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Genetics

Segmental determination in *Drosophila* conferred by hunchback (*hb*), a repressor of the homeotic gene Ultrabithorax (*Ubx*)

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ABSTRACT **The activity of homeotic genes in *Drosophila* cells determines segment-specific morphogenesis. Here, we provide evidence that the product of hunchback (*hb*), a segmentation gene, acts as a direct repressor or "silencer" of the homeotic gene Ultrabithorax (*Ubx*) and thus prevents ectopic activity of this gene: we show, by stable integration of reporter gene constructs, that hb protein binding sites are capable of repressing at a distance the activity of an embryonic *Ubx* enhancer outside the *Ubx* expression domain. This silencing activity is observed at advanced embryonic stages, at a time when the *hb* gene product is no longer detectable or required, and is dependent on the function of Polycomb (*Pc*). We propose a working hypothesis as to how hb protein in a "hit-and-run" fashion may effect stable and heritable silencing of the *Ubx* gene throughout advanced stages of development, thus mediating repression of this homeotic gene outside its realm of function.**

Drosophila cells become segmentally determined at an early embryonic stage and retain, without undergoing differentiation, their determined state through many cell divisions (1, 2). Segmental determination is conferred by the activity of homeotic genes such as Ultrabithorax (*Ubx*), whose product is necessary (3, 4) and to some extent sufficient (5, 6) to control the morphogenesis of segment-specific features. The *Ubx* gene is activated in a midembryonic region of the blastoderm embryo (7), and its continuous activity in the descendants of these embryonic cells is needed until differentiation starts (8).

Boundaries of early *Ubx* expression depend on the segmentation gene hunchback (*hb*) (9–11), whose product carries anteroposterior positional information by virtue of its restricted distribution in the early embryo: hb protein (HB) is found at high levels anteriorly as well as posteriorly in the blastoderm embryo (12), in embryonic regions that are near-complementary to the early *Ubx* expression domain (7). HB binding sites were found within *Ubx* control elements, and these apparently mediate repression of the *Ubx* gene at the blastoderm stage (13, 14). HB fades to undetectable levels soon after this stage (12), and *hb* function becomes dispensable from there onwards (10, 15, 16). Maintenance of *Ubx* expression boundaries throughout later stages depends on the function of a group of genes (4, 17, 18) of which Polycomb (*Pc*) is the best known member.

Previously, we found that *Ubx* expression boundaries at advanced embryonic stages are mediated by repressor control elements located in remote regions of the *Ubx* gene (19). These elements act at long distance to suppress outside the *Ubx* expression domain the activity of an embryonic *Ubx* enhancer element (termed BXD) which, in the absence of the repressor elements, confers a *Ubx*-like expression pattern in virtually every parasegment (ps) from head to tail. One of the repressor elements contains HB binding sites (14). Here, we provide evidence that HB acts directly to repress BXD-mediated activity. As this repression occurs at long distance, we shall refer to it as "silencing" (20). Moreover, we show that the silencing activity from HB binding sites is evident at late embryonic stages during which HB is no longer detectable or required. We discuss a mechanism by which HB may effect *Ubx* repression and thus confer segmental determination in a "hit-and-run" fashion.

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MATERIALS AND METHODS

Plasmids. The basic BXD construct (based on a Carnegie 20 transformation vector) containing the minimal BXD control fragment linked to the proximal *Ubx* promoter and a β-galactosidase (β-gal) gene has been described (19). Unique *Not* I and *Kpn* I sites were engineered between the BXD fragment and the proximal *Ubx* promoter so that fragments and oligonucleotides could be inserted and tested for their silencing capacity. Oligonucleotides (hb-mes, hb-ep, and hb-mut, a mutant derivative of hb-mes) were designed to contain all consecutive residues protected by HB binding (14) and flanking *Bam*HI or *Kpn* I/*Xba* I linkers (Fig. 1); for hb-eve, 10 nucleotide residues as published (21) were joined to 4 randomly chosen residues and *Bam*HI linkers. Oligonucleotides were cloned as tandom copies at random orientations into a Bluescript vector, and their sequence was confirmed by direct sequence analysis. Clusters of four to eight copies were inserted as *Kpn* I/*Not* I fragments into the basic BXD construct at a distance of 3.1 kilobases (kb) upstream of the *Ubx* transcription start site and >1 kb away from the active BXD enhancer elements (Bea Christen and M.B., unpublished data) within the BXD fragment. A derivative from hb-mes was made in which the proximal *Ubx* promoter was substituted by a 70-kDa heat shock protein (hsp70) TATA box (see ref. 19); however, there was virtually no silencing in transformants of this construct (only 1 of 11 lines showed a hint of silencing; the other 10 lines showed none).

Strains. *cn; ry*^{42} flies were used for *P* element transformation, and transformed lines were isolated and made homozygous for the transposon as described (22). The following loss-of-function alleles were used: *hb*^{7M} (23) and *Pc*^{3} (4). Individual transformant lines were crossed into mutant strains, homozygous mutant embryos were isolated at a frequency of one in four and recognized by their altered morphology of the germ band (*hb*$^{-}$; ref. 23) or of the gut (*Pc*$^{-}$; ref. 24). Homozygous mutant *osk*^{346} mothers were used in crosses with male transformants to produce offspring embryos containing moderately high levels of uniformly distributed HB (25).

Abbreviations: β-gal, β-galactosidase; HB, hb protein; ps, parasegment(s).

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Analysis of Transformed Embryos. Embryos were stained with anti-β-gal antibody (Cappell) as described (19, 24). *hb*⁻ embryos and embryos derived from *osk*⁻ mothers were also stained with anti-Ubx antibody (26) to compare *Ubx* and β-gal gene expression patterns.

RESULTS

Silencing Activity of HB Binding Sites. We designed oligonucleotides containing HB binding sites from two different cis regulatory regions: two (hb-mes, hb-ep) from an upstream *Ubx* control region mediating long-range repression (19) and one (hb-eve) from the even-skipped (*eve*) gene mediating activity of *eve* in stripe 2 and 7 (21). An additional oligonucleotide with a mutant HB binding site (hb-mut) was synthesized, a derivative of hb-mes containing two base changes in the middle of the conserved poly(A) stretch (21). We made four different constructs by inserting, in each case, four to eight tandem copies of a different oligonucleotide into the basic BXD construct, to test for their silencing activity (Fig. 1). For comparison, we used a BXD construct with a 0.6-kb *Ubx* fragment (containing hb-mes, hb-ep, and five additional HB binding sites) (14) inserted the same way (termed "06"). We isolated several independent transformant lines in each case and analyzed transformed embryos for β-gal expression.

The BXD expression pattern was described earlier (19). It became detectable at ≈3.5 hr of embryonic development and resembled embryonic *Ubx* expression in abdominal segments. In every single transformant line, it extended throughout ps2–14 (Fig. 2 *a* and *b*). In contrast, among the hb-mes, hb-ec, and hb-eve transformants, we obtained lines in which this BXD pattern was essentially confined to ps6–13. β-gal staining in hb-mes lines was virtually fully suppressed outside ps6–13 at early (Fig. 2*c*) and at late embryonic stages (particularly ventrally—e.g., in the ventral nerve cord of 14- to 16-hr-old embryos in which BXD-mediated activity continued to be strong; Fig. 2*d*). The same was true in some hb-ep and hb-eve transformants (Fig. 2 *e–h*), though we saw weak "ectopic" staining in these cases. The extent of suppression varied between lines (Fig. 1), a variability that commonly is observed among *Drosophila* transformant lines (27) and is attributable to interference from adjacent chromosomal sequences (compare ref. 19). However, the suppression effect was never observed in transformants of the basic BXD construct (see above) nor in hb-mut transformants: in each of nine hb-mut lines, the β-gal pattern was indistinguishable from a normal BXD pattern (Fig. 2 *i* and *j*). Thus, HB binding sites are evidently capable of silencing BXD activity outside ps6–13. This silencing activity can be as efficient as that of the whole 06 fragment (Fig. 2 *k* and *l*).

We expected the silencing activity of HB binding sites to be eliminated (or much reduced; see ref. 25) in *hb* mutant embryos. Indeed, we found that β-gal staining, similar to Ubx staining (9), extended from head to tail in these mutants at all embryonic stages (not shown). Furthermore, we previously found that β-gal expression mediated by an early-acting *Ubx* enhancer is completely suppressed in embryos derived from homozygous *osk*⁻ mothers (14), most likely due to maternally provided HB that is uniformly distributed at moderately high levels in these embryos at the beginning of development (25). However, we found that BXD-mediated β-gal staining, like *Ubx* staining (11), became detectable after ≈6 hr of development at the posterior end of embryos derived from *osk*⁻ mothers (and hb-mes fathers; not shown). It is likely that, by the time the BXD enhancer becomes maximally active, maternal HB has faded below a threshold concentration required to initiate silencing. Indeed, recent experiments suggest that four doses, but not two doses, of maternal HB are sufficient for permanent silencing of the *Ubx* gene (28).

We noticed that β-gal expression in hb-mes, hb-ep, or hb-eve but not in hb-mut transformants tends to be reduced in ps9 and in medial regions of the central nerve cord (most conspicuous in hb-eve lines; Fig. 2*f*). This was unexpected as it implies that HB binding sites mediate suppression of β-gal staining in cells of the embryo whose ancestors never did seem to contain measurable levels of HB. The result indicates that a protein other than HB can bind to HB binding sites.

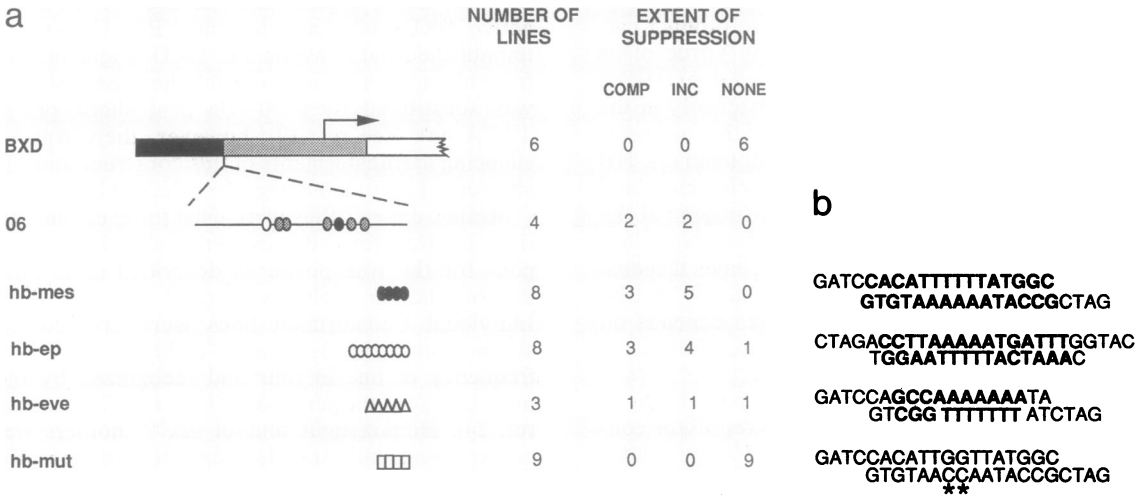

FIG. 1. Constructs and transformant lines. (*Left*) The top line shows the map (not to scale) of the basic BXD construct (19) containing the minimal BXD enhancer fragment (black), proximal *Ubx* promoter (stippled; arrow indicates direction of transcription), and β-gal coding region (white). Underneath the map is shown the point of insertion of the minimal 0.6-kb *Ubx* upstream fragment (06) conferring long-range repression (14) (all HB binding sites are marked as ellipses) and of oligonucleotides containing wild-type (△) or mutant (□) HB binding sites (four to eight copies). hb-ep (white ellipses) and hb-mes (black ellipses) were derived from regions within the 06 fragment and mediate epidermal and mesodermal expression, respectively (14). The total number of transformant lines obtained for each construct and the extent of suppression of the BXD pattern outside ps6–13 are given [virtually complete (COMP), incomplete (INC), or no suppression (NONE)]. (*b*) Sequences of HB binding sites and flanking linkers. Boldface letters are residues protected by HB or, in the case of hb-eve, conserved in HB binding sites [consensus sequence (G or C)(A or C)ATAAAAAA] (21). hb-mut is derived from hb-mes (mutant GC pairs are marked by asterisks). The orientation of sites is as in *Ubx* and *eve* genes (14, 21).

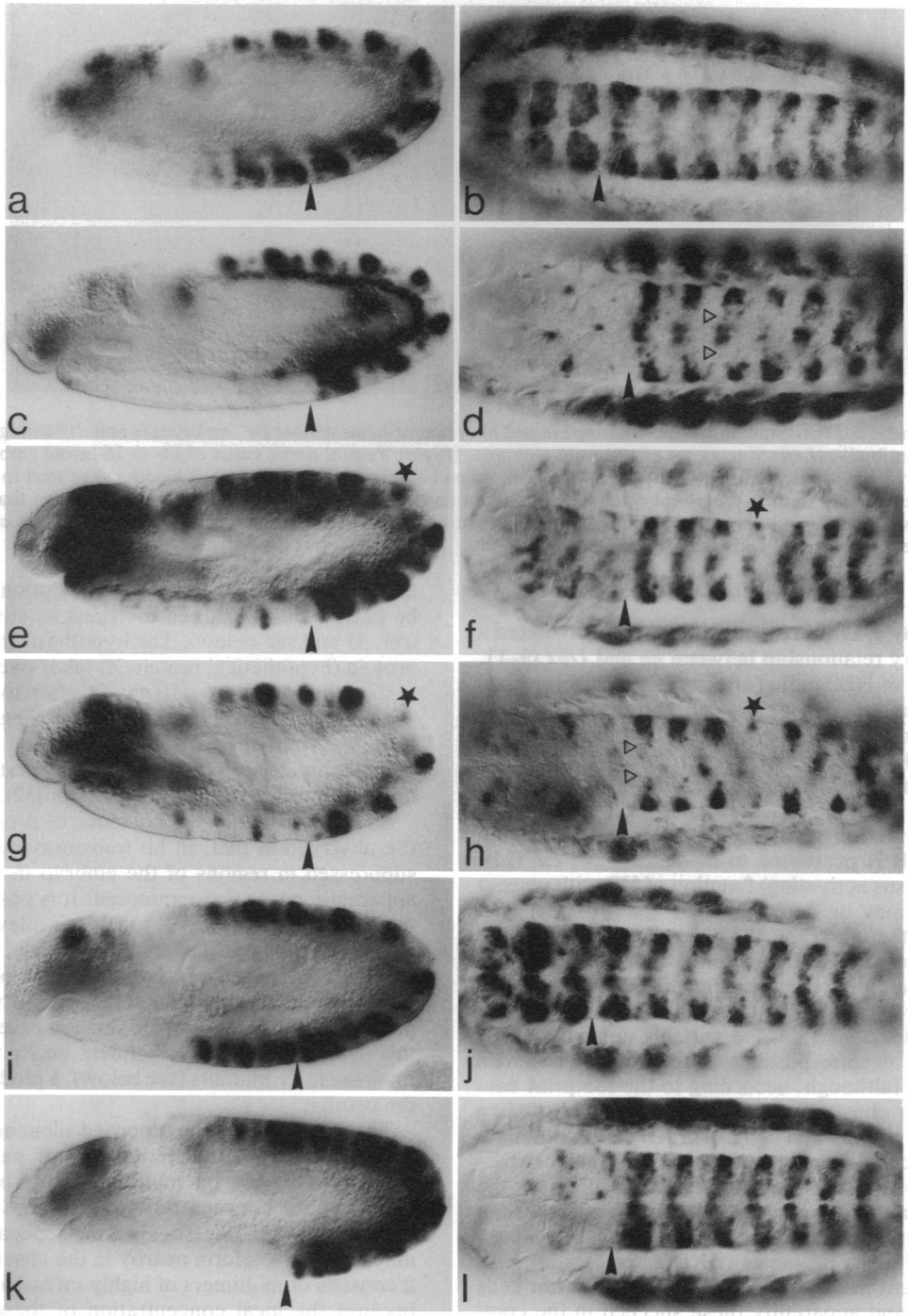

FIG. 2. Silencing mediated by HB binding sites. Embryos transformed with various constructs containing the BXD enhancer and stained with β-gal antibody. (*Left*) Side view of 6-hr-old embryos. (*Right*) Ventral view (ventral nerve cord) of 14- to 16-hr-old embryos (heads to the left). BXD transformants (*a* and *b*) show BXD pattern in ps2–14 of the ectoderm (expression in ps2 and ps14 is weak and cannot be readily discerned in the views and focal planes shown; in addition, there is some expression in the mesoderm as well as variable expression within heads). hb-mes (*c* and *d*), hb-ep (*e* and *f*), and hb-eve (*g* and *h*) transformants show BXD pattern, which is restricted to ps6–13, as do 06 transformants (*k* and *l*). Note the suppression of β-gal staining in ps9 (asterisks) and in medial regions of the ventral nerve cord (open triangles) of hb transformants (most obvious in *h* but also visible in *d* and *f*). The β-gal patterns in hb-mut transformants (*i* and *j*) are essentially indistinguishable from those in BXD transformants. Anterior limits of ps6 are marked by arrowheads.

Maintenance. Long-range repression mediated by *Ubx* control regions requires the function of *Pc* (19) and other *Pc*-like genes (J. Castelli-Gair and M.B., unpublished data), as does maintenance of *Ubx* expression boundaries at advanced embryonic stages (29). We therefore examined the β-gal staining pattern of hb-mes, hb-ep, and hb-eve transformants in homozygous Pc^- embryos. In each case, β-gal expression boundaries were lost progressively after ≈6 hr of embryonic development; by ≈10 hr of development, there was evenly strong β-gal expression throughout ps2–14 (including ps9 and medial regions of the ventral nerve chord; Fig. 3). Thus, *Pc* function is required for silencing mediated by HB binding sites—in other words, for the maintenance of expression boundaries initially conferred by these sites. Notably, the first *Pc* effects become visible at an embryonic stage at which HB has faded away almost completely (12).

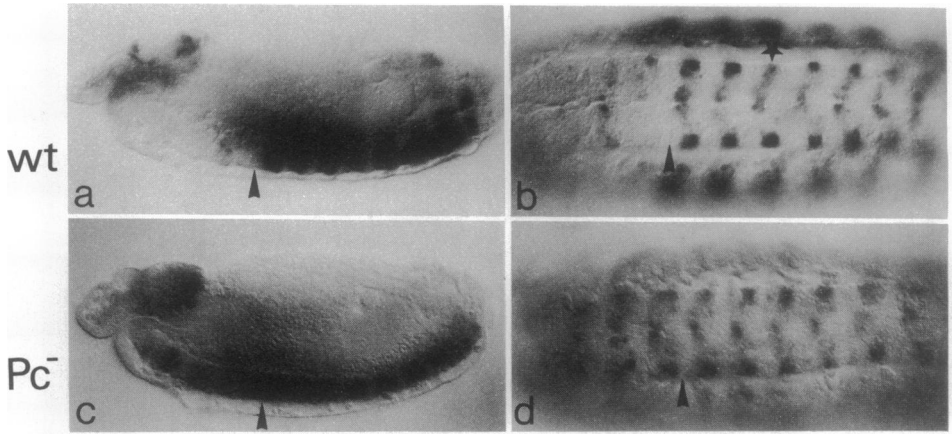

FIG. 3. Requirement for Polycomb function. Wild-type (*a* and *b*) or homozygous mutant Pc^- embryos (*c* and *d*) bearing an hb-mes construct stained with β-gal antibody. (*Left*) Side views of 10-hr-old embryos. (*Right*) Ventral nerve cords of 14- to 16-hr-old embryos. Anterior limits of ps6 are marked by arrowheads (anterior to the left). β-gal expression boundaries mediated by HB binding sites start to decay in *Pc* mutants after ≈6 hr of development, shifting progressively anteriorly, and a head-to-tail β-gal expression pattern similar to that in BXD or hb-mut transformants is observed after ≈10 hr of development. Note that the suppression effect in ps9 (asterisk in *b*) and, less clearly, the one in the middle region of the ventral nerve cord are reversed in *Pc* mutants.

DISCUSSION

Silencing Activity of HB. Previous studies have predicted a negative regulatory relationship between *hb* and *Ubx* (9–11, 15). The presence of HB binding sites in *Ubx* control regions (13, 14), their requirement for early repression (13), and our present results provide strong evidence that this relationship is direct: HB evidently acts as a direct repressor or "silencer" of the *Ubx* gene. Interestingly, silencing is conferred not only through HB binding sites derived from a *Ubx* control region but also through sites from an *eve* control region through which HB is presumed to mediate activation (21). The silencing (versus activating) function of HB with respect to the *Ubx* gene may be determined by the proximal *Ubx* promoter (rather than the type of HB binding site) since the latter cannot be substituted by an hsp70 TATA-box without loss of silencing activity (see *Materials and Methods*).

The anterior expression limits are the same in all three types of hb transformants, despite variable numbers of HB binding sites and despite possible differences of affinities between the sites [although, according to the footprint data (14, 21), the sites chosen may all be high-affinity binding sites]. The precise affinity and number of HB binding sites are evidently not crucial for the boundary position in our experiments. It is more likely that the exact position of the boundary is determined by activator proteins, acting through BXD, which compete with HB repressor for activity—e.g., for interaction with the promoter. BXD activators are presumed to be present in a particular pattern in posterior cells of every ps (19), and apparently these succeed in the competition with HB in ps6 but not in ps5 or more anterior ps—i.e., below a certain threshold concentration of HB. Indeed, the levels of HB are substantially lower in ps6 than in ps5 or more anterior ps (12). Recent results provide evidence that different thresholds of HB determine the positions of boundaries of *gap* gene expression and, probably, also of *Ubx* expression (28).

Maintenance of Silencing Activity. Silencing mediated by HB binding sites occurs at advanced embryonic stages at which HB is no longer detectable (12) or required (10, 15). This amounts to a "hit-and-run" mechanism. It is possible that HB initiates a particular chromatin structure, as suggested (29, 30), which prevents *Ubx* transcription and which is propagated in the absence of HB throughout subsequent development. We propose an alternative hypothesis in which HB initiates the formation of a silencing complex (which involves the proximal promoter and prevents its activation by enhancers)—i.e., of a stable transcription complex that may be heritable through cell divisions as envisaged previously (ref. 31 and see below). The hypothesis implies that another protein (hypothetical protein X) takes over the role of HB at a stage during which HB levels start to decline: HB may somehow tether protein X to HB binding sites, in an analogous way perhaps to a situation found for glucocorticoid receptor, whose efficient binding to certain DNA sites requires interaction with c-jun protein (32).

Some support for the existence of protein X comes from the observation that, in hb transformants, β-gal staining is suppressed in regions of the embryo in which the *hb* gene apparently never was expressed. It is possible that the need for HB to tether protein X to DNA is alleviated in a situation where HB binding sites are oligomerized and that protein X therefore can bind DNA in the absence of HB (particularly, if there were cooperative interactions between monomers of protein X; see below). Were this the case, we would expect this suppression to be dependent on *Pc* function and to be reversed in *Pc* mutants (see below), a prediction borne out by the results.

The heritability of the proposed silencing complex through cell divisions, as the heritability of particular chromatin states, might rely on multimerization of proteins and on cooperativity between different proteins (and may require multiple HB binding sites): after replication, the complex may be able to reform nearby in the absence of HB because it consists of multimers of highly interactive proteins and/or because the local concentration of these proteins is high. Such a complex might be expected to "linger" during replication and redistribute evenly on both daughter strands after replication, thereby indirectly memorizing HB binding to DNA. Thus, the memory function of the complex may consist of the complex' structure and its constituent proteins.

These constituent proteins (including protein X) may be ubiquitous proteins, and we do not expect their distribution in the embryo to reflect positional information. A candidate for such a protein may be the zeste protein, which self-associates to large aggregates (33), which is thought to mediate interactions between distal *Ubx* control regions and the proximal promoter by binding to these (34–36), and which appears to influence silencing (36). Another candidate may be the Pc protein since *Pc* function is required for continued silencing throughout late embryonic stages. Pc protein is ubiquitously expressed in the embryo (37) and does not bind DNA on its own (30). Finally, the complex may consist of other proteins encoded by the *Pc* group

of genes (18) of which, notably, Enhancer of zeste [*E(z)*] is a member (38). One of these Pc-like proteins may be protein X itself.

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