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The *Additional sex combs* gene of *Drosophila* encodes a chromatin protein that binds to shared and unique Polycomb group sites on polytene chromosomes

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SUMMARY

The *Additional sex combs* (*Asx*) gene of *Drosophila* is a member of the Polycomb group of genes, which are required for maintenance of stable repression of homeotic and other loci. *Asx* is unusual among the Polycomb group because: (1) one *Asx* allele exhibits both anterior and posterior transformations; (2) *Asx* mutations enhance anterior transformations of *trx* mutations; (3) *Asx* mutations exhibit segmentation phenotypes in addition to homeotic phenotypes; (4) *Asx* is an Enhancer of position-effect variegation and (5) *Asx* displays tissue-specific derepression of target genes. *Asx* was cloned by transposon tagging and encodes a protein of 1668 amino acids containing an unusual cysteine cluster at the carboxy terminus. The protein is ubiquitously expressed during development. We show that *Asx* is required in the central nervous system to regulate *Ultrabithorax*. ASX binds to multiple sites on polytene chromosomes, 70% of which overlap those of Polycomb, polyhomeotic and Polycomblike, and 30% of which are unique. The differences in target site recognition may account for some of the differences in *Asx* phenotypes relative to other members of the Polycomb group.

Key words: Polycomb group, *Additional sex combs* (*Asx*), Chromatin, Cysteine cluster, *Drosophila*

INTRODUCTION

Early in development, the homeotic genes of *Drosophila* are regulated by transiently expressed transcription factors encoded by the segmentation genes (White and Lehmann, 1986; Harding and Levine, 1988; Irish et al., 1989). Subsequently, the Polycomb group (PcG) genes are required to stably maintain repression of homeotic loci in cells where the target genes were initially repressed (Struhl and Akam, 1985; Wedeen et al., 1986; Jones and Gelbart, 1993; McKeon and Brock, 1991; Simon et al., 1992). The mechanism of PcG repression is not understood at the molecular level. Based on the similarity of a domain found in POLYCOMB and HP1, encoded by the *Su(var)205* locus, it was proposed that PcG proteins might regulate chromatin structure to make it more heterochromatin-like (Paro, 1990; Alberts and Sternglanz, 1990). However, other models have been proposed: (1) a compartmentalization model (Schlossherr et al., 1994; Strouboulis and Wolffe, 1996), (2) a looping model in which interactions between PcG proteins cause looping out of DNA that interferes with interactions between enhancers and promoters (Pirrotta, 1995), and (3) a repressor model in which PcG proteins interfere with basal transcription (Bienz, 1992).

About 15 PcG genes have been characterized (see Simon, 1995 for a review), although it has been estimated that there may be as many as 40 PcG genes (Jurgens, 1985; Landecker et al., 1994). Mutations in one PcG gene usually enhance the phenotypes of mutations in other PcG genes (Jurgens, 1985; Cheng et al., 1994; Campbell et al., 1995), suggesting that PcG proteins have similar functions, or are members of a complex (Locke et al., 1988; see Kennison, 1995 for a review). The cloned PcG genes encode chromatin proteins that bind chromosomes in a DNA-dependent manner. The distributions of Polycomb (PC), polyhomeotic (PH) and Polycomblike (PCL) protein binding completely overlap at about 100 sites on polytene chromosomes (Zink and Paro, 1989; DeCamillis et al., 1992; Lonie et al., 1994), and the distributions of Posterior sex combs (PSC) and Enhancer of zeste overlap at a subset of these sites (Rastelli et al., 1993; Carrington and Jones, 1996). In addition PC and PH coimmunoprecipitate, and PC and PH

bind PSC in a yeast two-hybrid assay and in vitro (M. K and H. W. B., unpublished data) providing molecular support for the suggestion that PcG proteins form a multimeric complex (Franke et al., 1992). Nevertheless, there must be discrete PcG complexes, because the distributions of PcG proteins on polytene chromosomes are not identical. Mammalian homologues of PcG genes have been cloned, and these proteins also form complexes and are required for regulation of homeotic genes (Alkema et al., 1995, 1997; Core et al., 1997; Akasaka et al., 1996).

Additional sex combs (Asx) shares many properties with other members of the PcG. It was first described by Jurgens (1985). Mutant alleles were recovered because males had a dominant extra sex combs phenotype and because of head defects in embryos. Embryos that are homozygous mutant for *Asx* exhibit relatively mild posterior transformations of thoracic and abdominal segments, and partial failure of head involution so that the cephalopharyngeal apparatus is more anterior and mouth hooks are more lateral than in wild-type embryos. Even though most segments are transformed posteriorly, the extent of the transformations is less severe than those seen in Pc^{-} embryos (Breen and Duncan, 1986). In mutant embryos derived from homozygous mutant mothers, the posterior transformations are more extensive. All the thoracic denticle belts partially resemble abdominal denticle belts, Keilin's organs are reduced or absent and the abdominal segments are transformed posteriorly. Nevertheless, even in embryos derived from maternal mutants, the extent of posterior transformation is not complete (Breen and Duncan, 1986; Soto et al., 1995). Like other dominant PcG mutations, mutations in *Asx* enhance the phenotypes of other dominant PcG mutations in doubly heterozygous adults (Campbell et al., 1995) and in embryos (Jurgens, 1985). Most *Asx* mutations appear to be gain of function, because their zygotic phenotypes are more severe than those of homozygous deficiency embryos (Sinclair et al., 1992).

Asx mutations also exhibit a number of phenotypes that are exhibited by a restricted subset of PcG genes, or that are unique to *Asx*. Even though zygotic *Asx* mutants ectopically express homeotic genes (McKeon and Brock, 1991; Simon et al., 1992), zygotic mutant embryos and embryos derived from mothers with Asx^{-} germlines exhibit tissue-specific derepression. SCR, UBX, ABD-A and ABD-B mis-expression occurs in epidermal and visceral mesoderm cells, but generally are not mis-expressed in the central nervous system (CNS) (Soto et al., 1995). For the PcG genes that have been studied, only *Pcl* mutants also exhibit tissue-specific misexpression of homeotic genes (Soto et al., 1995). These authors have speculated that ASX might act on different targets than other PcG proteins. Heterozygous *Asx* mutant adults, and homozygous mutant embryos exhibit segment defects that affect even-numbered segments more often than odd-numbered segments (Sinclair et al., 1992). Segmentation defects have also been reported in other PcG genes, including *super sex combs (sxc)* (Ingham, 1984), and *l(4)29/pleiohomeotic* (Breen and Duncan, 1986). Together, the evidence indicates that *Asx* has a specific role in gene regulation.

Two other features suggest that *Asx* differs from most PcG genes. First, the *P*-element-induced allele, Asx^{P1}, exhibits anterior as well as posterior transformations. In addition to posterior transformation of the fourth abdominal segment towards the fifth, homozygous flies show swollen halteres with occasional bristles similar to those seen in the triple row margin of wings, and the fifth abdominal segment shows unpigmented patches, consistent with transformation towards a more anterior tergite. Anterior transformations are characteristic of mutations in the trithorax Group (trxG), genes required for the continued activation of homeotic genes (Kennison, 1995). Their presence in an *Asx* mutant suggests that *Asx* might have a dual role in repression and in activation. Consistent with this idea, mutations in *Asx* act as enhancers of position-effect variegation, whereas most PcG mutations do not affect position-effect variegation (Sinclair et al., 1998). It might be expected that PcG mutations would suppress position-effect variegation if they function directly to regulate chromatin structure at target loci to repress gene activity. Because *Asx* mutations enhance position-effect variegation, similar to at least one member of the trxG (Farkas et al., 1994), it can be argued that ASX is required to maintain an open conformation of chromatin at variegating loci.

The features reported above make *Asx* a particularly interesting member of the PcG, as its analysis may shed light on the role of PcG proteins in segmentation, tissue-specificity, and its role in activation versus repression of target loci. To better understand the molecular basis for ASX function, we cloned the *Asx* locus. It encodes a putative 1668 amino acid protein with two domains that are conserved in mammals. The RNA and protein are ubiquitous, except that *Asx* mRNA does not appear to be deposited in oocytes. *Asx* is required for proper expression of *Ubx* in the central nervous system. The distribution of ASX on polytene chromosomes partially overlaps that of PC, PH and PCL, but there are many binding sites unique to ASX. These data rule out the possibility that tissue-specific regulation by *Asx* is achieved by tissue-specific distribution of the ASX protein, but support the hypothesis that unique *Asx* phenotypes arise at least partly as a consequence of a target specificity different from other PcG proteins.

MATERIALS AND METHODS

Fly strains, culture and genetic analysis

The Asx^{P1} strain and other *Asx* alleles were described in Sinclair et al. (1992). All flies were raised at 25°C on standard cornmeal sucrose medium containing tegosept as a mould inhibitor. Embryos were collected from cages on agar plates spread with yeast paste to obtain staged embryos for preparation of RNA, and from egg-laying chambers for in situ hybridization and antibody staining. Transformed lines containing the *bxd14* element were obtained from W. Bender.

Molecular procedures

General molecular procedures were carried out as described (Sambrook et al., 1989). The Asx^{P1} mutation is a *P* element insertion that fails to complement *Asx*. DNA flanking the insertion was recovered from a genomic library established from Asx^{P1} DNA using *P* element DNA as a probe, and used to screen a cosmid library supplied by J. Tamkun to recover cosmids spanning the insertion point. Reverse northern analysis was used to identify transcription units (Brunk et al., 1991). An imaginal disc library supplied by G. Rubin was screened to recover cDNAs corresponding to the 2 transcription units flanking the *P* element insertion site. Dideoxy sequencing was carried out manually on both strands of selected cDNAs, using protocols recommended by the suppliers of the DNA polymerases (Amersham, Pharmacia), and on the genomic DNA to

map introns, using a combination of directed deletions and primer walking.

RNA was isolated from staged embryos using the Trizol™ reagent (Bethesda Research Laboratories), and poly(A)$^+$ RNA was isolated by two rounds of chromatography on oligo(dT) cellulose. About 2 μg of RNA for each developmental stage was fractionated in 1.0% agarose gels containing formaldehyde, transferred to nylon filters, and probed with cDNA or with RP-49 genomic DNA to control for loading.

Analysis of *Asx* distribution in ovaries and embryos was carried out as described (DeCamillis and Brock, 1994).

Antibodies and immunostaining

A carboxy terminal antibody was raised in rabbits, and in mice, using an Asx C-terminal fragment containing amino acid residues 1590 to 1668, subcloned as a *Pst*I-*Kpn*I fragment into the *Sma*I site of pGEX-3X (Pharmacia) to generate a GST-ASX fusion. The fusion product was expressed in *E. coli* AD202, affinity purified over glutathione-agarose (Pharmacia) and injected into animals. Immunoglobulins were purified from serum using T-gel (Pierce Biochemical) and used directly for staining embryos and polytene chromosomes. An amino terminal antibody was raised in sheep against the peptide THSLRRHLPRIIVKPIPPEKKG beginning at amino acid 77 in ASX, and affinity purified on a column containing the peptide used as an immunogen and supplied to us by Chiron Mimotopes. An antibody against β-galactosidase was obtained from Jackson Laboratories.

Antibodies raised against both epitopes immunoprecipitated in vitro translated ASX protein. Neither the mouse antibody to the carboxy-terminus, nor the sheep anti-peptide antibody reacted with Asx null embryos, showing that they do not cross-react with other proteins (data not shown). We depleted the rabbit serum by reacting it with the original immunogen and this depleted serum gave no signal on polytene chromosomes, demonstrating that the polytene signals are specific to ASX and are not due to cross-reaction (data not shown). Antibodies were reacted with embryos (McKeon and Brock, 1991) and polytene chromosomes (DeCamillis et al., 1992), as described previously, except that some polytene nuclei were fixed in 1.0% instead of 3.7% paraformaldehyde for 15 seconds prior to squashing. The rabbit serum was used for mapping ASX-binding sites, but similar results were obtained with the sheep antibody.

RESULTS

Characterization of the Asx region

We reported previously the recovery of the *Asx^{P1}* mutation generated by *P* element insertion in region 51A5-6, and subsequent loss of the *P* element in revertants (Sinclair et al., 1992). Comparison of the DNA obtained from *Asx^{P1}* homozygotes and revertant flies by Southern analysis allowed us to identify the insertion site responsible for the *Asx* mutation. A restriction fragment containing the *P* element and the flanking DNA was isolated from genomic DNA, and the flanking DNA was used subsequently to screen a genomic cosmid library. Cosmids spanning about 55 kb were recovered. Reverse northern analysis was carried out using labelled cDNA prepared from embryo and adult RNA to identify which fragments were transcribed. Subsequently, these restriction fragments were used to probe northern blots of embryonic poly(A)$^+$ RNA.

The *P* element associated with the *Asx^{P1}* allele lies in the 4.5 kb *Eco*R1 fragment that hybridizes to two divergent, non-overlapping transcripts, a 7.0 kb transcript that extends distally, and a 4.4 kb transcript that extends proximally (Fig. 1A). We show elsewhere that the proximal transcript encodes the *Drosophila* homologue of cleavage and polyadenylation specificity factor (Salinas et al., 1998). The *P* element was inserted within the distal transcript, which seemed likely to correspond to *Asx*. To confirm this, 10 *Asx* mutants were analyzed for the presence of genomic rearrangements and for the presence of altered transcripts. *Asx^3* has an approximately 1.3 kb deletion that maps to the 2.3 kb *Sal*I fragment within the 6.0 kb *Eco*RI fragment immediately distal to the *P* element (Fig. 1B). As expected, northern analysis of this mutant shows the presence of a 5.7 kb band in mRNA prepared from *Asx^3* heterozygotes, which correlates with the deletion predicted from Southern analysis (Fig. 1B). No changes were observed in the other mutants, or in the proximal transcript in *Asx^3* mutants. Taking the *P* element insertion data and the mutant analysis together, we conclude that the distal transcript encodes *Asx*.

An imaginal disc cDNA library was screened for cDNAs corresponding to the distal transcript. Six cDNAs were recovered, providing a total of 6.6 kb. The cDNA was sequenced and compared to the genomic sequence. For most of development, *Asx* encodes a single transcript that contains

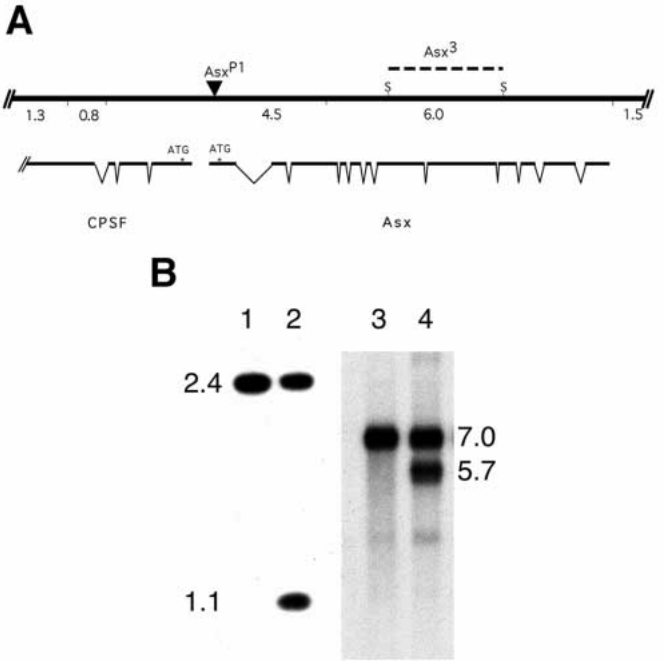

Fig. 1. Map of *Asx* locus, and mapping of *Asx^3* mutation. (A) The thick black line indicates genomic DNA, with the telomere on the right. Sizes of *Eco*RI restriction fragments are indicated below. The triangle marks the insertion site of the *P* element responsible for the *Asx^{P1}* mutation. The dotted line above shows the approximate limits of the deletion associated with *Asx^3*. Below are indicated the complete transcript of *Asx*, with locations of introns and the direction of transcription marked, and a partial cDNA of the proximal gene, which encodes dCPSF-160. (B) Southern and northern analyses of *Asx^3* mutants. Lanes 1 (wild-type) and 2 (*Asx^3/+)* show Southern analyses of genomic DNA cut with *Sal*I, and probed with the *Asx* cDNA, with size of bands indicated in kb at the left. Note the presence of a new band of 1.1 kb, corresponding to a 1.3 kb deletion in the *Asx^3* mutation. Lanes 3 (wild-type) and 4 (*Asx^3/+)* show northern analyses of embryonic poly(A)$^+$ RNA, with transcript sizes indicated on the right. Note the presence of a 5.7 kb RNA, corresponding to that expected from a 1.3 kb deletion in *Asx*.

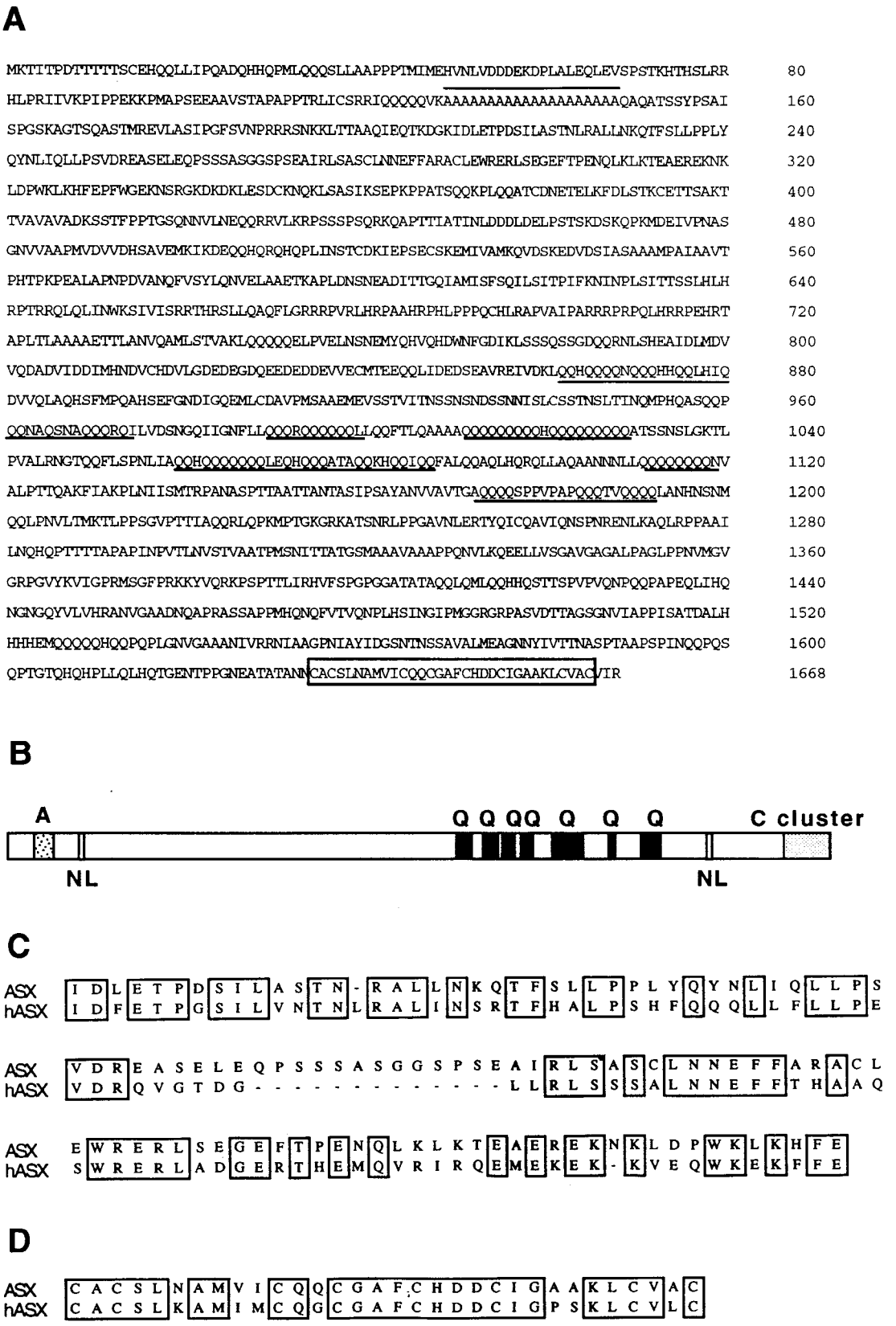

Fig. 2. Amino acid sequence of ASX and a cartoon of conserved domains. (A) ASX sequence. The 32 aa cysteine cluster at the C-terminal (aa 1633-1665) is boxed. The polyalanine stretch (aa 129-152) is overlined, and 7 glutamine repeats are underlined. The EMBL accession number is AJ001164. (B) Diagram of conserved domains. The domains mentioned above are indicated, as are the presence of two putative nuclear localization signals. (C) Comparison of conserved N-terminal domain of unknown function between ASX and human EST clone AA447511, labelled 'hASX'. (D) Comparison of conserved C-terminal cysteine cluster between ASX and human EST clone AA421697, labelled 'hASX'.

11 introns (Fig. 1A). The 3′-most cDNA does not contain a poly(A) tail, but 100 bp downstream in the genomic sequence is a canonical AATAAA, which likely indicates the 3′ end of the gene. To determine the 5′ end of the transcript, a 5′ RT-PCR reaction was used to extend the available cDNA by 200 bp. The total cDNA of 6.8 kb, added to the genomic DNA

including the polyadenylation signal gives a transcript of 6.9 kb, in good agreement with the northern data. The *P* element is inserted 182 bp downstream of the 5′ end of the cDNA, 256 bp upstream of the translation start. Only 350 bp separate the 5′ ends of the proximal and distal transcripts (Fig. 1A).

Analysis of ASX

The conceptual translation of *Asx* is presented in Fig. 2. The open reading frame begins 438 bp downstream of the putative transcription start and continues for 1668 amino acids. The protein has an estimated relative molecular mass of 182,000 and an estimated pI of 6.8. At the extreme carboxy terminus is a group of 8 cysteines with the structure $CxCx_7CxxCxxxCxxxCx_6CxxC$, which could be represented as two consecutive Zn fingers. Another notable feature is the distribution of specific amino acids, exemplified by a stretch of 20 alanines near the amino terminus of the protein and an A_3VA_3 stretch within the carboxy terminal region. There are at least 7 regions with glutamine repeats or a high percentage of glutamines. Glutamine and alanine together make up more than 24% of the amino acids, and proline, serine and threonine contribute 8.1, 7.8 and 6.6% respectively. Serine and threonine can occur in a localized area: 10/21 amino acids within residues 152-173 and 15/30 amino acids within residues 920-950 are S or T. These regions, plus two putative nuclear localization signals, are also marked on the diagram in Fig. 2B. Additional structural features include a nucleotide-binding motif at residues 1033-1039 (Koonin, 1993) and the RGRP tetrapeptide AT hook motif at residues 1502-1505 (Ashley et al., 1989).

Sequences in the mouse and human EST databases conserve the spacing of the cysteines in ASX and also conserve 25/32 amino acids, but this cysteine cluster is not related to any previously characterized cluster of cysteines in the database. A domain of unknown function near the amino terminus of ASX is conserved in sequences found in the mouse and human EST databases. The domain extends from residues 210-330 in ASX, but is somewhat shorter in the mammalian homologues. A comparison of mammalian and ASX conserved domains is shown in Fig. 2C,D. The mouse and human homologues of *Asx* have been cloned and characterized (F. R., unpublished data). It appears likely that ASX, like other PcG proteins, has a conserved function in flies and mammals.

Developmental analysis of Asx expression

To determine if *Asx* is developmentally regulated, we examined its steady-state RNA levels at different developmental stages using northern analysis. As shown in Fig. 3, high levels of Asx transcripts are present in embryos 0-1.5 hours post egg deposition, then levels fall sharply in 1.5-3 hour embryos, before rising through the remainder of embryogenesis. Transcript levels are low in larvae, and increase again in pupae and adults. Three transcripts are detected in adult males, which have not been further characterized, but which may indicate alternate splicing.

We also examined the spatial regulation of *Asx* transcripts. As shown in Fig. 4A, *Asx* RNA is abundant in nurse cells, but appears to be absent from stage 10 oocytes. Because newly laid embryos have high *Asx* RNA levels, it appears likely that *Asx* mRNA is deposited in oocytes late in oogenesis. At the blastoderm stage, *Asx* mRNA is more abundant in a broad band in the anterior region of the embryo, and in a narrower band in the posterior region (Fig. 4B). This pattern is similar to that seen with *polyhomeotic* mRNA in blastoderm embryos (Deatrick, 1992; DeCamillis and Brock, 1994). During the remainder of embryogenesis, *Asx* RNA is ubiquitous, although it rapidly becomes much more highly expressed in the neurectoderm, and later in the CNS (Fig. 4C).

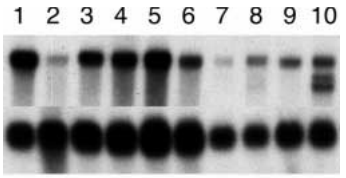

Fig. 3. Developmental northern analysis of *Asx*. The top panel was probed with the *Asx* cDNA. The bottom panel was probed with *RP49* as a loading control. Poly(A)$^+$ RNA was prepared from 0-1.5 hour, (lane 1); 1.5-3 hour (lane 2), 3-6 hour (lane 3), 6-9 hour (lane 4), 9-12 hour (lane 5), 12-18 hour (lane 6) embryos, mixed larvae (lane 7), pupae (lane 8), females (lane 9), and males (lane 10) respectively.

We used the mouse antibody described in the Materials and Methods to investigate ASX distribution in embryos. We did not detect a high concentration of ASX in nuclei prior to the cellular blastoderm stage (Fig. 4D) but, by the cellular blastoderm stage, ASX is detectable both in nuclei and cytopolasm. At this stage, there is a marginal increase in ASX in the anterior portion of the embryo, but this spatial regulation is not as easily seen for the ASX protein as for the mRNA. Later in embryogenesis, ASX is ubiquitous, but not uniformly distributed; protein is more heavily concentrated in the neurectoderm and the CNS than in other tissues (Fig. 4E,F), whereas staining levels are low in the amnioserosa.

We have noted previously that homozygous Asx^{P1} adults

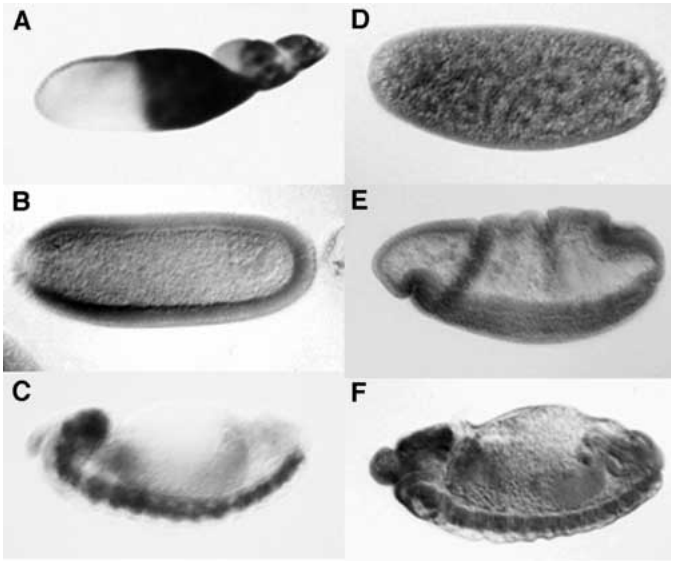

Fig. 4. Embryonic distribution of *Asx* mRNA and protein in oogenesis and embryogenesis. All embryos are mounted with anterior to the left and dorsal up. (A-C) RNA in situs. (A) Stage 10 oocytes; (B) cellular blastoderm embryo: note the increased hybridization in the anterior of the embryo; (C) stage 14 embryo, showing strong hybridization in the CNS: antibody staining; (D) stage 3 (preblastoderm) embryo; (E) stage 8 embryo; (F) stage 14 embryo.

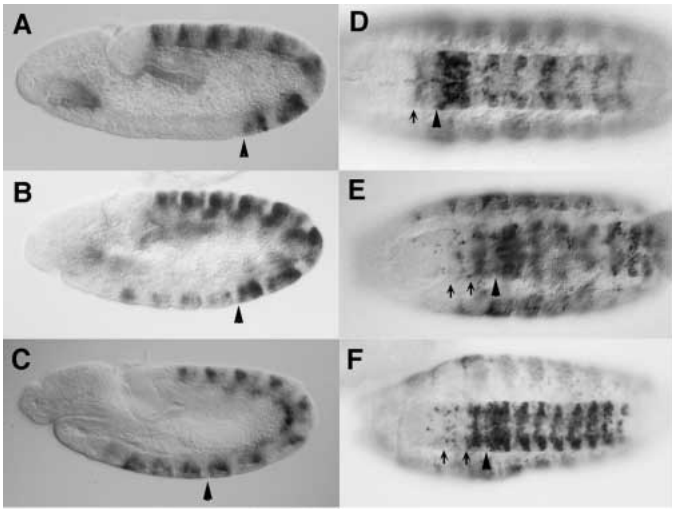

Fig. 5. Effects of *Asx* mutants on expression of *bxd-14* and *Ubx*. Embryos are mounted with anterior to the left and dorsal up and stained with antibodies (A-C) to β-galactosidase to detect activity of the *bxd14* reporter, or (D-F) to UBX to detect endogenous *Ubx* activity. The position of the border between parasegment 5 and 6 is marked with the arrowhead. Small arrows mark the positions of the parasegment 3-4 and 4-5 border, respectively. Embryos are homozygous for the *Asx* mutation and heterozygous for the *bxd14* reporter. Anti-β-galactosidase antibodies were used to monitor expression activity of the reporter. (A) Wild-type; (B) Asx^{P1}; (C) *Df(2R)trix*; (D) wild-type; (E) Asx^{3}; (F) *Df(2R)trix*.

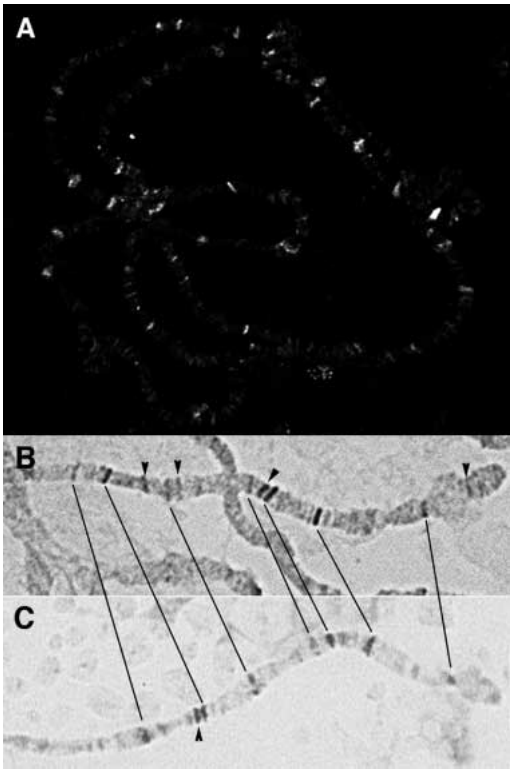

Fig. 6. ASX antibody staining of polytene chromosomes. (A) Sheep anti-peptide antibody on polytene nucleus from salivary gland. (B) ASX antibody staining of the distal portion of the X chromosome, compared to a similar region stained with PH antibody (C). Lines connect sites found with both antibodies and arrowheads mark unique binding sites.

show anterior as well as posterior homeotic transformations (Sinclair et al., 1992). Therefore it was of interest to determine if we could detect differences in the regulation of *Ubx* in Asx^{P1} embryos relative to embryos homozygous for a deficiency uncovering *Asx (Df(2R)trix)* or for a gain-of-function *Asx* (Asx^{3}) mutation. We monitored *Ubx* expression using the *bxd14* element, which carries a Polycomb Group Response Element (PRE) and is included in a 14.5 kb fragment from the *bxd* regulatory region of *Ubx* that regulates a *lacZ* reporter in parasegment 6-13 (Simon et al., 1990). This element has been used by Soto et al. (1995) to show that *Asx* is active in the CNS. As shown in Fig. 5, compared to wild-type, both Asx^{P1} and *Df(2R)trix* embryos show derepression of the *lacZ* reporter in the central nervous system in parasegments 2-5. Similar results were obtained with Asx^{3} (results not shown). We see no evidence of reduced expression of the *bxd14* reporter in parasegments 6-13. We conclude that Asx^{P1} mutants, like other *Asx* mutants, cause derepression of the *bxd14* reporter in the anterior CNS during embryogenesis.

Soto et al. (1995) have reported that in *Asx* mutants derived from homozygous mutant germlines, there is ectopic expression of endogenous ABD-B in the epidermis and visceral mesoderm, but not in the central nervous system. As noted above, they and we were able to detect a requirement for *Asx* in the central nervous system using the *bxd14* element that contains a *Ubx* regulatory element. Therefore, we decided to re-examine the requirement for *Asx* in the CNS for regulation of endogenous *Ubx*. Fig. 5 shows that *Ubx* is ectopically expressed in parasegment 3 in stage 15 embryos in two *Asx* mutants, and thus that *Asx* is required for regulation of the endogenous *Ubx* gene in the CNS. However, Asx^{P1} mutants have no effect on the regulation of the endogenous *Ubx* gene (data not shown).

Immunostaining of polytene chromosomes

All PcG proteins tested so far are chromatin proteins that bind to discrete euchromatic sites, many of which overlap among different PcG members. Asx has unique characteristics that indicate it may be functionally distinct from other PcG proteins, so polytene chromosomes were stained with the antibodies described above to determine if the ASX polytene staining pattern differed from that of other PcG proteins.

We reliably detected 90 sites of antibody staining on polytene chromosomes (Fig. 6A). Table 1 lists the cytological locations of the ASX-binding sites and compares them to the binding sites of PH/PC/PCL. Of the 90 sites, 27 are unique to ASX and 63 overlap with PC/PH-binding sites. PC and PH bind an additional 38 sites not recognized by ASX. A comparison of ASX and PH-binding sites is shown in Fig. 6 for part of the X chromosome. In addition to the differences in binding sites, there are also differences in staining intensity at specific sites. Sites 48A, 49EF and 100A all stain very intensely with antibodies to PH or PC but stain very weakly with ASX, whereas sites 35AB, 56C and 93E stain very intensely for ASX but weakly for PH or PC. Only two of the unique ASX sites overlapped sites unique to PSC, which also demonstrates about 70% overlap with PH/PC (Rastelli et al., 1993). One of the common ASX-binding sites is 51A, which

Table 1. Comparison of ASX with PH/PC protein binding sites on polytenes

ASX-binding sites	PH/PC-binding sites	ASX-binding sites	PH/PC-binding sites	ASX-binding sites	PH/PC-binding sites
X		*2L*		*3L*	
-	1A	21A	21A	-	61A
1D	-	22A	22A	61C	61C
1F	-	22B	22B	-	61D
2D	2D	-	22C	61F	61F
4C	4C	24A	24A	62F	62F
5A	5A	25EF	25EF	63A	-
5B	-	26F	26F	-	63F-64A
5D	5D	27B	-	64C	-
7A	-	28A	28A	-	65D
7B	7B	28D	-	66A	-
7D	-	-	29E	66C	-
8A	8A	30AB	30B	-	66E
-	8B	-	30C	67CD	67D
9A	9A	32EF	32EF	67E	67E
10A	-	-	33B	-	67F
10B	-	33F	33F	-	68A
12D	12D	-	34C	69C	69C
13E	13E	-	34D	69D	69D
14B	14B	35AB	35AB	70AC	70AB
-	16D	35CD	35D	70DE	70DE
17A	17A	-	36A	75D	-
-	17E	-	36B	-	76C
-	17F	36CD	-	77A	-
18CD	-	-37B	-	77E	
19D	19D	37D	-	-	78EF
19F	-	38C	-	79B	79B
		39F-40A	39EF		
		2R		*3R*	
		41C	41CD	-	82E
		-	43C	-	83C
		44A	44A	84B	84AB
		-	45C	84DE	84D
		46A	-	84F	84EF
		46CD	46C	85D	-
		47A	-	85EF	85E
		48A	48A	86C	86C
		49F	49EF	87B	87BC
		50A	-	88A	88A
		51A	51A	89B	89B
		-	51D	89C	89C
		56C	56C	89D	-
		-	57A	89E	89E
		57B	57B	90E	90E
		-	58CD	93E	93E
		-	58F	-	94DC
		-	59A	96A	-
		-	59C	96CD	96BC
		59F	59F	-	96F-97A
		60E	60E	97D	-
		-	60F	98BD	98CD
				99A	99AD
				99F	99F
				100AB	100A
				100F	100F

is the location of the *Asx* locus, suggesting that ASX, like other PcG genes (DeCamillis et al., 1992), may be autoregulated.

DISCUSSION

Comparison of ASX to other PcG proteins

The ASX protein shares structural features with other members of the PcG with respect to glutamine and serine/threonine repeats as in PH (DeCamillis et al., 1992), and a cysteine cluster like many PcG proteins including PSC, PCL, PH and SCM (Simon, 1995). The 32 amino acid cysteine cluster at the carboxy terminus of ASX is conserved at all 8 cysteine residues, and 25/32 residues overall relative to a mammalian EST sequence, suggesting that this domain has a conserved function. It is likely that the cysteine clusters are protein interaction domains, such as those found in the SV40 large T antigen (Loeber et al., 1991), or the double zinc finger motif human cysteine-rich protein (Feuerstein et al., 1994). One prominent feature of ASX is the presence of 20 consecutive alanines near the amino terminus. Enhancer of Polycomb (E(Pc)) contains a region with 18 alanines in a 21 amino acid stretch (K. Stankunas and H.W.B., unpublished data). In addition, *cramped*, a newly reported PcG gene contains a 36 aa sequence with 40% alanine (Yamamoto et al., 1997) and Sex combs on midleg contains a 29 aa region that is 52% alanine (Bornemann, 1996). Alanine-rich regions have been implicated in the repression functions of both yeast and *Drosophila* transcription factors (Tzamarias and Struhl, 1994 and references therein), although long uninterrupted runs of alanine have not been observed in these cases. The function of alanine repeats in PcG proteins remains to be demonstrated.

Anterior-posterior transformations of *Asx* mutations

Flies homozygous for Asx^{P1} alleles of *Asx* exhibit both posterior and anterior transformations, but mapping of the *P* element insertion site in Asx^{P1} mutants has not clarified the mechanism. The *P* element is inserted into the 5′ untranslated region of the gene and thus it should not interfere with the protein structure of ASX. The presence of the *P* element does not cause a change in RNA length, suggesting that the element is spliced out. Nor does the *P* element cause a reduction in the steady state concentration of *Asx* mRNA (D. A. R. S., unpublished observations). Heterozygous deficiencies for *Asx* do not exhibit anterior transformations, but do enhance anterior transformation phenotypes of heterozygous mutations in trithorax, as do Asx^{P1} mutations (T. A. M., unpublished data).

Asx^{P1} homozygotes show anterior transformations only in adults. We confirmed that Asx^{P1} homozygotes exhibit ectopic expression of the *bxd14* reporter, similar to that seen in other *Asx* mutations. Despite the strong effect that the Asx^{P1} mutation has on the regulation of the *bxd14* reporter gene, homozygous Asx^{P1} embryos displayed no defect in the regulation of the endogenous *Ubx* gene in the CNS, since we did not detect any reduction in *bxd14* reporter or endogenous *Ubx* expression in parasegments 6-13. It may be that *Asx* has a later function in imaginal tissues that is specific for activation of homeotic genes, or that the embryo assay with *bxd14* or *Ubx* expression is not sufficiently sensitive to detect reductions in expression.

Specificity of *Asx* phenotypes

The expression patterns of *Asx* mRNA and protein in ovaries and embryos is very similar to that reported for other PcG genes (Paro and Zink, 1992), (DeCamillis and Brock, 1994), (Martin and Adler, 1993; Lonie et al., 1994). One potential explanation for the observation that *Asx* regulates *Abd-B* genes in the epidermis, but not in the central nervous system (Soto et al., 1995), is that the expression of *Asx* is tissue-limited. Our observations do not support this hypothesis, however, because the expression of *Asx*, like that of other PcG genes, is ubiquitous.

The results showing tissue-specificity of ASX raise an interesting problem for the current view of how PREs function in the BX-C. There appears to be approximately one strong PRE per parasegmental domain, measured either by direct binding of PC to BX-C DNA (Chiang et al., 1995; Strutt et al., 1997), or by functional assays (Simon et al., 1993). It appears that each PRE can repress the various stage- and tissue-specific enhancers within the parasegment (Chan et al., 1994; Pirrotta et al., 1995), and also repress heterologous enhancers if experimentally manipulated (Simon et al., 1993), (Chiang et al., 1995). This implies that PREs do not provide specificity and that their activity depends on the state of activity when the PcG complex is formed (Poux et al., 1996). *Asx* mutations have no effect on the regulation of most of the homeotic loci in the CNS, but show derepression phenotypes in the epidermis that are as strong as many other PcG genes. This tissue specificity of *Asx* mutations implies that *Asx* may differentially mediate interactions between a PRE and specific enhancers, and thus in some way provide specificity of activity to the PRE. One possibility is that multiple PcG complexes assemble at each PRE, and that different complexes interact with different enhancers.

The *Ubx* gene is unusual in that it requires wild-type *Asx* function for proper spatial restriction in the CNS, but the CNS derepression phenotype of *Ubx* in *Asx* mutants is relatively mild and restricted to a few individually staining cells in PS3 and PS4. Conversely, the *bxd14* reporter shows strong derepression in the CNS in *Asx* mutants. Mutations in *Pc* and *esc* differ from *Asx* mutations in that they show approximately equal derepression phenotypes for the *bxd14* reporter and the endogenous *Ubx* gene. The fact that CNS expression of the *bxd14* reporter is more sensitive to *Asx* mutations than the endogenous *Ubx* gene indicates that at least in relation to *Asx* activity, functional interactions may exist between the *bxd14* element and other elements at the *Ubx* locus. Such interactions may help stabilize PRE activity at the endogenous locus, but when the *bxd14* element is isolated these stabilizing interactions are not present and thus it becomes more sensitive to *Asx* activity.

ASX binding to polytene chromosomes

Our polytene staining results show that ASX binds to the sites of the Antennapedia complex and to the bithorax complex (BX-C), consistent with the homeotic phenotypes, and reports of ectopic expression of homeotic genes in *Asx* mutants (McKeon and Brock, 1991; Simon et al., 1992; Soto et al., 1995). The apparent exception is that *Asx* does not appear to be required for regulation of *Scr, abd-A* or *Abd-B* in the CNS (Soto et al., 1995). Nevertheless, *Asx* is required for regulation of endogenous *Ubx* in the central nervous system, suggesting that it specifically regulates *Ubx*. This result implies that different PcG proteins can bind to different PREs within the BX-C.

We have shown that *Asx* mutations strongly enhance mutations in most other PcG genes (Campbell et al., 1995). One explanation for these data is that PcG proteins participate in multimeric complexes (Franke et al., 1992). Consistent with this idea, ASX protein partially co-maps with PH, PC and PCL proteins on polytene chromosomes. The finding that PSC binds to multiple PC/PH sites on polytenes but also has many unique binding sites shows that there are different PcG complexes that bind to different sites. The ASX-binding sites that differ from PC/PH-binding sites and from most PSC-binding sites indicate that ASX can bind to polytene chromosomes in the absence of PC, PH or PSC. This fact could explain some of the unique Asx phenotypes. Because many PC/PH-binding sites do not include ASX, ASX is not required in all cases for PC/PH activity. Conversely, there must be different classes of PH/PC complexes. One complicating factor is that there is no way of knowing if the PcG proteins bound at a given polytene site are members of functional repressive complexes. For example, all PcG proteins tested bind to site 2D, the site of the *ph* locus and yet *ph* is obviously not repressed in salivary glands. A similar argument can be made about site 51A, the location of the *Asx* locus. Nevertheless, our polytene binding data, combined with the previous results of others, argue strongly for heterogeneity of PcG complexes and for the possibility of discrete complexes with different functions. Because ASX binds to many unique sites, we argue that ASX is likely to have different functions than other PcG proteins.

Models of Asx function

Asx mutations have weaker homeotic phenotypes than mutations in some other PcG genes. Our results, and those of Soto et al. (1995), suggest that one reason is that homeotic genes are differentially sensitive to mutations in *Asx* within a given tissue; another reason may be that homeotic genes exhibit tissue-specific responses to *Asx*. One way to acount for these data is to postulate that *Asx* mediates interactions between specific enhancers and the complexes bound to a parasegmental PRE or PREs. In this view, ASX would not be an obligate member of repressing core PcG complexes containing PH and PC, but could associate with specific PcG complexes binding to a PRE, or with specific enhancers, to mediate interaction between proteins bound to the PRE and to the enhancer, determining whether a given enhancer was or was not repressed by PcG proteins. It will be interesting to determine if ASX protein binds the PRE and if its binding sites overlap with those of other PcG proteins.

Asx^{P1} mutations exhibit anterior as well as posterior transformations (Sinclair et al., 1992), and most *Asx* mutations tested enhance homeotic transformations of *trx* mutations (T. A. M and H. W. B, unpublished observations). However, Asx^{P1} and all other *Asx* alleles tested also enhance homeotic transformations of other PcG genes (Campbell et al., 1995; Jurgens 1985; T. A. M. and H. W. B., unpublished observations). This indicates that *Asx* has an important role in both activation and repression of homeotic loci. Interestingly, another PcG gene, *Enhancer of zeste* also appears to have a dual role in activation and repression of homeotic loci (LaJeunesse and Shearn, 1996). It is possible that ASX (and by extension E(Z)), is a member of both PcG and trithorax group complexes and thus has a dual role in activation and repression. Consistent with the idea that ASX functions as a mediator between protein complexes at the PRE, it is also possible that ASX mediates interactions between PcG and trithorax group proteins to determine if repression by PcG proteins or activation by trithorax group proteins dominates at target sites. ASX could be required for a step that precedes both activation and repression, and thus mutations in *Asx* could affect both processes. Whatever its function, the fact that ASX distribution on polytene chromosomes does not completely

overlap with other PcG proteins argues that ASX has a specific rather than a general role in repression and activation.

The PcG proteins are normally considered to be specific repressors, because PH and PC bind to about 100 sites on polytene chromosomes. Yet if the unique sites of PSC and ASX are considered in addition, the number of PcG targets is increased to about 160, suggesting by extension that many PcG targets remain to be identified. Our data argue strongly against the idea that there is only one way to construct a PcG complex. Instead, it seems likely that a group of related complexes, probably with analogous functions, can be assembled from different components, some of which are shared between complexes, and some of which are not. The shared proteins are likely to constitute a basic structural core and the unique proteins may provide the ability to modulate the activity or specificity of the complex. Because there are ASX-binding sites on polytene chromosomes not recognized by PH, PC, PCL or PSC, we predict that there must be complexes constructed from very different components, that likely have different core proteins. In this model, it may turn out that many genes can be repressed by PcG-like complexes, and that the original definition of PcG genes as repressors of homeotic loci will turn out to be too limited. It will be interesting to see if novel complexes containing a subset of PcG proteins function in repression. For the future, an important goal will be to understand the mechanisms that account for assembly of different PcG complexes at different targets, to determine the role of individual constituents of PcG complexes and to determine if different PcG complexes have different functions.

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