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 Stenglanz, 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 (Axx) 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 Axx 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 Axx enhance the phenotypes of other dominant PcG mutations in doubly heterozygous adults (Campbell et al., 1995) and in embryos (Jurgens, 1985). Most Axx mutations appear to be gain of function, because their zygotic phenotypes are more severe than those of homozygous deficiency embryos (Sinclair et al., 1992).

Axx mutations also exhibit a number of phenotypes that are exhibited by a restricted subset of PcG genes, or that are unique to Axx. Even though zygotic Axx mutants ectopically express homeotic genes (McKeon and Brock, 1991; Simon et al., 1992), zygotic mutant embryos and embryos derived from mothers with Axx− 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 Axx 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 Axx has a specific role in gene regulation.

Two other features suggest that Axx differs from most PcG genes. First, the P-element-induced allele, AxxP1, 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 Axx mutant suggests that Axx might have a dual role in repression and in activation. Consistent with this idea, mutations in Axx 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 Axx 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 Axx 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 Axx 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 Axx mRNA does not appear to be deposited in oocytes. Axx 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 Axx is achieved by tissue-specific distribution of the ASX protein, but support the hypothesis that unique Axx 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 AxxP1 strain and other Axx alleles were described in Sinclair et al. (1992). All flies were raised at 25°C on standard cornmeal sucrose medium containing teosol 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 bsd14 element were obtained from W. Bender.

**Molecular procedures**

General molecular procedures were carried out as described (Sambrook et al., 1989). The AxxP1 mutation is a P element insertion that fails to complement Axx. DNA flanking the insertion was recovered from a genomic library established from AxxP1 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 hybridized 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 PstI-KpnI fragment into the smem 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 and affinity purified on a column containing the peptide as described (DeCamillis and Brock, 1994). Comparison of the DNA obtained from Asx3/+ 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 AsxP1 allele lies in the 4.5 kb EcoRI 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. Asx3 has an approximately 1.3 kb deletion that maps to the 2.3 kb SalI fragment within the 6.0 kb EcoRI 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 Asx3 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 Asx3 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

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**RESULTS**

Characterization of the Asx region

We reported previously the recovery of the AsxP1 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 AsxP1 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 AsxP1 allele lies in the 4.5 kb EcoRI 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
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-CxxxCxxxCxxxCxxxC, 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_3/10 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).

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 cytoplasm. 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<sup>P1</sup> adults
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 \textit{Ubx} in \textit{Asx} \textit{P1} embryos relative to embryos homozygous for a deficiency uncovering \textit{Asx} (\textit{Df}(2R)\textit{trix}) or for a gain-of-function \textit{Asx} (\textit{Asx} \textit{3}) mutation. We monitored \textit{Ubx} expression using the \textit{bxd14} element, which carries a Polycomb Group Response Element (PRE) and is included in a 14.5 kb fragment from the \textit{bxd} regulatory region of \textit{Ubx} that regulates a \textit{lacZ} reporter in parasegments 6-13 (Simon et al., 1990). This element has been used by Soto et al. (1995) to show that \textit{Asx} is active in the CNS. As shown in Fig. 5, compared to wild-type, both \textit{Asx} \textit{P1} and \textit{Df}(2R)\textit{trix} embryos show derepression of the \textit{lacZ} reporter in the central nervous system in parasegments 2-5. Similar results were obtained with \textit{Asx} \textit{3} (results not shown). We see no evidence of reduced expression of the \textit{bxd14} reporter in parasegments 6-13. We conclude that \textit{Asx} \textit{P1} mutants, like other \textit{Asx} mutants, cause derepression of the \textit{bxd14} reporter in the anterior CNS during embryogenesis.

Soto et al. (1995) have reported that in \textit{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 \textit{Asx} in the central nervous system using the \textit{bxd14} element that contains a \textit{Ubx} regulatory element. Therefore, we decided to re-examine the requirement for \textit{Asx} in the CNS for regulation of endogenous \textit{Ubx}. Fig. 5 shows that \textit{Ubx} is ectopically expressed in parasegment 3 in stage 15 embryos in two \textit{Asx} mutants, and thus that \textit{Asx} is required for regulation of the endogenous \textit{Ubx} gene in the CNS. However, \textit{Asx} \textit{P1} mutants have no effect on the regulation of the endogenous \textit{Ubx} gene (data not shown).

\textbf{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. \textit{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 \textit{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 \textit{ASX}-binding sites and compares them to the binding sites of \textit{PH}/\textit{PC}/\textit{PCL}. Of the 90 sites, 27 are unique to \textit{ASX} and 63 overlap with \textit{PC}/\textit{PH}-binding sites. \textit{PC} and \textit{PH} bind an additional 38 sites not recognized by \textit{ASX}. A comparison of \textit{ASX} and \textit{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 \textit{PH} or \textit{PC} but stain very weakly with \textit{ASX}, whereas sites 35AB, 56C and 93E stain very intensely for \textit{ASX} but weakly for \textit{PH} or \textit{PC}. Only two of the unique \textit{ASX} sites overlapped sites unique to PSC, which also demonstrates about 70% overlap with \textit{PH}/\textit{PC} (Rastelli et al., 1993). One of the common \textit{ASX}-binding sites is 51A, which
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<sup>P1</sup> alleles of Asx exhibit both posterior and anterior transformations, but mapping of the P element insertion site in Asx<sup>P1</sup> 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 and protein in ovaries and embryos (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<sup>P1</sup> mutations (T. A. M., unpublished data).

Asx<sup>P1</sup> homozygotes show anterior transformations only in adults. We confirmed that Asx<sup>P1</sup> homozygotes exhibit ectopic expression of the bxd14 reporter, similar to that seen in other Asx mutations. Despite the strong effect that the Asx<sup>P1</sup> mutation has on the regulation of the bxd14 reporter gene, homozygous Asx<sup>P1</sup> 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.

### DISCUSSION

**Comparison of ASX to other PcG proteins**

The ASX protein shares structural features with other members of the PcG 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 and protein in ovaries and embryos (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<sup>P1</sup> mutations (T. A. M., unpublished data).

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

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<td>36B</td>
<td>75D</td>
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<tr>
<td>-</td>
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<tr>
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is the location of the Asx locus, suggesting that ASX, like other PcG genes (DeCamillis et al., 1992), may be autoregulated.
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 a complicated view of how PREs function, particularly in the context of the BX-C, and this view is not consistent with the notion that PREs are promiscuous in their interactions with specific enhancers. The apparent exception is that ASX does not appear to be required for regulation of Scr in Abdominal-B (Soto et al., 1995) 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 polytenes. 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 polytenes 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 determining 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, argues 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 account 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 alleles 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, AsxP1 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 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 polytenes 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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REFERENCES


Drosophila


