regulatory elements in the genome. We obtained 17 independent lines with the construct P[en3]. As stated above, P[en3] acts as an enhancer trap, and, as expected, β-gal expression patterns vary in each line, dependent on its position of insertion in the genome (data not shown). We examined the β-gal expression pattern in wing discs from P[en3] and P[en3]-both from most of the lines we recovered (15 lines). In seven of the 15 lines, we saw differences in β-gal expression patterns when the PSEs were deleted. The PSEs were important for activation or repression, dependent on the tissue. An example is shown in Fig. 6. In the proventriculus and in wing disks
from line P[en3]-9C, PSE1 and PSE2 act as silencing elements. There is very little β-gal expressed in a proventriculus or wing disc from P[en3]-9C larvae, some in P[en3]ΔPSE1-9C and expression everywhere in P[en3]Δboth-9C larvae. By contrast, PSE1 and PSE2 act as activators of expression in the larval brain. There is very little expression of lacZ in the P[en3]Δboth-9C larval brain, and this expression is increased in both P[en3]ΔPSE1 and P[en3]-9C larvae. Expression in the P[en3]-9C brain is variegated, suggesting a competition between activation and repression. These data suggest that PSE1 and PSE2 can act to either activate or repress transcription in a tissue-specific way and that they can mediate responses with enhancers and silencers at many genomic positions.

We wondered why we saw differences in lacZ expression patterns with the loss of PSEs in only a subset of the lines. We noticed a strong correlation between the presence of mini-white silencing in a line and changes in β-gal expression in embryos and/or larvae when the PSEs were deleted (see Table S1 in the supplementary material). In six out of seven lines that had mini-white silencing, changes in lacZ expression were observed when the PSEs were deleted (the one exception being P[en3]-en, where the endogenous PSEs are only 4 kb away). By contrast, in lines without mini-white silencing, deletion of PSE1 altered the expression pattern in only one line out of 8. Does the presence of a nearby PcG protein-binding site correlate with changes in expression on deletion of the PSEs? This is hard to assess as we do not know over how many kilobases a PSE can act, and this probably varies dependent on the region of the genome. Nevertheless, on average, lines where loss of PSEs alters the expression pattern tend to be closer to endogenous PcG-binding sites (see Table S1 in the supplementary material). These data are consistent with a model that in some chromosomal positions, the PSEs can interact with flanking DNA to alter expression patterns either of mini-white or lacZ, and that the presence of nearby PREs increases the tendency for this to happen. The PcG binding sites were determined in S2 and Kc cells, and we are examining expression in larvae and embryos where the distribution of the PcG proteins may be different. It will be interesting to revisit this issue when genome-wide PcG protein localization is available in embryos and larvae.

**Deletion of PSE2 from the endogenous en gene causes a loss of function phenotype**

We generated a deletion of 530 bp of en DNA, removing sequences from −942 to −412, including almost all of PSE2 and 366 bp of additional upstream sequences (Fig. 1A) from an imprecise excision of a P-element inserted at −412 (see Materials and methods). We called this mutation enΔ530. Our data suggest that the sequences deleted in enΔ530 are important for en activation. enΔ530 behaves genetically like a recessive double-mutant loss-of-function allele in en and inv, causing a defect in the posterior compartment of the wing (Fig. 7; Table 1). That is, although there is no phenotype when enΔ530 is put over a wild-type chromosome, crosses to either inv or en alleles gave flies with the same wing phenotype. This suggests that this small deletion may alter the expression pattern of both Inv and En. This is perhaps not that surprising as En and Inv have been previously reported to share regulatory DNA (Gustavson et al., 1996; Goldsborough and Kornberg, 1994). It is possible that this small deletion alters the chromatin structure of the en/inv region, causing a small decrease in the level of expression.

The penetrance of the wing phenotype was greatest in flies homozygous for enΔ530 or when enΔ530 was combined with an invΔ double mutant or a deficiency for the region. This suggests that either En or Inv can partially compensate for the loss of both in enΔ530. This is consistent with data that suggests En and Inv have redundant activities (Gustavson et al., 1996).

Because enΔ530 completely deletes PSE2, we examined embryos to see whether En or Inv antigen could be detected in cells between the stripes in enΔ530 homozygous embryos. We did not observe any misexpression of En or Inv in enΔ530 homozygous embryos. Thus, it appears that the repressive activity of PSE2 can be replaced by other PREs located throughout the en locus.
The phenotype of the en PRE deletion can be contrasted with phenotypes observed by deleting the iab-7 PRE (also called the Fab7-PRE) and the bxd PRE from the endogenous AbdB gene and Ubx genes, respectively. As stated above, previous studies on the iab-7- and bxd-PREs have shown that, in transgenes, these sequences can mediate both repression and persistent activation of a linked mini-white marker (Cavalli and Paro, 1998; Rank et al., 2002). The activation is thought to be mediated by the Trithorax group genes. This led to the idea that these PREs would be necessary for both activation and repression of AbdB and Ubx. However, deletion of the iab-7 PRE from within the AbdB gene and the bxd PRE from the Ubx gene in situ led to phenotypes that were consistent with a role only in repression (Mihaly et al., 1997; Sapos et al., 2007). Thus, for both the iab-7 PRE and the bxd PRE, no activating role could be seen in vivo. It cannot be ruled out that there are other TREs within the endogenous Ubx and AbdB gene that play redundant roles to those of the deleted fragments. These result also do not rule out the hypothesis that one role of the Trithorax group genes is to counteract the activity of the Polycomb group genes.

Are the en PREs also TREs?
We tested whether the en PREs could act as TREs in the vector pUZ in transgenic Drosophila. This vector has been used to show that fragments of DNA that include the bxd, Mcp, Fab7 and hedgehog PREs could act as both PREs and TREs for the expression of the linked marker gene mini-white (Rank et al., 2002; Mauzelle and Paro, 2002). We tested three versions of the en PRE in pUZ (the entire 2 kb fragment, PSE2 and the 530 bp fragment deleted in en2530) for their ability to mediate silencing and activation of mini-white in pUZ (data not shown). Although all three fragments mediated silencing of mini-white in this vector, none was able to activate mini-white expression (data not shown). Thus, we found no evidence that the en PRE could behave as a TRE in this assay.

As the β-gal expression level from P[en3]tou is relatively weak, we reasoned that we might be able to observe an effect of heterozygous mutations in trxG genes on its expression level. We checked the effect of heterozygous mutations in the trxG group genes brahma, trithorax and ash-1 on β-gal expression from P[en3]tou in discs and did not observe any effect (data not shown). Thus, we do not have any evidence that the positive regulatory effects of these fragments are due to trxG genes.

DISCUSSION
The ability of the PSEs to mediate long-range interactions is reminiscent of the promoter targeting sequences of AbdB (Zhou and Levine, 1999; Lin et al., 2004); however, we suggest that the activity is intrinsic to the PRE activity of the fragments. The pairing-sensitive silencing capacity of PREs has long suggested their ability to mediate interactions between physically separated DNA fragments. Furthermore, the Fab-7 PRE is able to mediate long-distance interactions between Fab-7-containing transgenes and the Abd-B locus (Bantignies et al., 2003), and to mediate looping to bypass insulators (Comet et al., 2006). Recent studies on the bithorax complex using 3C and FISH suggest that PREs interact with the promoter and other PREs in the inactive state, but not when a gene is being actively transcribed (Lanzuolo et al., 2007). Biochemical experiments showed that a Polycomb repressive complex could bring together two templates, and that this activity might be mechanistically distinct from repression (Lavigne et al., 2004). It is interesting that mutation of Dsp1-binding sites within both a Fab7 PRE and a 141 bp subfragment of PSE2 has been reported to change their activity from pairing-sensitive silencing to pairing-sensitive activation (Déjardin et al., 2005). This suggests that the ability to mediate interactions between regulatory sequences may be a common property of PREs, independent of their activating or silencing capacity.

The en PRE we are studying is able to activate or repress transcription from a distance, dependent on the context. Unlike the PREs in the bithorax complex, which are located tens of kilobases away from their promoters, the en PRE is located right next to the en promoter. We suggest that one of its activities is to bring together the promoter with enhancers or silencers, irrespective of the transcription state. The regulatory DNA of the en gene is spread over 70 kb and is intimately linked with inv. We suggest that en PREs are crucial for establishing the correct chromatin structure of this complex locus.

We thank Jeff Americo for construction of P[en1] and its derivatives, and for early studies on expression from these constructs. We thank Renato Paro for the pUZ vector and for hsGAL4 CyO flies; Jerome Déjardin and Giacomo Cavalli for hsGAL4 flies and detailed protocols; Tom Kornberg for fly stocks; and our colleagues Jim Kennison, Mark Mortin and Deb Hursh for many helpful suggestions throughout the course of this project. We thank Melissa Durant, Yuzhong Cheng, Mark Mortin, Jim Jaynes and Miki Fujioka for comments on this manuscript. This research was supported by the Intramural Research Program of the NIH, NICHD.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/4/669/DC1

Table 1. en(2530) is a loss-of-function mutation

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<tr>
<th>Genotype*</th>
<th>Wing phenotype†</th>
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<tbody>
<tr>
<td>en(2530)+</td>
<td>0% (0/60)</td>
</tr>
<tr>
<td>en(2530)/Df(2R)enX31</td>
<td>91% (91/100)</td>
</tr>
<tr>
<td>en(2530)/inv30/en9</td>
<td>91% (63/68)</td>
</tr>
<tr>
<td>en(2530)/inv30/en26</td>
<td>30% (28/94)</td>
</tr>
<tr>
<td>en(2530)/IIB86</td>
<td>22% (19/83)</td>
</tr>
<tr>
<td>en(2530)/en10L</td>
<td>60% (72/120)</td>
</tr>
<tr>
<td>en(2530)/inv17en26</td>
<td>21% (18/84)</td>
</tr>
<tr>
<td>en(2530)/en530</td>
<td>93% (54/58)</td>
</tr>
<tr>
<td>inv30/en(2530)/Df(2R)enX31</td>
<td>94% (64/68)</td>
</tr>
</tbody>
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†Df(2R)enX31 deletes both en and inv (Kuner et al., 1985). inv30/en9 and inv30/en10L are all en null mutations in inv and en; en(2530) and en10L are all en null mutations (Gustavson et al., 1996). inv17en26 is a hypomorphic inv allele generated in our laboratory.

‡The percentage of wings with a defect (number of wings with a defect/total number of wings scored).


