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Maintenance of the *engrailed* expression pattern by *Polycomb* group genes in *Drosophila*

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Summary

The stable maintenance of expression patterns of homeotic genes depends on the function of a number of negative *trans*-regulators, termed the *Polycomb* (*Pc*) group of genes. We have examined the pattern of expression of the *Drosophila* segment polarity gene, *engrailed* (*en*), in embryos mutant for several different members of the *Pc* group. Here we report that embryos mutant for two or more *Pc* group genes show strong ectopic *en* expression, while only weak derepression of *en* occurs in embryos mutant for a single *Pc* group gene. This derepression is independent of two known activators of *en* expression: *en* itself and *wingless*. Additionally, in contrast to the strong ectopic expression of homeotic genes observed in *extra sex combs*$^-$ (*esc*$^-$) mutant embryos, the *en* expression pattern is nearly normal in *esc*$^-$ embryos. This suggests that the *esc* gene product functions in a pathway independent of the other genes in the group. The data indicate that the same group of genes is required for stable restriction of *en* expression to a striped pattern and for the restriction of expression of homeotic genes along the anterior-posterior axis, and support a global role for the *Pc* group genes in stable repression of activity of developmental selector genes.

Key words: *Drosophila*, *Polycomb* group, *engrailed*, homeotic, imprinting, maintenance.

Introduction

Molecular and genetic studies have uncovered the basis for pattern formation in *Drosophila* (St Johnson and Nüsslein-Volhard, 1992; Ingham, 1988). Early interactions between maternal and transiently expressed zygotic transcription factors result in the subdivision of the embryo into a series of segments, each composed of an anterior and posterior population of cells. Segmentation requires expression of the *engrailed* (*en*) gene in the posterior group of cells in each segment (Lawrence and Morata, 1976; Kornberg et al., 1985), while segmental identity is specified by the selective expression of the homeotic genes of the bithorax and Antennapedia complexes (Lewis, 1978; Kaufman et al., 1980; Sanchez-Herrero et al., 1985; Harding et al., 1985). Unlike the early regulators, that are expressed transiently, *en* and the homeotic genes are expressed in spatially restricted patterns throughout development. Maintenance of patterned expression involves controls distinct from those that guide pattern establishment (Heemskerk et al., 1991; Struhl and Akam, 1985).

The controls that govern stable expression of selector genes are especially interesting, because they may identify the mechanisms responsible for stable determination of developmental fate. Genetic approaches have identified a group of negative regulators, the *Polycomb* (*Pc*) group genes (Duncan, 1982; Jürgens, 1985), that are essential for maintaining spatial restrictions of homeotic gene expression. The *Pc* group currently includes eleven genes classified according to their similar homeotic phenotypes. In *Pc* group mutant embryos, the apparently correct initial expression of homeotic genes decays into global expression. This results in transformation of most body segments towards a more posterior segment fate.

If the *Pc* group genes play a general role in stable determination of cell fate, we might expect them to control the stable pattern of *en* expression. There is some suggestive evidence that they might do so. Most directly, ectopic expression of *en* was observed in *polyhomeotic*$^-$ (*ph*$^-$) mutant embryos (Dura and Ingham, 1988; Smouse et al., 1988). Furthermore, Busturia and Morata (1988) observed derepression of an *en-Lac-Z* fusion gene in *Pc*$^-$ clones in mosaic wings of adult animals. Finally, larval cuticle defects seen in embryos lacking both maternal and zygotic *Polycomb-like* (*Pcl*) or *pleiohomeotic* (*pho*) function are consistent with an effect of these genes on segmentation gene expression (Breen and Duncan, 1986). Nonetheless, an involvement of *Pc* group genes in *en* regulation has not been generally accepted because of three factors. (1) *ph* mutant embryos exhibit pleiotropic phenotypes beyond those observed in embryos mutant for other *Pc* group genes (Dura et al., 1987); therefore, the observed derepression of *en* cannot be generalized to other members of this group, (2) Busturia and Morata (1988) failed to detect derepres-

sion of *en* in embryos mutant for *Pc* itself, and (3) segmentation defects are slight, or nonexistent even in *Pc* group mutant backgrounds that give severe homeotic transformations. To explore this issue directly, we examined the *en* expression pattern in embryos mutant for single or multiple *Pc* group genes. In contrast to a nearly normal expression pattern in most single mutant embryos, we find strong ectopic *en* expression in double and triple *Pc* group mutant embryos. The requirement for *Pc* group function in repression of *en* supports a global role for *Pc* group genes in maintaining the repressed state of developmental regulators.

Materials and methods

The following mutant alleles were analyzed in this study: Pc^3 and *Df (3L) Pc-Mk*; Scm^{XF24}, Scm^{D1}, and Scm^{D2}; Pcl^{E90}, and Pcl^{D5}; Psc^1, and *Df (2R)* vg^D; Asx^{XT129}, Asx^{IIF51}, Asx^{XF23}, and *Df (2R) trix*; *E(Pc)*; pco^{25}; ph^{503}; l(4)29^b (*pho*); esc^{10} and esc^2; and wg^{cx4}. All mutant alleles have been previously described (Duncan, 1982; Jürgens, 1985; Breen and Duncan, 1986; Dura et al., 1987; Phillips and Shearn, 1990; Brunk et al., 1991; Baker, 1987). Recombinant chromosomes and other mutant combinations were made using standard genetic crosses. The triple mutant on the second chromosome was obtained from Jürgens (described by Jürgens, 1985). Psc^1 was lethal in combination with Pc^3. Instead of Psc^1, we used *Df (2R)* vg^D (a deletion of the *Psc* locus) in combination with Pc^3. esc^- mutant embryos were collected from esc^{10}/esc^2 adult flies. esc^{10}/esc^2 adults were obtained by crossing esc^{10} males to females heterozygous for esc^2 and a duplication containing the *esc* locus exactly as described by Struhl (1983). esc^{10} is a deletion of the *esc* locus and esc^2 is an apparent null allele (for further details see Struhl, 1983). Wild-type embryos were of the sevelen strain.

For immunological and in situ detection embryos were collected and fixed following standard procedures. The *en* protein was detected using a monoclonal antibody that recognizes the *en* and *invected* proteins (Patel et al., 1989). Detailed protocols for reaction of embryos with antibodies and detection using the peroxidase reaction were those described by Kellerman et al. (1990). In situ hybridization using a digoxigenin-labeled *Abd-B* cDNA was as described previously (Tautz and Pfeifle, 1989). This cDNA probe hybridizes to all of the transcripts of Abd-B (Kuziora and McGinnis, 1988).

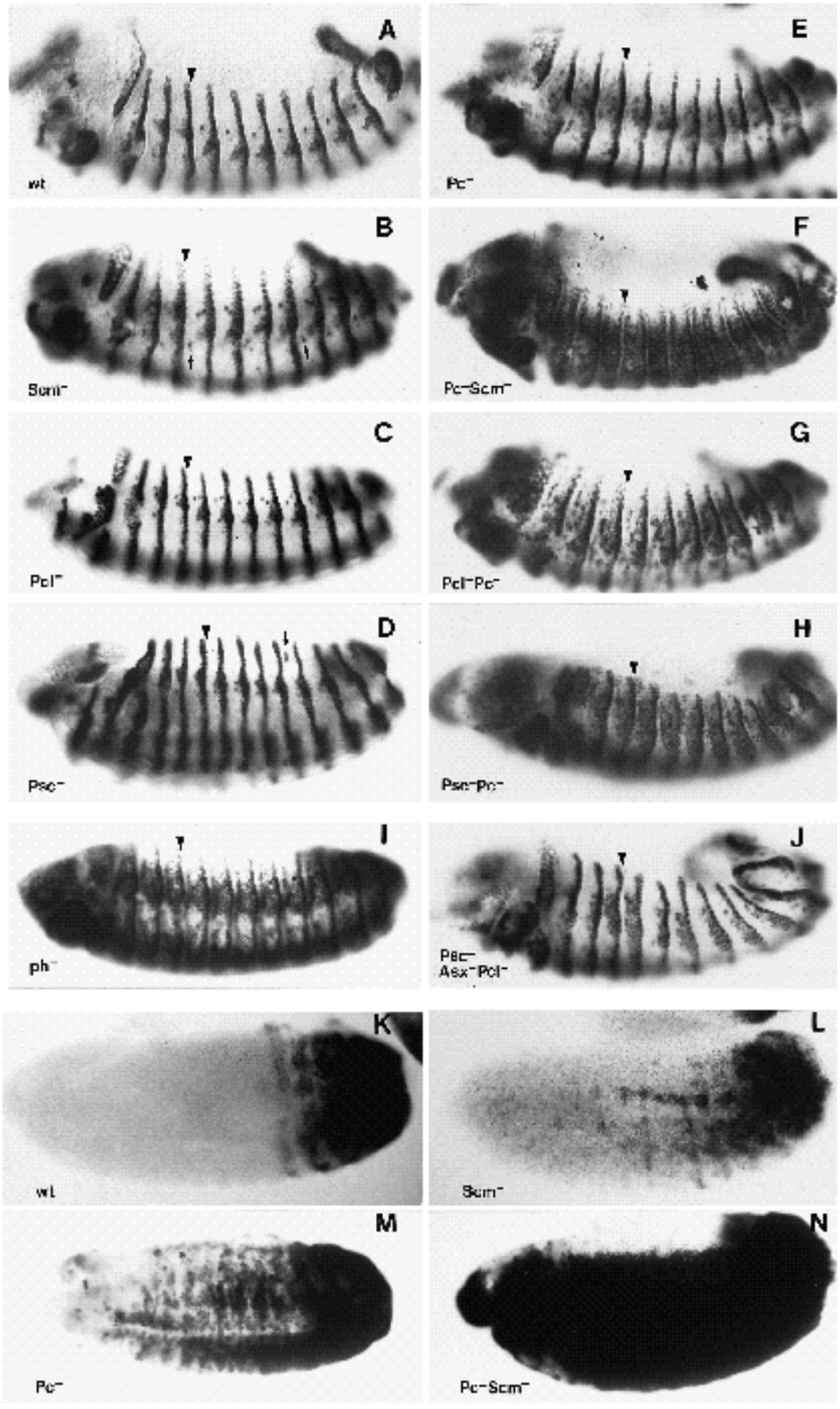
Results and discussion

We used an antibody to the *en* protein to examine its expression in embryos mutant for the *Pc* gene itself, or mutant for other members of the *Pc* group. In addition to the striped expression found in the wild type (Fig. 1A; DiNardo et al., 1985), in Pc^- embryos ectopic *en*-expressing cells are found scattered in the anterior of every segment, mainly in the lateral region of the epidermis (Fig. 1E). It is not surprising that this derepression was previously missed (Busturia and Morata, 1988), as it occurs in relatively late embryos and sensitive staining is required to detect it. Embryos mutant for other *Pc* group genes also show defects in *en* expression that range in severity. Examples include subtle defects, such as duplication of the lone *en*-positive cell in the anterior compartments of the first through the seventh abdominal segments (A1-A7) in *Sex combs on mid-legs*$^-$ (*Scm*$^-$; Fig. 1B) and *Pcl*$^-$ (Fig. 1C) embryos, as well as patchy expression in the anterior of some segments in *Posterior sex combs*$^-$ (*Psc*$^-$, Fig. 1D), *pleiohomeotic* (*pho*$^-$), and *polycombeotic*$^-$ (*pco*$^-$, also called *Enhancer of zeste*, Jones and Gelbart, 1990) embryos (data not shown).

The effects of *Pc* group mutations are synergistic in that embryos homozygous for two or three weak mutations show strong homeotic transformation (Jürgens, 1985). To test whether such synergy might enhance some of the weak effects on *en* expression, we stained embryos homozygous for two or three different *Pc* group mutations. All mutant combinations tested gave extensive ectopic *en* expression (Fig. 1F-H, J). The degree of derepression depends on the particular combination of mutant alleles. The double mutants Pc^-Scm^- and Psc^-Pc^- (Fig. 1F, H) provide dramatic examples of synergy, but in all combinations derepression is invariably stronger than the sum of the effects in single mutants (compare Fig. 1B,C,D, and E with F,G, and H; note that derepression in these backgrounds is not due to homeotic transformations, see Fig. 3 and below). Synergy also appears to apply to phenotype: the double and triple mutant embryos have frequent defects in their larval cuticles (D. M., unpublished). However, segmentation still occurs. We believe that the subtlety of segmental defects is due to the late occurrence of widespread ectopic *en* expression in these mutant embryos. The earliest ectopic expression, observed in 5-6 hour embryos, occurs with low penetrance and is restricted to a small number of cells primarily in the dorsal ectoderm region. Derepression increases and spreads progressively until it encompasses much of the embryo by 9-10 hours of development.

As in the case of homeotic genes (Struhl and Akam, 1985; Riley et al., 1987; Kuziora and McGinnis, 1988; Jones and Gelbart, 1990; Simon et al., 1992), the initial *en* pattern in the *Pc* group mutant embryos appears normal. Ectopic expression is only detectable after the completion of the early tiers of regulation that establish and refine the *en* striped pattern (DiNardo et al., 1988; Heemskerk et al., 1991). Thus, *Pc* group genes encode factors required for maintenance of the expression pattern of *en*, a segment polarity gene, as well as maintenance of expression patterns

Fig. 1. Localization of *en* (A-J) and *Abd-B* (K-N) in wild-type and *Pc* group mutant embryos. The *en* protein and the *Abd-B* transcripts were detected as described in Materials and methods. In wild-type embryos (A), *en* is expressed in a series of stripes about 1-2 cells wide at this stage of development (~9.5-10.5 hours; DiNardo et al., 1985). Mid-laterally a spur of *en* expression extends anteriorly. The first through the seventh abdominal segments (A1-A7) also contain a lone *en*-positive cell in their anterior domains; arrowheads provide a reference point between the third thoracic (T3) and the first abdominal (A1) segments. Mutant embryos (B-J) show different degrees of ectopic *en* expression (see text). Arrows in B and D point to examples of subtle ectopic expression. The embryo in H is about 20 minutes younger than the rest. *Abd-B* transcripts are expressed in parasegments 10-14 in wild type (K; Kuziora and McGinnis, 1988) and ectopically in the more anterior parasegments in mutant embryos (L-N). Representative mutant alleles are shown here: Pc^3 (E,F,G, H,M,N), Scm^{XF24} (B,F,L,N), Pcl^{E90} (C,G), Psc^1 (D), and *Df (2R)* vg^D (H,J); Asx^{XF23} (J), ph^{503} (I).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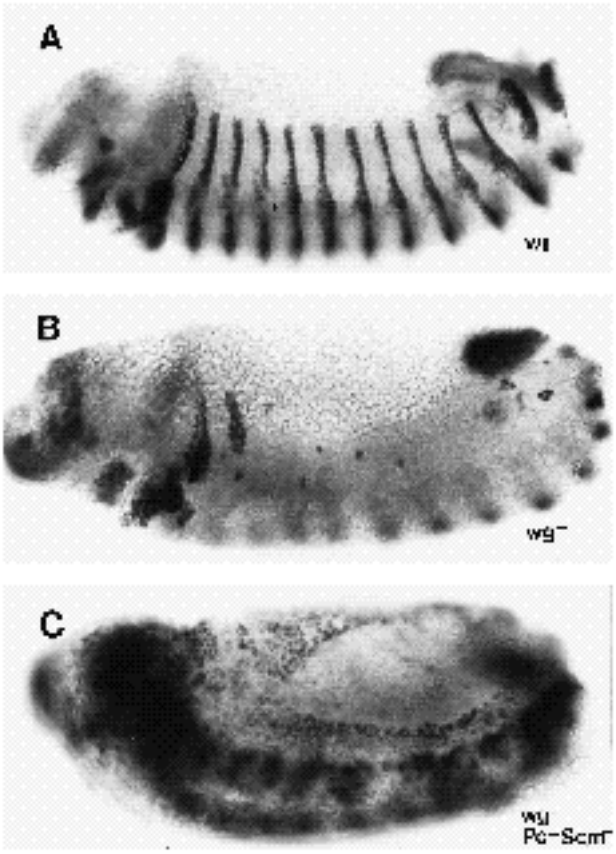

Fig. 2. *wg*-independent derepression of *en*. (A) wt; (B) wg^{-}; (C) wg^{-}; $Pc^{-}Scm^{-}$. The *en* protein was detected as described in Materials and methods. Embryos are the progeny of wg^{cx4}/+;$Pc^{3}Scm^{XF24}$/+ heterozygotes.

of the homeotic genes. This suggests a general role for the *Pc* group in maintaining expression patterns of developmental regulators in *Drosophila.*

Following its initial induction by pair-rule gene products, continued *en* expression in the embryo requires input from at least two other pathways (DiNardo et al., 1988; Martinez-Arias et al., 1988; Heemskerk et al., 1991). These involve autoregulation and cell-cell communication. The latter requires expression of the segment polarity gene *wingless* (*wg*) in a row of cells adjacent to the *en* stripe. We asked whether the ectopic *en* expression in *Pc* group mutant backgrounds is also dependent on these pathways for either its initial activation or maintenance. To test this, we examined the pattern of *en* expression in $wg^{-};Pc^{-}Scm^{-}$ mutant embryos. By germ band shortening, in wg^{-} embryos ectodermal *en* expression has completely decayed (Fig. 2B; DiNardo et al. 1985). But in $wg^{-};Pc^{-}Scm^{-}$ triple homozygous embryos, we detect a lawn of *en*-expressing cells throughout the ectoderm (Fig. 2C). Since extensive cell death occurs in wg^{-} mutant embryos, the *en*-positive cells in these embryos probably represent most of the surviving ectodermal cells. Similar results were obtained with $en^{-};Pc^{-}Scm^{-}$ mutant embryos (using the en^{cx1} allele and a polyclonal antibody capable of recognizing the truncated *en* protein produced by en^{cx1}; D. M., unpublished). Therefore, in *Pc* group mutant backgrounds ectopic *en* expression occurs independently of functions of *en* and *wg*, two activators of *en* expression. Derepression is most likely also independent of pair-rule activators of *en*, as it occurs at a time when most pair-rule gene products have decayed (for example, see Kellerman et al., 1990). Derepression probably results from the action of positive regulators such as *trithorax* (*trx*) that are known to antagonize *Pc* group mutant phenotypes (Ingham, 1983; Kennison and Tamkun, 1988; Shearn, 1989). However, we note that removal of the zygotic function of *trx* itself does not suppress *en* derepression in two *Pc* group mutant combinations that we have tested so far: ectopic *en* expression in $trx^{-}Pc^{-}$ and $trx^{-}Pc^{-}Scm^{-}$ mutant embryos is indistinguishable from the derepression observed in Pc^{-} and $Pc^{-}Scm^{-}$ embryos, respectively (D. M., unpublished).

If *Pc* group genes play related roles in the control of *en* and homeotic genes, we expect a parallel in their effect on expression of these genes. Using in situ hybridization to examine the expression of *Abd-B*, a homeotic gene that controls the developmental fate of the more posterior abdominal segments (Sanchez-Herrero et al., 1988), we found a general correlation in the degree of its derepression in *Pc* group mutant backgrounds with that of *en*. For example, both *en* and *Abd-B* are derepressed weakly in Scm^{-} (compare Fig. 1B and L), moderately in Pc^{-} (compare Fig. 1E and M), and very strongly in $Pc^{-}Scm^{-}$ double mutant embryos (compare Fig. 1F and N). A similar relationship also holds for other mutant backgrounds that we have tried (ph^{-}, Psc^{-}, Asx^{-}, Pcl^{-} single mutants, and the triple mutant $Psc^{-}Asx^{-}Pcl^{-}$; data not shown). However, there is one exception to the correlation. In embryos in which both the maternal and zygotic functions of *esc* have been removed, *en* is unaffected (other than changes due to homeotic transformations, see Fig. 3B and legend), whereas *Abd-B* is strongly derepressed (Fig. 3D). Thus, the remaining *Pc* group genes can still restrict *en* expression in the complete absence of the *esc* gene product. This supports the hypothesis (Struhl, 1983) that *esc* functions in a pathway independent of the other genes in the group.

The segmentally repeated expression of *en* is fundamentally different from the segment-specific expression pattern of homeotic genes. The domain of expression of each homeotic gene is confined by an anterior parasegmental boundary; *en* on the other hand is expressed in the posterior of *every* segment. Lewis (1978) suggested that the anterior boundary of the homeotic expression domains could be defined by a gradient of *Pc* repressor, each homeotic gene having a different sensitivity to this repressor (Fig. 4A). While it was subsequently shown that (1) the *Pc* transcript is uniformly distributed in the embryo (Paro and Hogness, 1991) and (2) the patterns of homeotic gene expression are initially established correctly in esc^{-} and Pc^{-} mutant embryos (Struhl and Akam, 1985; Kuziora and McGinnis, 1988), it still remained possible that maintenance of homeotic gene expression patterns relies on a gradient of *Pc* protein or a gradient in the level of product of one of the other *Pc* group genes. However, the *Pc* group gene products are required for repression of *en* in the anterior domain of *every* segment. The similar level of this requirement in each segment suggests that *Pc* group function is uniformly present in the embryo.

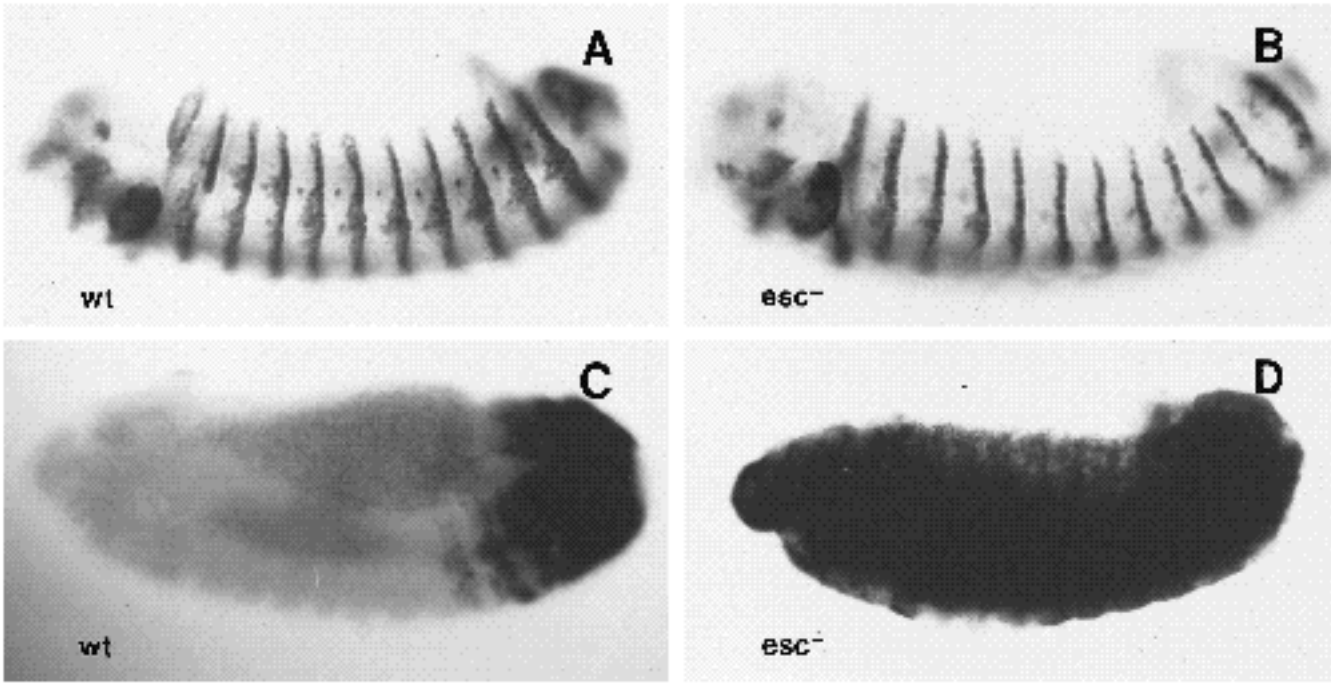

Fig. 3. Comparison of expression of *en* (A,B) and *Abd-B* (C,D) in *esc*⁻ mutant embryos. Note that although *en* is not derepressed in *esc*⁻ mutant embryos, the morphology of all stripes resembles that of an A8 stripe. *esc*⁻ mutant embryos were collected from esc^{10}/esc^{2} adult flies constructed as described in Materials and methods.

Finally, it is important to note that genes regulated by a common set of *Pc* group repressors display opposite states of activity within the same cell (Fig. 4). For example, in the anterior of A5, *Pc* group repression maintains *en* in the off state but allows *Abd-B* expression, whereas in the posterior of A3, it maintains *Abd-B* in the off state but allows *en* expression (Fig. 4B). If, as we have argued, *Pc* group function is uniformly present in the embryo, an intriguing question remains: how is repression controlled so as to allow opposite states of activity of the loci it regulates? One possibility is that the *Pc* group genes encode passive ubiquitous co-factors whose activity is dictated by other, as yet unknown, regulators. These putative regulators would have to be expressed in patterns that complement the expression patterns of *en* and each of the homeotic genes. However, this hypothesis is not satisfying, because extensive genetic analysis has failed to uncover any such regulators, and because it fails to explain two features of regulation by the *Pc* group genes: (1) derepression of *en* in some *Pc* group mutant embryos (e.g. *Pc*⁻ and *Psc*⁻ embryos, Fig. 1D and E) is stochastic in nature and appears independent of patterned regulators, and (2) *Pc* group genes maintain the patterns of expression precisely as established by earlier-acting transiently expressed regulators. Even defective patterns occurring in embryos mutant for some of the early regulators are stably maintained (Kellerman et al., 1990), presumably in a *Pc* group-dependent fashion. We therefore favor another possibility involving imprinting as proposed by Paro and co-workers (Paro, 1990; Franke et al. 1992). Imprinting would provide a means for the stable maintenance of the off states of expression, as set up by the early regulators, in a manner analogous to maintenance of methylation patterns in mammalian cells (Allen et al., 1990).

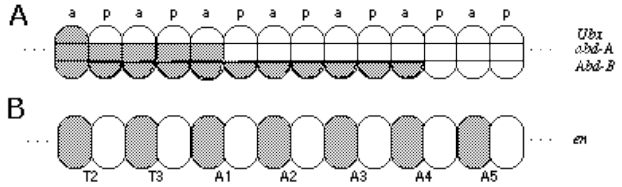

Fig. 4. Requirement for the *Pc* group in maintaining the off state of *en* and the homeotic genes suggests that *Pc* group function is uniformly present in the embryo. Ovals represent anterior (a) or posterior (p) cell nuclei of the second thoracic (T2) through the fifth abdominal (A5) segments in the epidermis along the anterior-posterior body axis. In A, each nucleus is divided into three parts, which designate the different homeotic loci that are simultaneously controlled by the *Pc* group. B shows the state of expression of *en* in nuclei corresponding to those in A. Shaded areas denote a locus under *Pc* group repression and unshaded areas show the regions where a locus is active. A schematic diagram depicting the graded repressor model as envisioned by Lewis (1978)is shown in A. All of the genes in the bithorax complex are inactive in the more anterior segments of the embryo (e.g. anterior T2) where repressor activity is high. Conversely, in the more posterior segments of the embryo (e.g. posterior A4), where repressor activity is low, all of the genes in the complex are active. As shown, the sensitivities of *en* (B) and the homeotic genes (A;*Ubx, abd-A, and Abd-B*) are independently controlled in a fashion that cannot be reconciled with genes having a different level of sensitivity to a common graded repressor. *Ubx, Ultrabithorax; abd-A, abdominal-A; Abd-B, Abdominal-B*.

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