

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

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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-1*, 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; Maura 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 *bxd* 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 (Hogga 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 *bxd* PRE actually contributes to transcriptional silencing (Petruck 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 (Moazed 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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–395 bp) that is bound by PcG proteins in tissue culture cells, embryos and adults (Strutt and Paro, 1997; Négre 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-bxd*-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 *Sph*I. This resulted in a singly cut vector at a natural *Sph*I site located at –395 upstream of the transcription start site of *en*. The next contiguous upstream 8 kb *Sph*I fragment was cut out of the *en* lambda phage clone E8 (Kuner et al., 1985) and cloned into *Sph*I-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 *Sph*I fragment was cut with *Cl*aI, generating a 7.5 kb *Sph*I-*Cl*aI fragment. A PCR fragment containing the 181 bp deletion was created using the primers GTCTGGCAAATCGATATTCGA (which contained the upstream natural *Cl*aI site), and GCGGCATGCTTTCCACAGACACTTTCA, which added a synthetic *Sph*I site at –576 bp upstream of the *en* transcription start site. The 7.5 kb *Sph*I-*Cl*aI fragment, *Cl*aI-*Sph*I cut PCR fragment and *Sph*I cut construct H were ligated. The resulting clone was sequenced to confirm the 181 bp deletion. For P[Δboth], the 8 kb *Sph*I fragment was cut with *Xba*I and a 5 kb fragment was isolated. The 2 kb deletion present in P[Δboth] was generated by PCR using the primers CTCTATCTAGATAACTATTCTGTATCC, which contains the natural *Xba*I site, and GCGGCATGCGAA-TTCAAATGATGAGAATATAAGATAAGC, which added a synthetic *Sph*I site before the *Eco*RI site present at –2.407 kb. The 5 kb *Sph*I-*Xba*I fragment, *Xba*I-*Sph*I cut PCR fragment and *Sph*I cut construct H were ligated. The resulting clone was sequenced around the *Xba*I junction and in the area of the deletion. P[Δboth] contains two *Sph*I sites, one at –395 and one at –8 kb. A 10 kb *Bam*HI, *Sac*II subfragment of P[Δboth] that contained only the *Sph*I site present at –395 was cloned into an intermediary vector where it would be the only *Sph*I site (clone SRK1). For P[en2], PCR fragments were generated to flank PSE1 by *Lox*P sites and PSE2 by FRT sites. The PSE1 *Lox*P primer had a 5' *Sph*I site, and the 3' primer had an *Asp*718 site. For PSE2, the 5' primer had an *Asp*718 site, and the 3' primer had a *Sph*I site. The primers used were: 5' *Lox* P, GCGGCATGCGATAACTTCGTATAA-TGTATGCTATACGAAGTTATAATTCGGTTGATATGATGCGC; 3' *Lox* P, GCGGGTACCATAACTTCGTATAGCATACATTATACGAAGTTATTTCCACAGACACTTTTCAT; 5' FRT, GCGGGTACCGAAGTTCCCTA-TACTTTCTAGAGAATAGGAAGTTTCGAGATGGCATGTGGCTCT; and 3' FRT, GCGGCATGCGAAGTTTCCTATTCTCTAGAAAGTATAGGAA-CTTCCCATGCTGGAGCTGTGAGCC. *Sph*I-*Asp*718 cut PCR fragments were cloned into *Sph*I-cut SRK1. The resulting clone was cut with *Bam*-*Sac*II to yield a 12 kb band and put back into *Bam*HI-*Sac*II P[Δboth] vector. P[en2] was completely sequenced in the region of the PCR fragments. P[en3] was generated by cutting Construct H with *Sph*I and cloning in the *Sph*I fragment from P[en2] that contained PSE1 and PSE2 flanked by *Lox* P and FRT sites.

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]*, *99B* 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, then sequentially for Δboth lines. The presence of the deletions 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[ry⁺Δ2-3]99B* were crossed to *Sco/CyO*; *ry⁵⁰⁶/ry⁵⁰⁶* virgins. *Sp⁺*; *ry⁻* that were either *CyO* or *Sco* (*ry⁻* derivatives of the *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 acted 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 (Négre 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/Psq, 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 PSE and PRE 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.

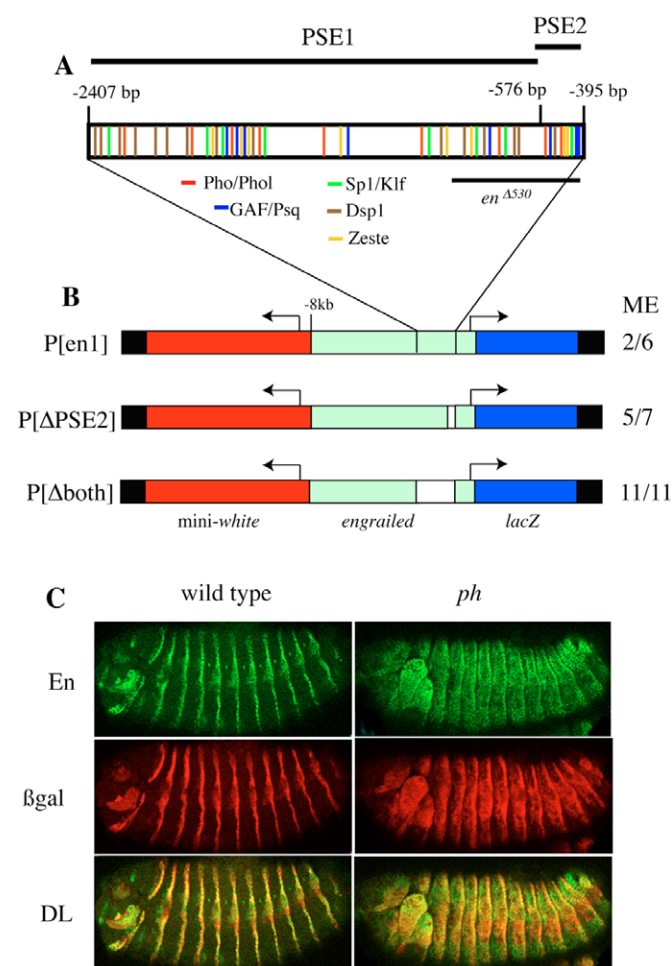


Fig. 1. Eight kilobases of upstream *en* regulatory DNA includes sequences with PRE activity. (A) Consensus binding sites for DNA-binding proteins implicated in PRE activity are shown (Brown et al., 2005) along with the location of PSE1 and PSE2. The line below shows the extent of deletion present in the *en* mutant, *en*^{Δ530}. (B) DNA constructs used in these experiments. Arrows indicate the start of transcription. Black boxes indicate P element ends. Green boxes indicate *en* DNA. Red boxes indicate *mini-white* DNA. Blue boxes indicate *lacZ* DNA. White boxes indicate DNA deleted from the construct. Sequences deleted from ΔPSE2 extend from –395 to –576 bp upstream of the start of *en* transcription. Sequences deleted from Δboth extend from –395 bp to –2407 bp. Misexpression (ME) is defined as detection of β-gal protein between the stripes. Number of lines with misexpression/total number of lines stained. Misexpression in P[en1] and P[ΔPSE2] lines was much less extensive than that seen in Δboth (see Fig. 2). (C) En (green) and β-gal protein (red) in a P[en1] line in a wild type and a *ph-d⁴⁰¹ ph-p⁶⁰²* double mutant embryo. Anterior is leftwards, dorsal is upwards. Ten hours AEL (after egg laying). DL, double label. En is present in a single PNS cell between the stripes, but β-gal is not.

1C). 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],

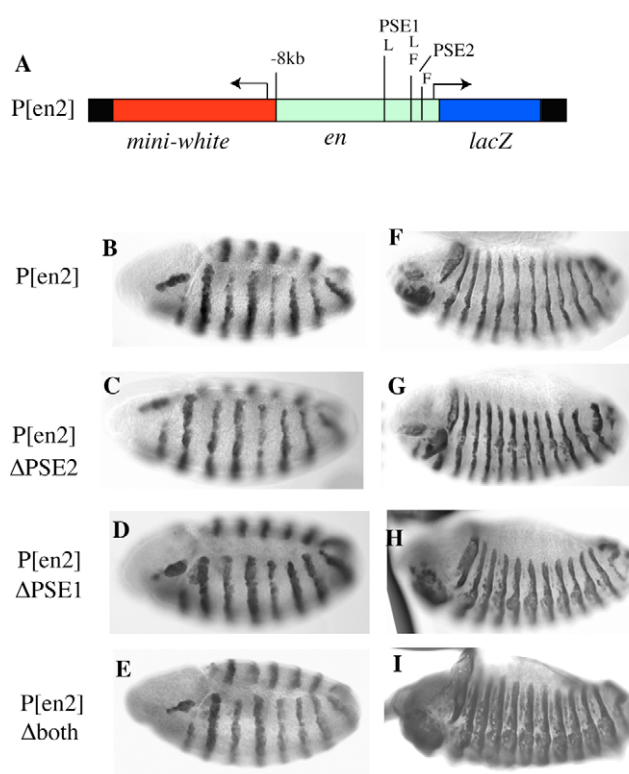


Fig. 2. Both PSE1 and PSE2 contribute to PRE activity.

(A) Construct P[en2] contains 8 kb of upstream *en* sequences with PSE1 and PSE2 flanked by LoxP (L) and FRT (F) sites, respectively. (B–I) Immunoperoxidase staining detecting β-gal protein in embryos from a P[en2] line and its derivatives obtained by treating with Flp and/or Cre recombinase. Embryos shown are 6 hours AEL (B–E) and 10 hours AEL (F–I); anterior is leftwards, dorsal is upwards. All are lateral views, except C, which is a ventrolateral view. Expression is initiated correctly in all lines, then β-gal is misexpressed in cells between the stripes in ΔPSE2, ΔPSE1 and Δboth lines. Deletion of PSE2 causes relatively minor misexpression (G). Deletion of PSE1 is more extensive (H), and deletion of both causes the most misexpression between the stripes (I).

Fig. 1B). We examined expression from multiple lines from each construct. If β-gal is being accurately expressed, β-gal protein is only detected in the regions of *en* stripes in embryos (Fig. 1C). Detection of β-gal in cells between the stripes indicates that expression is not accurate (misexpression, ME, Fig. 1B). Misexpression began at about 7 hours of development, when PcG repression normally occurs. In two out of six P[en1] chromosomal insertion sites, a few cells between each stripe expressed β-gal at similar levels to those seen in Fig. 2G. When PSE2 was deleted in P[ΔPSE2], five of seven lines had a small amount of misexpression. By contrast, all 11 lines generated from P[Δboth] gave a large amount of misexpression, similar to what is shown in Fig. 2I and to what is seen when β-gal is expressed from P[en1] in a *ph* mutant background. These data showed that the fragment extending from –2.4 to –395 has PRE activity, but did not allow us to determine accurately the role of PSE2 in PcG repression.

In order to further delineate the role of PSE1 and PSE2 as *en* PRE elements, we looked at the effect of deleting PSE1 and PSE2 from the same insertion site. We made a construct (P[en2], Fig. 2A) that contains the same 8 kb of upstream sequences, but where PSE2 is flanked by FRT sites and PSE1 is flanked by loxP sites, allowing us to remove either one or both fragments at a single genomic location.

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] Δ both at three out of four insertion sites (misexpression in the fourth line was already massive in the Δ PSE1 and Δ PSE2 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] Δ PSE1 (Fig. 2H), which contains PSE2, with the much more extensive misexpression seen in P[en2] Δ both (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 insertion 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; N  gre 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.

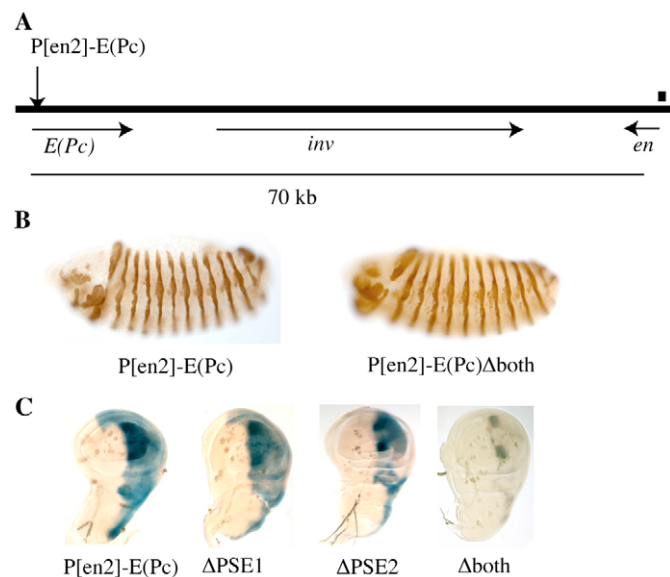


Fig. 3. PRE action over a distance. (A) Location of P[en2] in line P[en2]-E(Pc). Thick line indicates genomic DNA. Arrows below the line indicate the extent and direction of transcription of *E(Pc)*, *inv* and *en*. Black box above the line indicates the location of the PSE1 and PSE2 DNA in the genomic *en* gene. Arrow above the line denotes the location of P[en2] in the *E(Pc)* gene. (B) Immunoperoxidase staining showing β -gal protein in the starting line P[en2]-E(Pc) and in P[en2]-E(Pc) Δ both. β -Gal is expressed in only a few cells between the stripes in P[en2]-E(Pc) Δ both, in contrast to the massive misexpression seen at other chromosomal locations (see Fig. 2I). Embryos are anterior leftwards, dorsal upwards, 10 hours AEL. (C) X-gal staining of wing discs from P[en2]-E(Pc) and its Flp and/or Cre recombinase-treated derivatives. β -Gal activity in the posterior compartment (where *en* is normally expressed) is largely dependent on the presence of PSE1 or PSE2, although a small amount of β -gal activity remains in the Δ both derivative.

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 P[en3] 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* from P[en3]-*en* (Fig. 4C). Enhancer traps in *tou* are usually not expressed like *en*, but expression from P[en3]-*tou* showed many aspects of *en* 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 trxB 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 at ~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 led 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

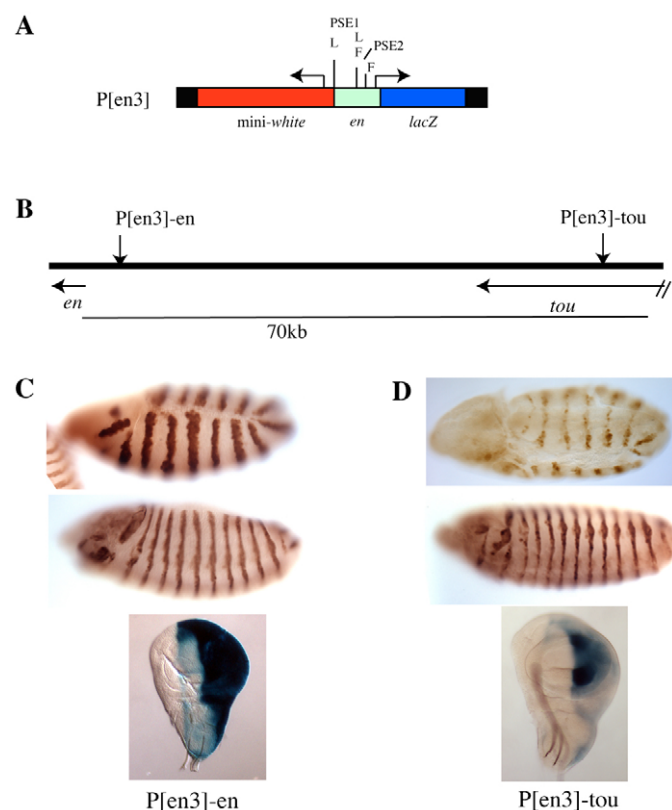


Fig. 4. P[en3] inserted in *tou* is expressed in an *en*-like manner.

(A) The P[en3] construct contains *en* DNA from -2407 bp to +188 bp. PSE1 is flanked by LoxP sites (L), PSE2 is flanked by FRT sites (F). (B) Thick line indicates genomic DNA. Arrows below the line indicate the transcription units and the direction of transcription of *en* and *tou*. The start of *tou* transcription is 10 kb upstream of what is shown. Arrows above the line indicate the position of insertion of P[en3] in two independent lines, P[en3]-*en* and P[en3]-*tou*. (C,D) Immunoperoxidase staining to detect β -gal protein in embryos and X-gal staining to detect β -gal activity in wing discs of lines P[en3]-*en* (C) and P[en3]-*tou* (D). Embryos are oriented with anterior leftwards and dorsal upwards. Upper embryos are ~6 hours AEL. Lower embryos are ~10 hours AEL. β -Gal expression in stripes is not initiated until about 6 hours AEL in P[en3]-*tou* embryos, and staining is apparent within individual cells of stripes before the entire stripe is stained. Wing discs were stained for equivalent amounts of time.

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] Δ PSE1-*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] Δ both-*tou* discs. The activating effects of PSE1 and PSE2 in larvae are also seen when examining expression from P[en2] inserted in *E(Pc)* (Fig. 3C). Although the β -gal expression pattern in wing discs from P[en2]-*E(Pc)* is remarkably similar to *en*, deletion of both PSE1 and PSE2 from this line drastically lowered the expression level. Thus, although PSE1 and PSE2 are not required for striped embryonic expression from P[en2]-*E(Pc)* (Fig. 3B, because P[en2] has 8 kb of upstream DNA and carries the embryonic enhancers), they are necessary to activate *lacZ* expression in larvae, where activity is coming from endogenous *en/inv* enhancers. These data clearly show that both PSE1 and PSE2

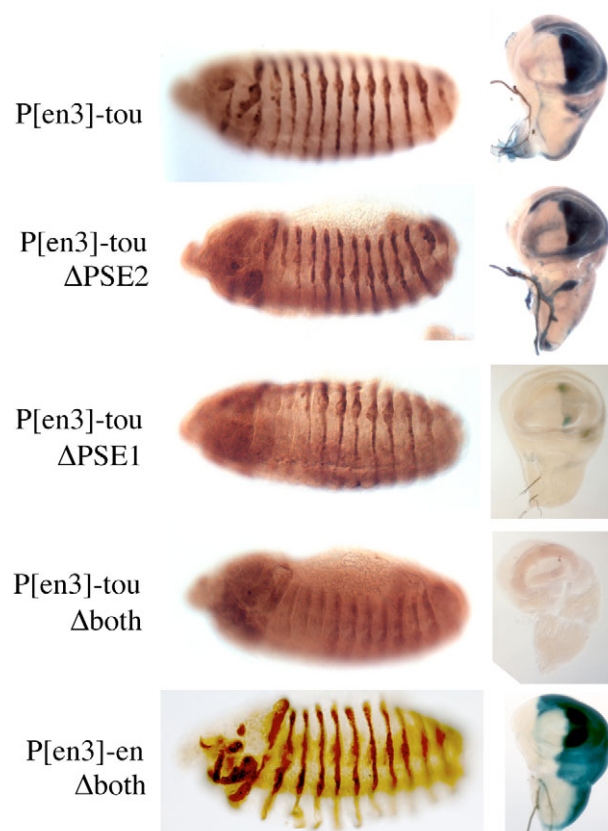


Fig. 5. PSE1 and PSE2 can act as positive regulatory elements. Left column shows immunoperoxidase staining to detect β -gal protein in line P[en3]-*tou* and its derivatives and from P[en3]-*en* Δ both (embryos 10 hours AEL; anterior is leftwards, dorsal is upwards). Right column indicates wing discs stained for β -gal activity. Two things happen in embryos when the PSEs are removed from P[en3]-*tou*: (1) the intensity of the stripes decreases and (2) the β -gal background levels increase. In wing discs, removal of PSE2 causes some misexpression of β -gal in the anterior compartment in the wing pouch in P[en3]-*tou* Δ PSE2 wing discs. Similar results were seen in other discs. Deletion of PSE1 from P[en3] 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.

are able to mediate activation by *en* enhancers that are located many kilobases away from the insertion sites of P[en3]-*tou* and P[en2]-*E(Pc)*.

PSE1 and PSE2 can interact with many regulatory elements in the genome

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 of P[en3] and P[en3] Δ 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 discs

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] Δ PSE1-9C and expression everywhere in P[en3] Δ both-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] Δ both-9C larval brain, and this expression is increased in both P[en3] Δ PSE1 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 PSEs 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 *en* ^{Δ 530}. Our data suggest that the sequences deleted in *en* ^{Δ 530} are important for *en* activation. *en* ^{Δ 530} 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 *en* ^{Δ 530} 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 *en* ^{Δ 530} or when *en* ^{Δ 530} was combined with an *en/inv* double mutant or a deficiency for the region. This suggests that either *En* or *Inv* can partially compensate for the loss of both in *en* ^{Δ 530}. This is consistent with data that suggests *En* and *Inv* have redundant activities (Gustavson et al., 1996).

Because *en* ^{Δ 530} completely deletes PSE2, we examined embryos to see whether *En* or *Inv* antigen could be detected in cells between the stripes in *en* ^{Δ 530} homozygous embryos. We did not observe any misexpression of *En* or *Inv* in *en* ^{Δ 530} homozygous embryos. Thus, it appears that the repressive activity of PSE2 can be replaced by other PREs located throughout the *en* locus.

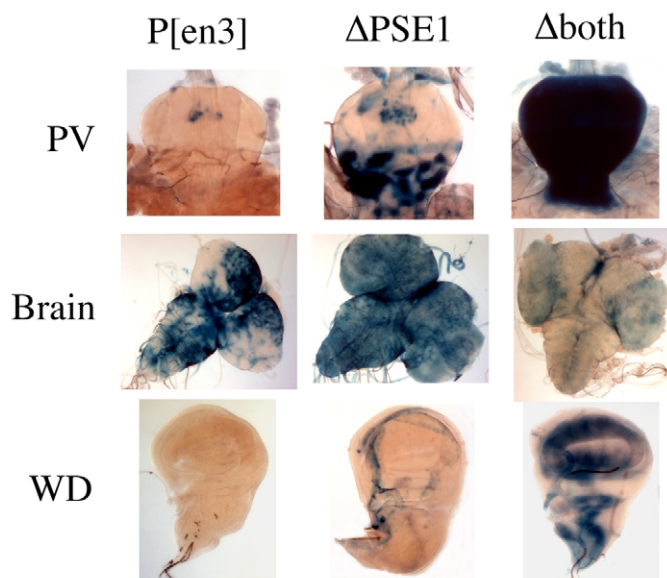


Fig. 6. *en* PSEs can mediate both positive and negative signals from many enhancers and silencers. P[en3] is inserted into the *capulet* transcription unit. β -Gal activity in proventriculus (PV), brain and wing disc (WD) is shown. In the PV, loss of both PSE1 and PSE2 leads to ubiquitous expression of β -gal, whereas in the brain, loss of both PSE1 and PSE2 decreases the expression. PSE2, which is present in Δ PSE1, seems to repress transcription in the proventriculus and wing disc, but activates it in the brain.

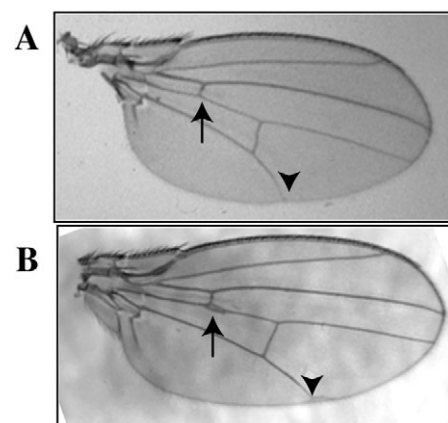


Fig. 7. Deletion of 530 bp including PSE2 in the endogenous *en* gene leads to abnormalities in the posterior compartment in wings. (A) Wild-type wing. (B) Wing from *en* ^{Δ 530} homozygotes. Arrows indicate the intersection of the anterior crossvein with the IV longitudinal vein where a defect is seen in the *en* ^{Δ 530} homozygotes. Arrowheads indicate the intersection of longitudinal vein with the wing margin. This vein splits before the intersection with the margin in *en* ^{Δ 530} homozygotes.

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; Sipos 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; Maurange 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 *en*^{Δ530}) 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

Table 1. *en*^{Δ530} is a loss-of-function mutation

Genotype*	Wing phenotype†
<i>en</i> ^{Δ530} /+	0% (0/60)
<i>en</i> ^{Δ530} /Df(2R) <i>enX31</i>	91% (91/100)
<i>en</i> ^{Δ530} / <i>inv</i> ³⁰ <i>en</i> ⁹	91% (63/68)
<i>en</i> ^{Δ530} / <i>en</i> ^{1B86}	30% (28/94)
<i>en</i> ^{Δ530} / <i>en</i> ^{1M99}	22% (19/83)
<i>en</i> ^{Δ530} / <i>en</i> ^{10L}	60% (72/120)
<i>en</i> ^{Δ530} / <i>inv</i> ^{17en26}	21% (18/84)
<i>en</i> ^{Δ530} / <i>en</i> ^{Δ530}	93% (54/58)
<i>inv</i> ^{17en26} /Df(2R) <i>enX31</i>	94% (64/68)

*Df(2R)*enX31* deletes both *en* and *inv* (Kuner et al., 1985). *inv*³⁰*en*⁹ are null mutations in *inv* and *en*; *en*^{1B86}, *en*^{1M99} and *en*^{10L} are all *en* null mutations (Gustavson et al., 1996). *inv*^{17en26} 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).

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 *en* 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.

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Supplementary material

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

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