Genetic and Molecular Analysis of the *Drosophila melanogaster* Polycomb Group Gene *Additional Sex Combs*

by

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ABSTRACT

Proteins of the trithorax group (trxG) and the Polycomb group (PcG) maintain the spatially restricted active and repressed states respectively of the homeotic genes throughout development. Mutations in PcG genes cause homeotic genes to be ectopically expressed which generally results in posterior homeotic transformations. Mutations in trxG genes have the opposite effect. They reduce homeotic gene expression and generally cause anterior transformations. Mutations in trxG and PcG genes mutually suppress each others homeotic transformations, suggesting that the two groups of proteins either act antagonistically or that they have opposite and independent functions. *Additional sex combs* (*Asx*) can enhance both trxG and PcG homeotic mutations, suggesting that *Asx* is important for both activation and repression of the homeotic loci. In addition, *Asx* has both tissue and stage specific effects on homeotic gene regulation.To gain further insight into *Asx* function, I used antibodies to the Asx protein to examine its expression pattern in embryos and to show that Asx protein binds to sites on polytene chromosomes containing other PcG proteins, but that it also binds to some unique target sites. Asx protein is probably part of a large PcG complex that binds to many target sites but the fact that it can bind to some unique target sites also indicates that it may be a member of other non-PcG protein complexes. To identify and clone genes important for *Asx* activity, interactor cDNAs from a yeast 2 hybrid screen were mapped to specific chromosomal sites. A large deletion (*Df(3L)ZN47*) that removes the locus of the *z40* interactor genetically interacts with *Asx* and shows target specific homeotic regulatory defects. Also, the PcG gene *super sex combs* (*sxc*) shows a strong genetic interaction with *Asx* and an attempt was made to transposon tag and clone *sxc*. These results suggest that *Asx* is a component of both repression and activation and that its stage and tissue specific activities are modulated by interactions with specific protein subsets such as z40.

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I would like to dedicate this thesis to my wife Isabella.

General Introduction

Overview

The significance of the Polycomb group (PcG) of genes in *Drosophila melanogaster* was recognized by Lewis in 1978 when he suggested that the segmental transformations observed in *Polycomb* (*Pc*) mutants were the result of the misregulation of homeotic genes. Although at the time the Polycomb 'group' was not yet a group as there were only two clearly identified members, this initial observation was important in recognizing that separate genes existed which did not contribute to the final morphological differentiation of the adult fly except by ensuring that the homeotic genes were expressed within their proper expression boundaries.

Drosophila development occurs in three basic stages; i) embryogenesis, ii) the larval stages and iii) pupation. The process of pupation eventually produces the adult fly. During pupation, most adult tissues are derived from a small set of disc-like tissues termed the imaginal discs that in embryogenesis segregate away from cells that give rise to the larval tissues. PcG proteins are important components of a system that maintains cell memory throughout these developmental stages by regulating developmentally important target genes (Struhl and Akam 1985a; Wedeen et al. 1986b; Riley et al. 1987a; Glicksman and Brower 1988a; Dura and Ingham 1988b; Glicksman and Brower 1990b; McKeon and Brock 1991a; Simon et al. 1992b; Moazed and O'Farrell 1992; Pelegri and Lehman 1994; Serrano et al. 1995). PcG proteins appear to be required for the regulation of multiple target genes but the homeotic loci are their best characterized targets (Struhl and Akam 1985b; Wedeen et al. 1986a; Riley et al. 1987b; Glicksman and Brower 1988b; Dura and Ingham 1988a; Glicksman and Brower 1990a; McKeon and Brock 1991b; Simon et al. 1992a; Soto et al. 1995), and are perhaps of the most importance. The homeotic loci determine the anterior-posterior morphological polarity of the developing organism.

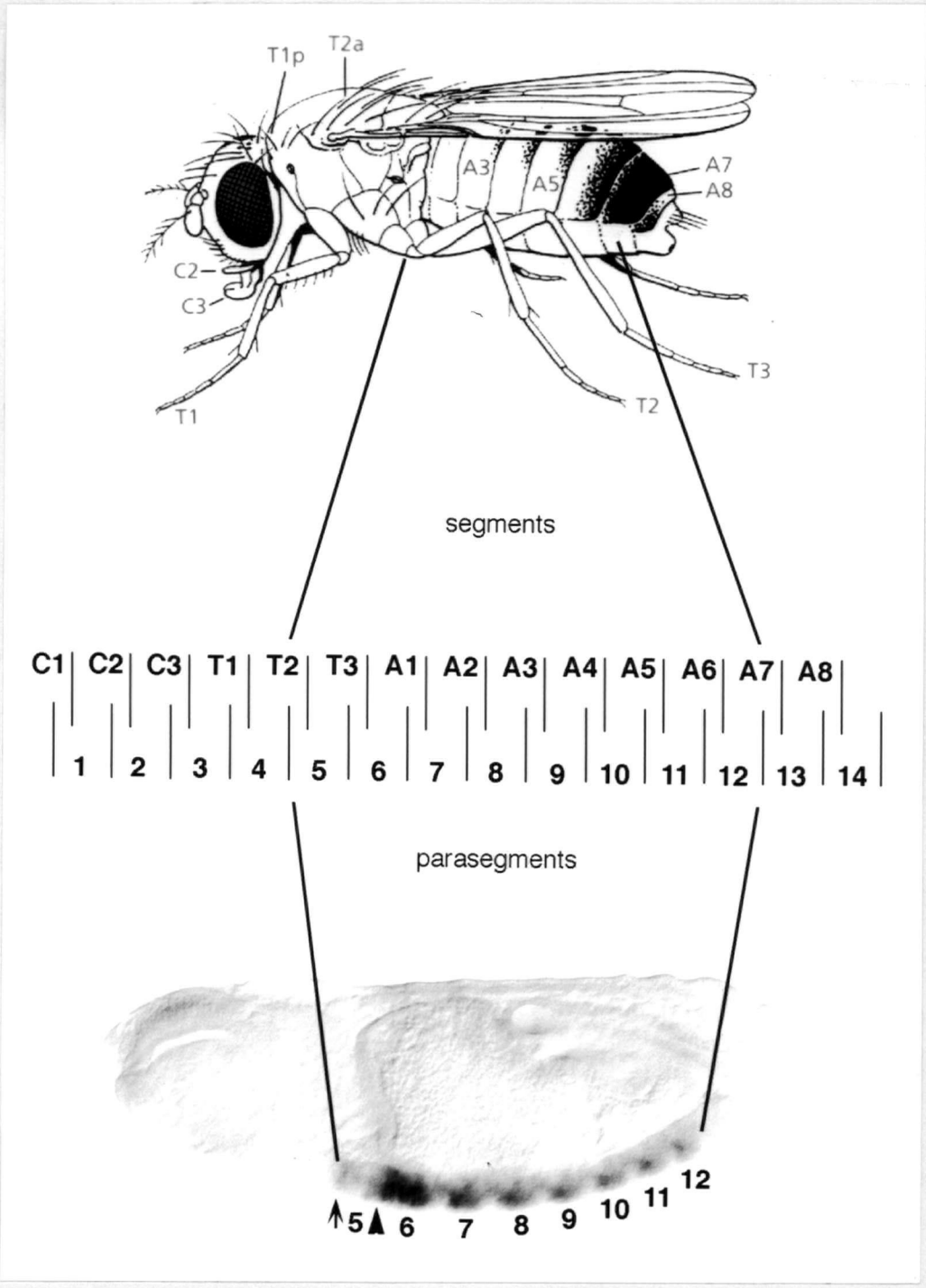
The homeotic genes

The 8 homeotic genes are arranged in two gene complexes (the HOM-C): the Antennapedia complex (ANT-C), which determines the proper development of the anterior regions of the body including the anterior of the thorax, and the bithorax complex (BX-C), which determines the proper development of the posterior thorax and the abdomen (Lewis 1978; Struhl 1982; Bender et al. 1983; Sanchez-Herrero et al. 1985; McGinnis and Krumlauf 1992). The ANT-C contains the homeotic genes *labial* (*lb*), *proboscipedia* (*pb*), *Deformed* (*Dfd*), *Sex combs reduced* (*Scr*) and *Antennapedia* (*Antp*) (McGinnis and Krumlauf 1992), and the BX-C contains the genes *Ultrabithorax* (*Ubx*), *abdominalA* (*abdA*), and *AbdominalB* (*AbdB*) (McGinnis and Krumlauf 1992). The order in which the genes appear in the HOM-C reflects the order in which the genes are expressed in the developing embryo. Starting from the 3' end of the complex, the HOM-C genes are activated with progressively more posterior domains of expression moving in a 5' direction through the complex (McGinnis and Krumlauf 1992). This incredible correlation between the position of a HOM-C gene in the complex and its domain of expression in the embryo is also highly conserved in the mammalian homeotic (or *Hox*) gene complexes (Duboule and Dolle 1989; Graham et al. 1989; Krumlauf 1994) .

A developing *Drosophila* embryo is divided into discrete units termed parasegments. Embryonic parasegments are slightly offset from but roughly correlate with the segments of the adult fly (see Fig. 1). Each of the homeotic genes listed has a unique embryonic expression pattern that is restricted to a specific set of parasegments (see Fig. 1 for the *Ubx* expression pattern). The parasegmental expression pattern of a particular homeotic gene correlates with the morphological determination of the corresponding set of segments in the adult fly (Lewis 1978; Bender et al. 1983; Sanchez-Herrero et al. 1985; Beachy et al. 1985; Carroll et al. 1986; Akam 1987; McGinnis and Krumlauf 1992). An alteration in the parasegmental expression domain of a particular homeotic gene can result in the morphological transformation of one segment into another (Lewis 1978; Struhl 1982;

Fig.1

Comparison of the embryonic expression pattern of the homeotic gene *Ubx* with the corresponding adult fly segments. Parasegments 1-14 in the embryo roughly correlate with the three head segments (C1-C3), three thoracic segments (T1-T3), and eight abdominal segments (A1-A8) of the adult fly. *Ubx* embryonic expression in parasegment (PS) 5 is faint and the protein accumulates in the anterior region of PS5 which corresponds to the posterior of the second thoracic segment (T2). The anterior boundary of PS5 is marked by an arrow. Ubx protein accumulates to high levels throughout PS6 (the PS6 anterior boundary is marked by an arrowhead) and at moderate levels in parasegments 7 to 12. PcG proteins maintain repression of the *Ubx* gene in PS1-4. A PcG mutation can cause the PS5 restricted boundary of *Ubx* expression to break down, thus causing *Ubx* expression to expand into more anterior (para)segments. *Ubx* expression in more anterior segments causes transformations of anterior segments into more posterior ones such as the transformation of the wing (a T2 structure) towards the haltere (a T3 structure). The embryo is positioned laterally and oriented with anterior to the left, posterior to the right, ventral down and dorsal up. The fly drawing is taken from page 4 in Lawrence 1992.


T1p
T2a
A3
A5
A7
A8
C2
C3
T1
T2
T3
segments
C1 C2 C3 T1 T2 T3 A1 A2 A3 A4 A5 A6 A7 A8
1 2 3 4 5 6 7 8 9 10 11 12 13 14
parasegments
5 6 7 8 9 10 11 12

Bender et al. 1983; Beachy et al. 1985; Carroll et al. 1986; Duncan 1987; McGinnis and Krumlauf 1992).

For example, if *Ubx* is ectopically expressed in parasegment 4, the second thoracic segment (T2) can be transformed towards the third (T3). This would be visible as a transformation of the T2 wing towards the small wing-like structure known as a haltere found on T3 (Lewis 1978). A T2 to T3 transformation is an example of a posterior homeotic transformation, where a more anterior structure is transformed towards a more posterior one. Posterior transformations are seen with gain of function (GOF) regulatory mutations of the homeotic genes where homeotic genes are ectopically expressed beyond their normal expression boundaries (Struhl 1981; Duncan 1987; McGinnis and Krumlauf 1992; Shimell et al. 1994). Mutations in PcG genes also produce GOF homeotic transformations (Slifer 1942; Duncan 1982; Duncan and Lewis 1982; Ingham 1984; Jurgens 1985; Dura et al. 1985). This indicates that the PcG are negative regulators required for the repression of the homeotic genes outside of their normal expression domains. Loss of function (LOF) regulatory mutations of the homeotic genes have the opposite effect, they cause anterior transformations (Struhl 1981; Duncan 1987; McGinnis and Krumlauf 1992). Another set of proteins, the trithorax group, (trxG) are required to maintain normal levels of homeotic gene expression within their spatial expression domains (Kennison 1993). Thus trxG mutations reduce expression and generally cause LOF or anterior homeotic transformations (Kennison 1993).

Early versus late regulation of the homeotic genes

Homeotic gene regulation is divided into an early initiation stage and a late maintenance stage. Early developmental regulation of the homeotic genes is achieved by the segmentation genes. Products of the segmentation genes set up an early regulatory heirarchy in the embryo that reflects the clear metameric development of later stages (Akam 1987; Ingham 1988). Disruption of homeotic gene regulation during early embryogenesis

has an eventual effect on imaginal disc morphogenesis and thus directly effects the determination and final morphology of the adult structures. Since segmentation gene expression patterns are transitory and fade away early in development, other factors are required to maintain homeotic gene expression domains in later stages of the developing embryo and in the imaginal discs. In PcG mutants, early expression of the homeotic genes is normal, it is only at later embryonic stages (stage 10 or 11) that homeotic gene regulation breaks down resulting in the ectopic expression of the homeotic genes (Struhl and Akam 1985a; Wedeen et al. 1986b; Riley et al. 1987a; Glicksman and Brower 1988a; Dura and Ingham 1988b; Glicksman and Brower 1990b; McKeon and Brock 1991a; Simon et al. 1992b; Soto et al. 1995). This indicates that the PcG are not required for the initial repression of the homeotic genes but are required for the maintenance of homeotic gene repression. The trxG gene products are also required at this stage for maintenance of homeotic gene activation within their normal expression domains (Kennison 1993). Thus the activity of these two groups of proteins maintains the tight homeotic gene expression patterns that are set up by the early acting segmentation genes.

PcG Homeotic Phenotypes

Most homeotic genes are expressed with restricted anterior expression boundaries. In PcG mutants, these restricted boundaries break down and homeotic genes are ectopically expressed in more anterior regions (Struhl and Akam 1985a; Wedeen et al. 1986b; Riley et al. 1987a; Glicksman and Brower 1988a; Dura and Ingham 1988b; Glicksman and Brower 1990b; McKeon and Brock 1991a; Simon et al. 1992b; Soto et al. 1995). The ectopic expression of homeotic genes in PcG mutants is also referred to as derepression. Derepression of the homeotic genes produces distinctive GOF homeotic transformations such as the transformation of the wings to the halteres (small 'pseudowing' structures located posterior to the wing), the transformation of antenna to leg, the transformation of the 4th abdominal segment (A4) to the 5th (A5, most easily seen in males), and the

transformation of the first abdominal segment (A1) into more posterior segments (Slifer 1942; Duncan 1982; Duncan and Lewis 1982; Ingham 1984; Jurgens 1985; Dura et al. 1985).

One exception to the above is the extra sex combs phenotype which results from a break down in the posterior expression boundary of the ANT-C gene *Sex combs reduced* (*Scr*) (Glicksman and Brower 1988a). In PcG mutants, *Scr* is ectopically expressed in the second and third legs transforming them towards the first leg. The first legs of male *Drosophila melanogaster* have unique structures about midway down the leg termed sex combs. Transformation of the second and third legs into the first results in the appearance of sex combs on the second and third legs. Many of the PcG members were originally identified due to this distinctive phenotype, and it is a characteristic of many PcG genes that they are haplo-insufficient with regards to the sex comb phenotype. That is, heterozygous PcG mutants display sex combs on the second and sometimes the third legs of male flies, indicating that a loss of one copy of the gene is enough to disrupt its normal function. Double heterozygous combinations between different PcG mutations tend to enhance the severity of the extra sex combs phenotype and other PcG homeotic phenotypes (Hannah-Alava 1958; Kennison and Russell 1987; Kennison and Tamkun 1988; Adler et al. 1989; Cheng et al. 1994; McKeon et al. 1994; Campbell et al. 1995)

The Polycomb group genes

All the PcG members identified so far maintain repression of the homeotic genes outside their normal expression domains. To date, about 15 PcG members have been identified; *extra sex combs (esc), Polycomb (Pc), polyhomeotic (ph), Posterior sex combs (Psc), Enhancer of zeste (E(z)), Polycomb-like (Pcl), Additional sex combs (Asx), Sex combs on midleg (Scm), Sex combs extra (Sce), super sex combs (sxc), pleiohomeotic (pho), multi sex combs (mxc), Suppressor of zeste 2 (Su(z)2) , Enhancer of Polycomb (E(Pc))* (Simon 1995) and *cramped (crm)* (Yamamoto et al. 1997). Most members have

been identified either by their strong adult homeotic transformations (Slifer 1942; Lewis 1978), cuticle defects (Nusslein-Volhard et al. 1984) or enhancement of PcG or GOF homeotic phenotypes (Botas et al. 1982; Kennison and Russell 1987). It is likely that most of the stronger members of the group have already been identified. However, deletion analysis of the genome looking for enhancers of PcG homeotic phenotypes has produced estimates that there may be up to 40 PcG genes in total (Jurgens 1985; Landecker et al. 1994).

There is strong evidence that PcG proteins form large functional complexes with one another. Polycomb (Pc) and polyhomeotic (ph) proteins co-immunoprecipitate as members of a large MW complex (Franke et al. 1992). The Sex combs on midleg (Scm) and ph proteins physically interact *in vitro* (Peterson et al. 1997) and domains from Pc, ph and Posterior sex combs (Psc) proteins also interact *in vitro* (Strutt and Paro 1997; Kyba and Brock 1998). Additionally, antibody binding indicates that Pc, ph, and Polycomblike (Pcl) proteins completely overlap at about 100 sites on the large polytenized chromosomes from the larval salivary glands (Franke et al. 1992; Lonie et al. 1994). Scm overlaps with these proteins at all sites examined (Peterson et al. 1997, a complete list of Scm binding sites is not yet published) while Psc, Suppressor of zeste 2 (Su(z)2), and Enhancer of zeste (E(z)) overlap at a subset of these polytene binding sites as well as binding to a number of unique sites (Rastelli et al. 1993; Carrington and Jones 1996). All these PcG proteins bind to the homeotic loci. This suggests that many of the PcG proteins form at least one functional complex *in vivo* and complex formation is probably required at a large number of chromosomal target sites.

PcG proteins act on specific target loci through regulatory elements termed Polycomb Group Response Elements (PREs). PREs were originally identified as regulatory maintenance elements that were required to maintain homeotic gene expression patterns late in embryogenesis (Simon et al. 1990; Zink et al. 1991; Muller and Bienz 1991). Enhancer regions from various BX-C and ANT-C genes have been shown to drive

the expression of reporter constructs in a homeotic gene specific expression pattern (Simon et al. 1990; Zink et al. 1991; Muller and Bienz 1991). However, these highly regulated expression patterns begin to break down at about 3-6 hrs., producing ectopic expression of the reporter constructs. Maintenance of spatially restricted expression patterns late in embryogenesis only occurs when a PRE is present in conjunction with the gene enhancer elements (Zink et al. 1991; Muller and Bienz 1991; Busturia and Bienz 1993; Simon et al. 1993; Pirrotta et al. 1995; Chan et al. 1994; Chiang et al. 1995). Maintenance of these spatially restricted expression domains is abolished in PcG mutants (Zink et al. 1991; Muller and Bienz 1991; Busturia and Bienz 1993; Simon et al. 1993; Pirrotta et al. 1995; Chan et al. 1994; Chiang et al. 1995) indicating that these PRE maintenance elements are dependent on functional PcG activity .

PREs have also been identified by their PcG dependent variegated repressive effects on a *white* reporter gene (Fauvarque and Dura 1993; Kassis 1994; Gindhart and Kaufman 1995; Hagstrom et al. 1997). Not all PREs necessarily act as maintenance elements, but all maintenance elements that have been identified are PREs. For the sake of simplicity, PRE maintenance elements will simply be referred to as PREs.

Transgenes with a PRE present produce an ectopic PcG binding site on polytene chromosomes (Chan et al. 1994; Chiang et al. 1995), indicating that PREs probably contain binding sites for PcG proteins. *In vitro* analysis of the bxd5.1 element, a minimal PRE from the regulatory region of the homeotic gene *Ubx*, has identified several high and low mobility complexes that contain the PcG protein ph and bind to distinct subelements within the 5.1 element (Hodgson and Brock 1998). Thus, at least in the case of the *bxd* PRE, maintenance elements bind large complexes containing PcG proteins that function in maintaining the repression of target loci late in development.

Models of PcG function

The specific mechanism of PcG mediated repression of target loci is unknown. Several models of PcG function have been suggested; 1) that PcG proteins repress target loci by compacting the DNA into a heterochromatin-like structure (Paro 1990), 2) that PcG proteins target DNA segments to repressive "compartments" in the nucleus (Schlossherr et al. 1994), 3) that PcG proteins inhibit transcription initiation (Bienz 1992), or 4) that PcG proteins interfere with the interaction between enhancer elements and the promoter by "looping" out the DNA (Pirrotta 1995). The main problem with all of these models is that none of them have any strong data to support them.

The heterochromatin model was originally suggested because the PcG protein Pc and the chromatin protein HP1 share a functional domain in common. HP1 is encoded by a Suppressor of position effect variegation (Su(var)), *Su(var)205* . Su(var)s appear to be modifiers of heterochromatin (Henikoff 1990). However, most PcG mutations that have been tested are not modifiers of PEV and of those that are, only *E(Pc)* acts as a Su(var) (Sinclair et al. 1998a). As well, alterations in regulation of the homeotic loci do not affect restriction enzyme accessibility suggesting that the DNA is not in a compacted state (Schlossherr et al. 1994; McCall and Bender 1996). The compartment idea has not been disproven but it does not seem likely to be true. PcG proteins, in general, bind to about 100 sites on both polytene and mitotic chromosomes (Franke et al. 1992; Buchenau et al. 1998) and these sites are not localized to a specific region of the nucleus, so one would have to argue that there are at least 100 separate repressive compartments in the nucleus. PcG proteins do not seem to interfere with basal transcription since transgenes inserted within the homeotic loci are still transcribed even in regions where the endogenous homeotic gene is repressed (McCall and Bender 1996). Thus if they do interfere with transcriptional initiation, it must be through promoter specific elements and not through the general transcriptional machinery.

Finally, there is no evidence for the existence of looping structures. Presumably, the looping model would predict that PREs isolate enhancer elements and make them

unavailable for enhancer-promoter interactions. Constructs that are flanked by PREs are still subject to position specific alterations in reporter gene expression (Zink and Paro 1995) suggesting that the enhancer and promoter elements have not been isolated from interacting with external elements. However, (Muller 1995) has shown that stable PcG repressive complexes can be assembled at artificial target sites if at least one PcG protein is targeted to the site and if there is an enhancer element and promoter from the endogenous *Ubx* locus present. This indicates that there is specificity in PcG mediated repression of enhancer-promoter interactions and suggests that PcG activity is somehow dependent on PRE-enhancer-promoter cross talk. That PREs can crosstalk with each other is supported by the observation that transgenes containing a PRE show stronger repression of the reporter when they are paired in *trans* (Fauvarque and Dura 1993; Kassis 1994; Chan et al. 1994; Zink and Paro 1995; Hagstrom et al. 1996; Hagstrom et al. 1997) than when they are present as single elements.

One possibility is that weak PcG binding sites in enhancer regions somehow crosstalk with strong PcG binding sites located on PREs and interfere with the function of activation complexes. Double mutations of PcG and trxG genes generally lead to the suppression of the homeotic transformations seen with mutations in either group alone (Ingham 1983; Capdevila et al. 1986; Kennison and Tamkun 1988). This has led to the suggestion that PcG and trxG proteins either have opposite and independent functions (Ingham 1983) or they function antagonistically to one another, possibly involved in a stoichiometric competition for repression or activation at specific target loci (Jones and Gelbart 1993). Thus another model of PcG mediated repression is that binding of PcG proteins to multiple regulatory sites within a locus directly interferes with the activity of trxG proteins and prevents them from activating loci in specific embryonic domains.

The *Additional sex combs* (*Asx*) gene

The PcG gene *Additional sex combs* (*Asx*) shares many general attributes with other PcG genes, but it has some distinctive aspects that may shed light on PcG repressive activity. First of all, most PcG mutations show the same extent of homeotic gene derepression in all embryonic tissues. *Asx* , however, has some very strong tissue specific effects. It is required for homeotic gene regulation in the epidermis but has only a minor effect on regulation in the central nervous system (CNS) (McKeon and Brock 1991a; Soto et al. 1995). This suggests that unlike other PcG genes, *Asx* activity is targeted in both a parasegment specific and a tissue specific manner in the embryo. Secondly, *Asx* mutations generally display weak PcG homeotic transformations, but the allele Asx^{P1} strikingly displays both trxG and PcG homeotic transformations (Sinclair et al. 1992). This suggests the possibility that *Asx* could have a role in mediating both PcG repression and trxG activation of the homeotic loci. Finally, many *Asx* mutations display segmentation defects (Sinclair et al. 1992), a phenotype that is usually associated with mutations in early patterning events. This possibly implicates *Asx* as having a possible role in initiation as well as maintenance. Taken together, an analysis of *Asx* could tell us 1) possible ways of targeting PcG repressive activity in a tissue specific manner, 2) the relationship between PcG mediated repression and trxG mediated activation at target loci, and 3) possible implications for PcG function in initiation as well as maintenance.

Both molecular and genetic approaches were used to analyze basic *Asx* function in relation to the activity of other PcG genes. Since different *Asx* mutations may disrupt different aspects of *Asx* function, the regulation of two different homeotic loci and a *Ubx* PRE were examined for allele specific requirements for *Asx* activity in the embryo. Antibodies raised to the Asx protein were used to look for possible relationships between the distribution of Asx protein both in the embryo and on polytene chromosomes and the tissue and target specific requirements for *Asx* activity that were observed. To examine the possibility that *Asx* could function as both a member of the PcG and the trxG, *Asx* mutations were tested for their ability to enhance both PcG and trxG homeotic mutant

phenotypes. And finally, two different approaches were used to try to identify interacting proteins that were important for *Asx* function. First of all, interacting proteins identified in a yeast-2 hybrid screen were analyzed for possible effects on homeotic gene regulation. Secondly, an attempt was made to clone the PcG gene *super sex combs* (*sxc*), a PcG gene that shows a strong genetic interaction with *Asx* . The results of these experiments are discussed within the context of *Asx* function in particular and PcG function as a whole.

Materials and Methods

Fly strains

The fly strains used in this study are described in Lindsley and Zimm 1992 and on Flybase (http://flybase.bio.indiana.edu/). Some additional specific references are also given below.

Asx^{1}, *Asx*^{3}, *Asx*^{8}, *Asx*^{9}, and *Asx*^{13} are all *Asx* homozygous lethal alleles that are described in Jurgens (1985) or Sinclair et al. (1992). Because these *Asx* mutations have stronger phenotypes than a deletion of *Asx*, they are probably gain of function *Asx* alleles. The *Asx* deficiency *Df(2R)trix* is described in Breen and Duncan 1986 and is probably the only known null allele of *Asx*. *Df(3R)red* is a large deletion the removes the *trx* locus and is therefore a null. The *trx*^{B11} allele has a deletion in the coding region of *trx* and is probably a null and *trx*^{Z11} is a point mutation in the SET domain, a protein domain conserved with some other regulatory proteins (Stassen et al. 1995). *Pc*^{4} is a strong, probably null allele. *Pcl*^{2} is a strong *Pcl* hypomorph (Jurgens 1985).

The *Asx*^{P1} allele is homozygous viable and is described in Sinclair et al., 1992. The *Asx*^{P1} chromosome carries the recessive eye mutations *cn* and *bw* so homozygous *Asx*^{P1} flies are identifiable by their white eyes.The *Asx*^{P1}*cn bw/Asx*^{P1}*cn bw ; Ly/TM3* stock was constructed in the following manner. *Asx*^{P1} *cn bw/Asx*^{P1} *cn bw ; +/+* female flies were crossed to a *+/+ ; Ly/TM3* stock. *Asx*^{P1} *cn bw/+ ; Ly/+* and *Asx*^{P1} *cn bw/+ ; TM3/+* male flies were collected from the F1 and separately back crossed to the *Asx*^{P1} *cn bw/Asx*^{P1} *cn bw ; +/+* stock. *Asx*^{P1} *cn bw/Asx*^{P1} *cn bw ; Ly/+* and *Asx*^{P1} *cn bw/Asx*^{P1} *cn bw ; TM3/+* flies were collected from the F2 and crossed to each other. *Asx*^{P1} *cn bw/Asx*^{P1} *cn bw ; Ly/TM3* flies were collected from the F3 and used to make a stock.

Df(3R)ZN47 and all other interactor deficiency strains were acquired from the Bloomington stock center. Transformed lines containing the *bxd14* element were obtained

from W. Bender and are described in Simon et al 1990 and 1993. All *sxc* alleles are described in Ingham, 1984.

Immunostaining of mutant and wild type embryos

Embryos were collected by allowing flies of the appropriate genotype to lay overnight at 25° on 2% agar plates that were coated with a thin film of 5% ethanol, 5% acetic acid and 5% yeast. Plates were also supplemented with a sprinkle of dry bakers yeast. Embryos were fixed as follows. Overnight collections were rinsed with tap water and strained through a sieve. Embryos in the sieve were placed in a 50% bleach solution for 3 min. to remove the cuticle. Embryos were then placed in an eppendorf tube containing 700µl of heptane, 630µl of PBS (pH 7.0) and 70µl of paraformaldehyde and rotated for 20 min. at room temperature. The PBS/paraformaldehyde layer was removed and 700µl of methanol was added. The embryos were then vortexed for 2 min. and any embryos that did not sink to the bottom of the tube were removed along with all liquid. Embryos were then rinsed 3X with methanol and stored in methanol at -20° for up to two months or until needed.

Immunostaining was performed by washing fixed embryos of the desired genotype 3X with a PBT solution (0.1% TritonX, 0.2% BSA in PBS), 1 X 30 min. with PBT, 1 X 30 min. with PBT + 2% normal serum, and then incubating with the primary antibody at the appropriate dilution rotating overnight at 4°. The next day, embryos were again washed 3X with PBT, then 4 X 30 min. with PBT, 1 X 30 min. with PBT + 2% normal serum, and then incubated with a secondary antibody.

For HRP (Horse Radish Peroxidase) staining, the secondary antibody was either conjugated directly to HRP or conjugated to biotin. Secondary antibody was added at the appropriate dilution (1/200-1/500 for an HRP conjugated secondary and 1/5000 for a biotinylated secondary) and the embryos were incubated for 2-4 hrs. at room temperature or at 4° overnight. After incubation with the secondary, embryos were washed as above

omitting the PBT + 2% normal serum wash. If amplification was being used, avidin conjugated to HRP was incubated at a dilution of 1/1000 at room temperature for one hour. Washes were then repeated as above. The HRP signal was detected by incubating the embryos with 0.3 mg/ml DAB (Diamnobenzidine) in PBT for 10 min. 30% H_2O_2 was added at a concentration of 1/1000 and the reaction was allowed to proceed for 10 min. After the reaction was complete, embryos were washed with PBS then washed with a dilution series of ethanol (30%,70% then 95%). All ethanol was removed and the embryos were then mounted in Gary's Magic Media (GMM), a 1:1 mixture of Canada Balsam and methyl salicylate. Embryos were viewed using Nomarski optics and photographed using Kodak Ektachrome 160T slide film (exp. +1).

For immunofluorescence, the secondary was conjugated to Texas Red and incubated in the dark at a concentration of 1/200 for 2 hrs. at room temperature. PBT washes were carried out as before, embryos were rinsed in PBS and then 1:1 DABCO:PBS was added (DABCO = 2.5mg/ml 1,4-diazobicyclo-(2,2,2)-octane in 90% glycerol, used to retard photobleaching). Embryos were allowed to settle, the DABCO:PBS was removed and DABCO was added. The embryos were mounted on slides and fluorescent images were collected using a Bio-Rad 600 confocal microscope. Confocal images were projected using NIH image and the projected images were arranged using Photoshop 4.0.

The following primary antibodies were used. The monoclonal antibody FP3.38 was used at 1/10,000 with amplification (1/1000 without) to detect Ubx protein. The monoclonal antibody 8C11 was also used at 1/10,000 with amplification to detect AntP protein. A monoclonal antibody to Scr protein was used at 1/100 and a monoclonal antibody to AbdB protein was used at 1/10 with no amplification. The axon specific monoclonal antibody BP102 was used at 1/100 to detect the CNS of *Asx* mutant embryos. An anti-ß gal antibody was used at a dilution of 1/2,000 without amplification to detect lacZ staining in embryos. Mouse and Sheep anti-Asx antibodies were used at a dilution of 1/200.

All embryos were staged according to Campos-Ortega and Hartenstein 1985. Homozygous *Asx* and other PcG mutant embryos were identified by their homeotic misexpression phenotypes, except for *Df(2R)trix* embryos stained with the axon specific antibody BP102 which were identified by double staining a *Df(2R)trix/CyOßelav-lacZ* stock with BP102 and an anti-ßgal antibody. Homozygous *Df(2R)trix* embryos stained only with BP102 and did not show the *ßelav lacZ* expression pattern. This was also used to identify *Df(2R)trix* homozygotes that were double stained with anti-ßgal and a mouse anti-Asx antibody. Asx^3 mutants were also stained with BP102 and all embryos were scanned equally for possible CNS defects. *Df(3L)ZN47* homozygotes were identified by their morphological defects.

Immunostaining of polytene chromosomes

20 pairs of wild type or Asx^{P1} *cn bw*/Asx^{P1} *cn bw* flies were allowed to lay for two days on standard cornmeal sucrose medium containing tegosept as a mold inhibitor. Dry yeast was added periodically and the larvae were raised at 17°. Late stage third instar larvae were picked off the sides of the bottles and salivary glands were dissected out of them in a 0.1% Triton-X/PBS solution. Glands were then transferred to a droplet of pre-fixative for 6-20 sec. for Asx and 15-20 sec. for Pc. The glands were then transferred to a droplet of fixative on a siliconized coverslip and left for 2-3 min. Two different pre-fixative and fixative solutions were used. A 1% paraformaldehyde(Pf)/1% Triton-X in PBS pre-fix with a 1% Pf/45% acetic acid fix or similar solutions with an increased Pf concentration of 3.7%. It was found that the lower (1%) Pf concentration improved chromosome morphology without reducing the amount of signal. The coverslips were picked up with poly-L-lysine treated slides and the glands were broken up with a pencil tip and squashed using thumb pressure. Coverslips were removed using liquid N_2 and a razor blade and the slides were stored in PBS. Chromosomes with good morphology were used immediately for immunostaining.

Chromosomes were washed in a blocking buffer (10% nonfat dry milk, 1% BSA, 0.2% NP40 and 0.2% Tween) for 30 min. at room temperature. The primary antibody was added at the appropriate dilution (1/20 for sheep and rabbit Asx antibodies and 1/50 for the rabbit Pc antibody, diluted in blocking buffer) and incubated in a moist chamber at 4° overnight. The next day, slides were washed 2 X 15 min. in wash buffer (300mM NaCl, 0.2% NP40,0.2% Tween 20 in PBS) and incubated with the secondary antibody.

For immunofluorescence, the slides were incubated at room temperature in the dark in a moist chamber for 1-4 hrs. The secondaries used were either conjugated to FITC (used at a dilution of 1/100) or Texas Red (used at a dilution of 1/50). Slides were then washed as before and mounted in DABCO. Fluorescent images were collected using a Bio-Rad 600 confocal microscope, except for Pc binding to Asx^{P1} chromosomes where photographs were taken using Kodak 400ASA color slide film on a Zeiss Axiophot Photomicroscope.

For HRP staining, slides were incubated for 1-2 hrs at room temperature with a biotin conjugated secondary. Washes were performed as before and preincubated biotin-avidin complexes from the Vectastain kit were added to the slides. The complexes were allowed to incubate with the slides for 40 min. at room temperature and then the slides were washed in 2XPBS. The HRP reaction was allowed to proceed by adding 0.5 mg/ml DAB and 1/1000 H_2O_2. Chromosomes were either counterstained with 5% Giemsa which stains them a blue-gray or with 0.1 mg/ml DAPI, a flourescent dye that is quenched by HRP staining. Photos were taken using both bright field and phase contrast optics with Kodak 160T color slide film (exp. +1).

Crosses

All crosses were performed at 25°. Flies were raised on standard cornmeal sucrose medium containing tegosept as a mold inhibitor. 15-20 females were each crossed to 15-20 males of the appropriate genotypes. Crosses were turned over once after allowing them to

lay for four days. Parents were discarded after four days and the F1 was allowed to eclose. F1 flies were then scored at two day intervals over a 10 day period.

Asx^{P1} homozygous crosses were performed in the following manner. A stock of *Asx*^{P1}*cn bw/Asx*^{P1}*cn bw ; Ly/TM3* flies were crossed to balanced homozygous lethal mutants on the third chromosome (ie. *Df(3L)ZN47*, *Pc*^{4} and various *trx* alleles). Males of the genotype *Asx*^{P1}*cn bw/+ ; mutant/TM3* or *Ly* were collected from the F1. Since there is no crossing over in males, loss of the chromosomal markers at this stage was not a problem. *Asx*^{P1}*cn bw/+ ; mutant/TM3* or *Ly* flies were then crossed to a homozygous *Asx*^{P1}*cn bw/Asx*^{P1}*cn bw ; +/+* stock. The genotypes of the F2 progeny were identified in the following manner. *Asx*^{P1}*cn bw/Asx*^{P1}*cn bw ; mutant/+* flies were white eyed (due to homozygosity of the recessive *cn bw* eye markers) but otherwise wild type. *Asx*^{P1}*cn bw/Asx*^{P1}*cn bw ; +/TM3* or *Ly* flies were white eyed but also carried a second dominant marker (either *Ly* or *Sb*). *Asx*^{P1}*cn bw/+ ; mutant/+* flies had wild type eyes and no dominant markers while *Asx*^{P1}*cn bw/+ ; +/TM3* or *Ly* flies had wild type eyes plus a dominant marker. Crosses with the *Asx*^{P1} revertant line *Asx*^{R11} were done the same way.

To test for interactions between homozygous lethal *Pc* and *Asx* alleles, mutant balanced stocks were crossed to one another and the transheterozygous adults in the F1 were scored for the penetrance of extra sex combs on the second and third legs and for the penetrance of posterior abdominal transformations (scored as patches of pigment in abdominal segments 2-4). The penetrance of PcG homeotic phenotypes was compared to sibling single *Pc* and *Asx* mutants. Single versus double mutants were easily distinguished via independant assortment of the mutant alleles in relation to dominant markers carried on the second and third chromosome balancers.

To test for interactions between homozygous lethal *trx* and *Asx* alleles, mutant balanced stocks were crossed to one another and the transheterozygous adults in the F1 were scored for overall penetrance of adult homeotic transformations of either the haltere to the wing or the fifth abdominal segment to a more anterior segment. Haltere to wing

transformations were scored as positive if the haltere was bloated or if triple row margin bristles were present. Anterior transformations in the abdomen were scored as patches lacking pigment in the 5th and 6th abdominal segments. Transheterozygous mutants were compared to their siblings that contained single mutant alleles for either *trx* or *Asx* . Single versus double mutants were easily distinguished via independant assortment of the mutant alleles in relation to dominant markers carried on the second and third chromosome balancers.

A Chi square test was perfomed on all *Asx/trx* crosses to determine if there was a statistically significant increase in the penetrance of trxG homeotic mutations in the transheterozygotes compared to the single *trx* mutants. In each case, the number of single *trx* mutant individuals with trxG homeotic mutations was used to calculate the number of individuals we would expect to see in the double heterozygotes if there was no enhancement. For example, in the cross Asx^1 females crossed to *Df(3R)red* males, 31/97 double heterozygotes and 16/113 single *trx* mutants had trxG phenotypes. 16/113=0.1416, 0.1416 * 97=13.7. Therefore, if there was no enhancement, we would expect about 14/97 double mutants to have trxG phenotypes. The actual number seen was 31. The Chi square calculation was performed as usual to yied a p value of 20.6. The Chi square table p value for 5% at 1 df was 7.879 indicating that in this case, the hypothesis that there was no enhancement was rejected.

The *sxc* screens were conducted at 25° on standard media as above. All crosses were *en masse* except for the final selection crosses which were single pair matings between one male and 5-10 females. Otherwise, screens were performed as described in the results section of Ch.4.

Mounting and photographing flies

Fly abdomens were cut dorsally with a razor blade and mounted on slides in GMM (1:1 methyl salicylate : Canada Balsam). Slides were cleared at 65° for 2-5 days. Thoraxes

were prepared by first boiling the flies for 10 min. in 10% KOH. The flies were then washed with distilled water and then a dilution series of ethanol (30%, 70%, 95%, 100%). The dorsal thorax was dissected using a pair of dissecting scissors and mounted in GMM.

All fly parts mounted in GMM were photographed using bright field optics and Kodak Tech Pan film (exp. -1,0,+1). Whole flies were photographed using a dissecting scope and Kodak 160T slide film. Scanning electron microscope (SEM) images were collected with no sample preparation. Flies were mounted on tabs and the SEM image was collected immediately.

DNA in situ hybridization to polytene chromosomes

20 pairs of wild type flies were allowed to lay for two days on standard cornmeal sucrose medium containing tegosept as a mold inhibitor. Dry yeast was added periodically and the larvae were raised at 17°. Late stage third instar larvae were placed in a droplet of 0.8% NaCl solution and then into a droplet of 45% acetic acid. Salivary glands were dissected and transferred to a droplet of fixative (3:2:1 Acetic acid:water:lactic acid) on a siliconized coverslip. Fixation was allowed to occur for 4-5 min. and then the glands were broken up by tapping the coverslip with the end of a pencil. The pencil was used to spread the chromosomes by trailing it across the coverslip in a zig zag motion and the slides were left at 4° overnight. This allows the fixative to evaporate and flattens the chromosomes. Coverslips were removed by plunging the slide into liquid nitrogen and flipping them off with a razor blade. Slides were plunged into 95% ethanol for 10 min., air dried, and stored at room temperature until needed.

A standard lab protocol was followed for the labelling and hybridization of probes to polytene chromosomes. The general approach is outlined below.

Interactor cDNA probes were labelled using a Boehringer-Mannheim digoxigenin (DIG) random prime labelling kit. 1μg of gel purified template DNA was used for each reaction and the protocol was followed as stated in the kit. Once the reaction was complete,

the labelled probe was precipitated along with 40µg of carrier DNA (sonicated salmon sperm DNA, denatured) using NaOAc and ethanol. The DNA pellet was resuspended in water, precipitated again and then resuspended in 150 µl hybridization buffer. (0.6M NaCl, 0.05M $NaPO_4$ pH 7.0 buffer, 1x Denhardt's reagent, 5% dextran sulphate and 50% formamide)

Slides were heat treated at 65° for 30 min. in 2XSSC, 1X10min. in 2XSSC, and then rinsed in 70 % then 95% ethanol. The chromosomes were then treated with 0.07 NaOH for 2.5 min. and then rinsed as before. The probe was denatured by heating it to 70° for 10 min. and 10 µl was added to $22mm^2$ coverslips. The coverslips were picked up by each slide and the edges were sealed with rubber cement. The slides were incubated in a moist chamber at 37° overnight. The next day, the rubber cement was removed and coverslips were rinsed off of the slides and slides were washed in 2XSSC at 65° and followed by multiple washes at room temperature. The slides were then washed in a blocking buffer (100mM Tris-HCl, pH 7.5, 150 mM NaCl plus 1% block from BM kit) and an AP (alkaline phosphatase) conjugated anti-DIG antibody (from the BM kit) was added to 22X40 coverslips at a dilution of 1/5000. Incubation with the antibody was in a moist chamber for 60 min. Slides were rinsed twice in a wash buffer (100mM Tris-HCl, pH 7.5, 150 mM NaCl) and the AP reaction was detected using standard NBT and X-phosphate substrates. Slides were examined under the microscope using phase contrast optics. Black and white photos were taken with Kodak Tech Pan film (exp. +1) and color photos were taken with Kodak 160T slide film.

Northern and Southern Blotting

Genomic DNA was recovered from 6-10 adult flies by first homogenizing the flies in 200 µl of a buffer containing 0.1M Tris (pH 9.0), 0.5% SDS, 50mM EDTA, 5% sucrose and 100µg/ml protease K and incubating them for one hour at 50°. 50µl of 5M potassium acetate was added and mixed by inversion and the mixture was left on ice for 10

min. The mixture was centrifuged and the supernatant was removed and placed in a new tube. The supernatant was extracted with phenol/CIA (95% chloroform, 5% Iso amyl alcohol) and followed by a CIA extraction and DNA was ethanol precipitated out of the solution (no extra salt was added). The DNA pellet was redissolved and digested with the appropriate restriction enzyme and subjected to agarose gel electrophoresis for blotting. The gel was blotted to Hybond N filter paper in 10X SSC using a standard gravity blot set up.

DNA probes were radioactively labelled with a Boehringer-Mannheim random prime kit to a specific activity of at least 1 x 10^8 cpm/μg. A standard protocol for hybridizing probes to genomic blots was used with the following alteration. A phosphate buffer containing 0.1M NaH_2PO_4, 0.05M $Na_4P_2O_7 \cdot 10\ H_2O$, 1mM EDTA, 7% SDS and 100μg/ml Salmon sperm DNA was used for pre-hybridization and hybridization.

A blot containing embryonic, larval, pupal and adult poly $(A)^+$ mRNAs (Hugh Brock and Kryn Stankunas) was hybridized as described above for Southern blots.

Chapter 1

Introduction

Additional sex combs (*Asx*) was originally identified independently in a screen for embryonic lethal mutations that caused pattern defects in the larval cuticle (Nusslein-Volhard et al. 1984; Jurgens 1985), and as a mutation that enhances the dominant sex combs phenotype of *Pc* mutants (Dura et al. 1985; Jurgens 1985). Homozygous *Asx* mutants die at the end of embryogenesis with severe head defects and mild posterior transformations in the cuticle of the abdomen (Jurgens 1985; Breen and Duncan 1986). Because the posterior transformations were less severe than those normally seen with other PcG mutations, *Asx* was categorized as a weak member of the PcG. The cuticle phenotype of a PcG gene reflects the state of homeotic gene regulation during embryogenesis. Specifically, posterior transformations are a consequence of ectopic expression of homeotic genes outside of their normal domains of expression. Consistent with the above observation, homeotic derepression phenotypes in the CNS (central nervous system) are less severe in *Asx* mutant embryos compared to most other PcG genes (McKeon and Brock 1991a; Simon et al. 1992b) even when the maternal contribution of *Asx* is removed (Soto et al. 1995).

Most PcG mutations show equal levels of homeotic gene derepression in all tissues (Soto et al. 1995; McKeon and Brock 1991a; Simon et al. 1992b). Although *Asx* mutations have only weak effects in the CNS, they are unusual in that they have a much stronger homeotic derepression phenotype in the epidermis (Soto et al. 1995; McKeon and Brock 1991a; Simon et al. 1992b). *Polycomblike* (*Pcl*) is the only other PcG member that has clear tissue specific effects, but it is the opposite of *Asx* in that homeotic genes are strongly derepressed in the CNS but only weakly derepressed in the epidermis. This tissue specific pattern of homeotic gene derepression in *Asx* mutants suggests that *Asx* may not be globally required like most of the other PcG proteins, and instead may have a highly

specific role in homeotic gene regulation. One possibility is that *Asx* mediates crosstalk between different PREs and tissue specific enhancers.

As well as its tissue specific effects, one allele of *Asx*, Asx^{P1}, is unusual in that it occasionally displays trxG mutant phenotypes as well as typical PcG mutations in the adult fly (Sinclair et al. 1992). This allele implicates *Asx* in both trxG and PcG activity and therefore a possible role in the maintenance of activation, as well as its role in the maintenance of repression.

In this chapter, I set out to determine: 1) if *Asx* would regulate endogenous homeotic loci and a reporter construct containing a *Ubx* specific PRE in the same tissue specific manner; 2) if the tissue specific activity of *Asx* would be reflected in a tissue specific expression pattern of Asx protein in the embryo; 3) if Asx protein bound to the same chromosomal target sites as other PcG proteins or if the differences in *Asx* activity would also be reflected in the binding of Asx to unique target sites; and 4) possible mechanistic explanations for the effect of the Asx^{P1} mutation. It was found that although there are stage and tissue specific requirements for *Asx* activity, Asx protein is expressed with no tissue or stage specificity at all. Asx binds to multiple target sites that contain other PcG proteins but also binds to some unique sites. These unique binding sites may reflect some of the unique aspects of *Asx* activity. Finally, the binding of Asx to Asx^{P1} mutant chromosomes was examined but found to be completely wild type. This suggests that the Asx^{P1} mutation does not reduce the accumulation of Asx protein or affect its ability to bind to chromosome target sites.

Results

Asx mutations have tissue specific effects on homeotic gene regulation in the embryo

In the developing *Drosophila* embryo, each homeotic gene is expressed with a unique parasegmental register. Fig.2 (A-C) compares the expression of the homeotic gene

Ultrabithorax (*Ubx*) in the *Asx* mutant *Df(2R)trix* (a deletion that removes the entire *Asx* locus) with the PcG mutant Pcl^2. *Pcl* is considered to be only a moderately strong member of the PcG, but the derepression of *Ubx* in the CNS is far more extensive than that seen in an *Asx* mutant (compare Fig.2 B and C).

Previous studies with *Asx* have shown that there are tissue specific differences in *Asx* dependent homeotic gene regulation. *Asx* is required to maintain the proper anterior expression boundaries of the homeotic genes *AbdB* and *Ubx* in the epidermis of the developing embryo (McKeon and Brock 1991a; Simon et al. 1992b; Soto et al. 1995). However, the *Asx* mutation *Df(2R)trix* only slightly affects these boundaries in the CNS (McKeon and Brock 1991a; Simon et al. 1992b) and the *Asx* mutation Asx^3 does not affect these boundaries in the CNS at all (Soto et al. 1995). To determine if these differences in the literature reflect an actual difference in the effect of these two *Asx* mutations on homeotic gene regulation in the CNS, the *Df(2R)trix* and Asx^3 alleles were re-examined for alterations in the proper regulation of the homeotic genes *Ubx* and *AbdB*.

Fig.3 C and D confirms that both the *Df(2R)trix* and Asx^3 mutations show a break down in regulation of *AbdB* in the epidermis, but contrary to Simon et al (1992), there is no alteration in the pattern of *AbdB* expression in the CNS (compare to wild type, Fig. 3A). Contrary to Soto et al (1995), Asx^3 was found to cause a breakdown in the regulation of *Ubx* in the CNS which is similar to that seen in *Df(2R)trix* mutants (Fig.3 G and H). The extent of ectopic *Ubx* expression in the CNS of *Asx* mutants is variable and is sometimes seen extending into parasegment (PS) 4 only (see Fig.2 B), into both PS3 and PS4 (Fig.3 H), or sometimes as far as PS2 (Fig.3 G).

These results show that *Asx* function is required for *Ubx* regulation in the CNS, although it is not as important as other PcG genes such as *Pcl* (see Fig.2 A-C for a comparison). *Asx* function is not required in the CNS for the regulation of *Abd B* but it is essential for both *Abd-B* and *Ubx* regulation in the epidermis. No other PcG gene shows this particular pattern of tissue specific regulation of the homeotic genes.

Fig.2

A comparison of the CNS derepression phenotype of the homeotic gene *Ubx* between *Asx* and the moderately strong PcG gene *Pcl*. Embryos are positioned laterally and oriented with anterior to the left, posterior to the right, ventral down and dorsal up. In Fig.1A, the CNS is situated ventral to the midgut (a) and the hindgut (long thin tube-like structure, b). In wild type embryos (Fig.1 A), *Ubx* is normally expressed in a very strong, spatially restricted pattern in the CNS. *Ubx* expression is faint in parasegment (PS) 5 (the PS 5 anterior boundary is marked by an arrow), strong in PS 6 (the PS 6 anterior boundary is marked by an arrowhead) and at moderate levels in parasegments 7 to 12 (Fig. 1 A). In an *Asx* mutant embryo, the PS 5 anterior boundary breaks down and misexpression of *Ubx* is seen in PS4 (Fig. 1 B). *Pcl* mutant embryos show a much stronger breakdown of *Ubx* regulation and *Ubx* expression is seen all the way into the posterior region of the brain (Fig. 1 C).

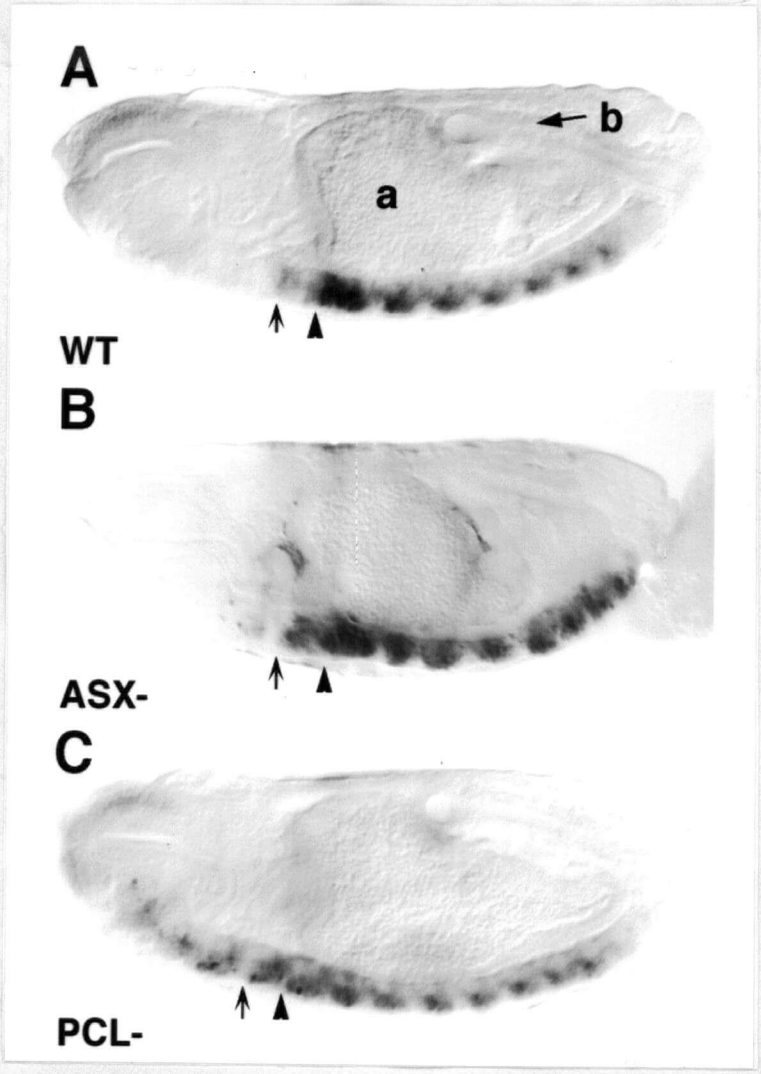
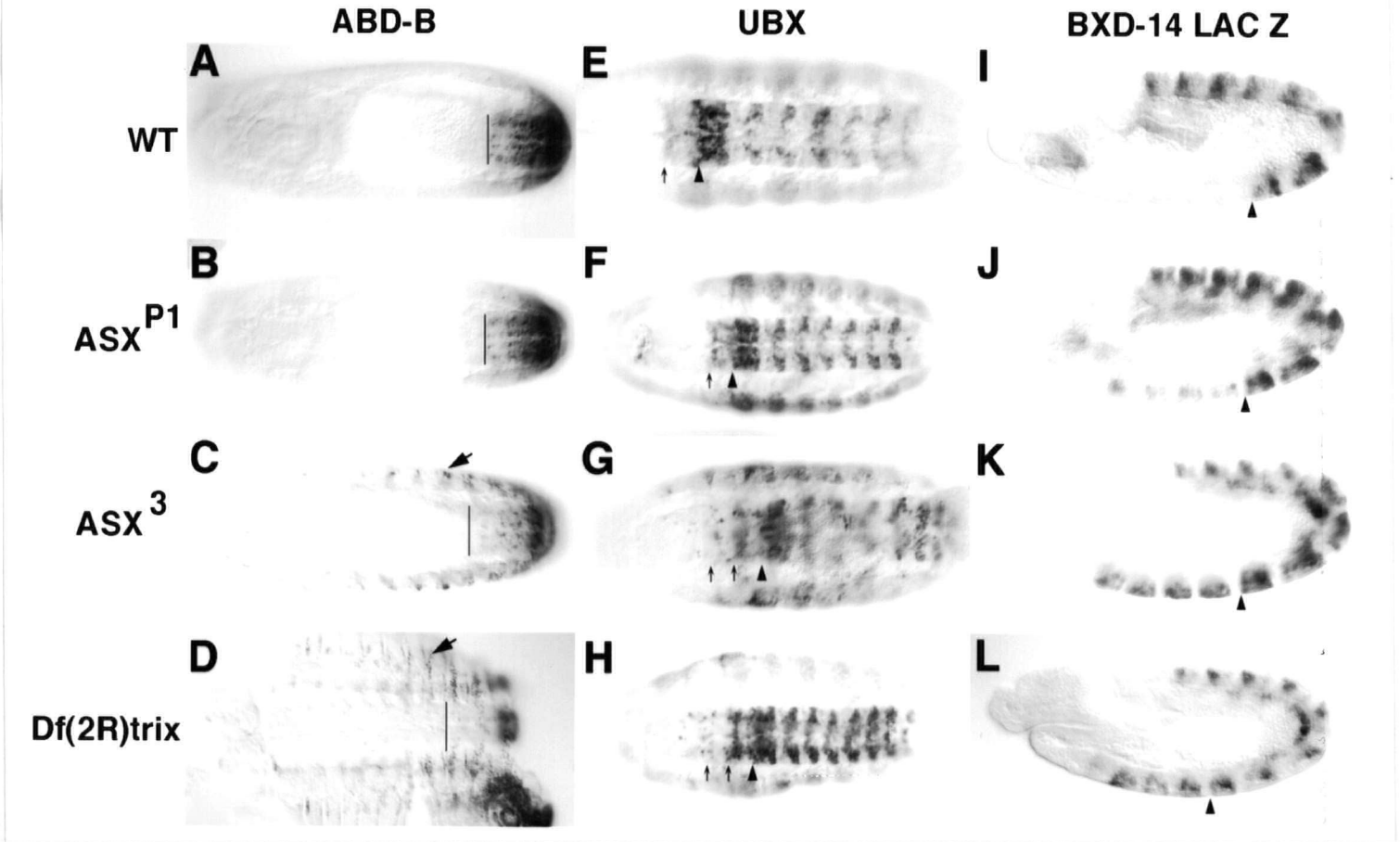

A
b
a
WT
B
ASX-
C
PCL-

Fig.3

The regulation of the homeotic genes *Ubx* and *AbdB* and the bxd14 maintenance element in *Asx* mutant embryos. The embryos in A-H are late stage 15 (~ 12-13 hrs. old) and are oriented with anterior to the left, posterior to the right and with the ventral region facing the viewer so as to make comparisons between the CNS and epidermal expression patterns easier. They are stained with either an anti-AbdB (A-D) or an anti-Ubx (E-H) primary antibody which is then detected with an HRP conjugated secondary antibody. The embryos in I-L are positioned laterally with anterior to the left and posterior to the right. They are stained with an anti-βgal antibody which is then detected with an HRP conjugated secondary. The embryos in I-K are at late stage 9 (~ 4 hrs. old) and the embryo in L is at stage 11 (~ 7 hrs. old). **A**, wild type Abd-B expression. Abd B is normally expressed only in the extreme posterior of the embryo with a CNS expression boundary at PS 10 (marked with a straight line in the CNS). **B**, Abd-B expression in Asx^{P1} mutants is wild type. **C** and **D**, Abd-B expression in an Asx^{3} and a *Df(2R)trix* mutant respectively. The PS 10 boundary in the CNS is maintained but ectopic expression in the epidermis is apparent (large arrow points to ectopic expression in the epidermis of PS 11). The *Df(2R)trix* mutant has been dissected dorsally in order to show the epidermal expression of Abd-B more clearly. **E**, wild type expression of Ubx, described in detail in Fig.1. **F**, Ubx expression in Asx^{P1} mutants is wild type. **G** and **H**, Ubx expression in an Asx^{3} and a *Df(2R)trix* mutant respectively. Ectopic Ubx expression is seen in PS 4 and 3 (G and H) as well as occasionally as far as PS2 (G). The PS 6 boundary is marked with an arrowhead and PS 3 and PS 4 boundaries are marked with small arrows. **I**, lacZ expression of the bxd14 reporter construct in a wild type embryo. It has a boundary of expression at PS6 (arrowhead). **J-L**, expression of the bxd14 transgene in an Asx^{P1} , an Asx^{3} and a *Df(2R)trix* mutant respectively. In all cases the PS 6 boundary breaks down and the transgene is expressed up to PS 2.


ABD-B
UBX
BXD-14 LAC Z
A
E
I
WT
B
F
J
ASX P1
C
G
K
ASX 3
D
H
L
Df(2R)trix

The Asx^{P1} mutation does not affect endogenous homeotic gene expression in the embryo but is required for regulation of a *Ubx* transgene

Asx^{P1} displays both trxG mutant phenotypes in the adult fly (abdominal segment 5 and 6 transformed towards a more anterior segment and transformation of the haltere towards the wing) as well as typical PcG mutations (anterior abdominal segments transformed towards more posterior segments). These adult homeotic phenotypes are a direct consequence of alterations in expression of the homeotic genes *Ubx*, *AbdA* and *AbdB*. TrxG homeotic transformations are caused by a reduction in the expression of the homeotic genes within their normal expression domains. To see if the adult phenotypes observed in Asx^{P1} mutants were a consequence of homeotic misexpression phenotypes in the embryo, Asx^{P1} homozygous mutant embryos were examined for alterations in the regulation of the homeotic genes *Ubx* and *Abd-B*.

500-800 homozygous Asx^{P1} embryos were examined for homeotic misexpression phenotypes. All embryos were completely wild type for *AbdB* (Fig. 3 B) and *Ubx* (Fig.3 F) expression both in the CNS and the epidermis. Therefore either the Asx^{P1} mutation does not affect *Ubx* or *AbdB* regulation in the embryo and the effect of the Asx^{P1} mutation must occur during imaginal disc development, or Asx^{P1} has only subtle effects on homeotic gene regulation in the embryo that are not detectable with this assay.

The bxd14 transgene is a 14.5 kb regulatory element from the *Ubx* gene that is sufficient for the maintenance of lacZ expression from PS6-13 for up to 12 hrs during embryogenesis. It has been shown to contain a minimal 5.1 kb PcG response element (PRE) based on the fact that it binds PcG proteins both *in vivo* and *in vitro* and it fails to maintain its proper expression boundaries in PcG mutants (Simon et al. 1990; Simon et al. 1993; Chan et al. 1994; Chiang et al. 1995; Hodgson and Brock 1998). Derepression of the bxd14 in specific PcG mutants is usually comparable to the level of derepression seen at the endogenous *Ubx* locus in those same mutants. Despite the fact that the endogenous *Ubx* gene has only a minor requirement for *Asx* activity in the CNS, the Asx^{3} mutation

causes a very strong breakdown in the bxd14-lacZ expression boundary relatively early in embryogenesis (Soto et al, 1995 and Fig.3 K). *Df(2R)trix* and Asx^{P1} were both tested for their effects on maintenance of the bxd14 element and they also cause a breakdown of the bxd14-lacZ expression boundary in the CNS (Fig.3 J and L). The ectopic staining in the Asx^{P1} mutants (Fig.3 J) is less intense than the other two Asx alleles (Fig. 3 K and L), but no reduction of bxd14-lacZ expression in PS6-13 was seen in the Asx^{P1} mutants.

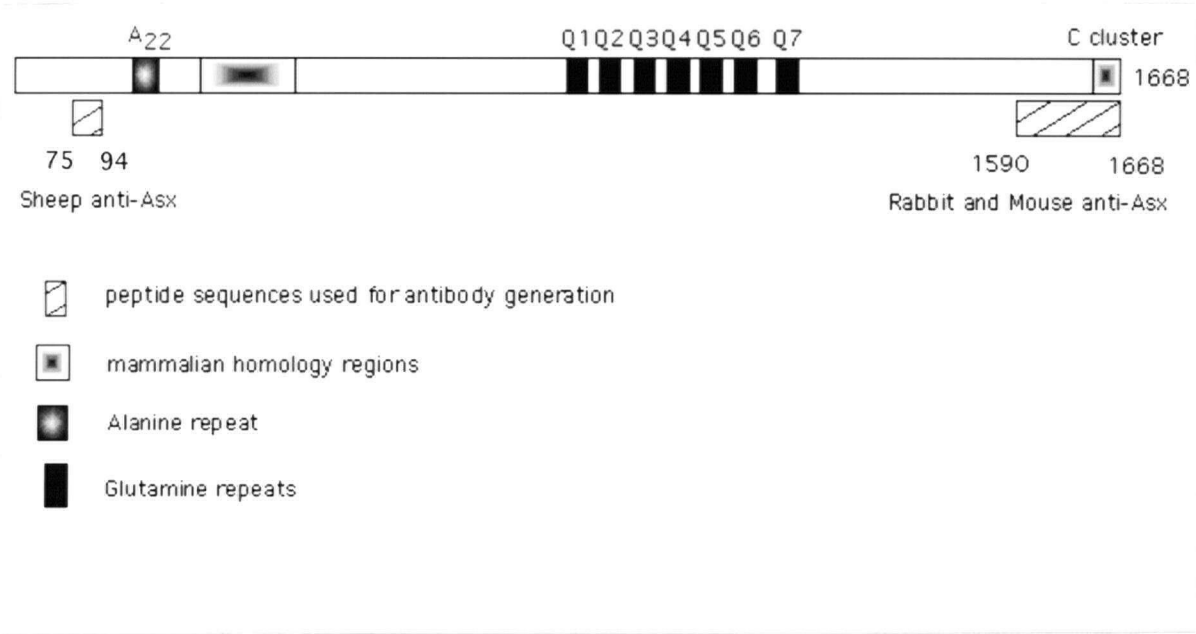
The derepression of the bxd14 construct in Asx^{P1} mutants indicates that the Asx^{P1} mutation does have some embryonic activity. The Asx^{P1} mutation acts like a typical *Asx* allele in the embryo in that it causes derepression of the bxd14 element but it does not reduce expression of the element in PS6-13. It is possible then that the trxG adult homeotic phenotypes seen in Asx^{P1} mutants are not the result of misregulation of the homeotic genes in the embryo; but are the result of a mutant effect at the level of imaginal disc development. The fact that *Asx* mutations have a much stronger effect on bxd14 regulation in the CNS than the endogenous *Ubx* locus also indicates two possibilities: i) other response elements must exist in the *Ubx* regulatory region that can direct proper maintenance of the *Ubx* gene or ii) the bxd14 element interacts with other *Ubx* regulatory sequences to stabilize its activity.

Asx is expressed ubiquitously in all stages of embryogenesis

One possible mechanism for the tissue specific regulatory effect of *Asx* is that it has a tissue specific expression pattern, for example, strong expression in the epidermis and reduced expression in the CNS. The *Asx* gene has recently been cloned and sequenced and three different antibodies were raised to Asx (Sinclair et al. 1998b). A diagram of the Asx protein showing most of the major identifiable domains is shown in Fig.4. Asx contains a region of 22 repeating alanines, multiple glutamine repeats and two domains homologous to a putative human Asx protein, including a putative pair of zinc fingers at the carboxy terminus. A sheep antibody was raised to a peptide sequence in the amino terminus of Asx

Fig.4

Sites of antibody generation on the Asx protein. A sheep antibody was raised to a sequence in the amino terminus of Asx while a rabbit and a mouse antibody were raised to the carboxy terminus.


A22
Q1Q2Q3Q4Q5Q6 Q7
C cluster
1668
75 94
Sheep anti-Asx
1590 1668
Rabbit and Mouse anti-Asx
peptide sequences used for antibody generation
mammalian homology regions
Alanine repeat
Glutamine repeats

and a mouse and a rabbit antibody were raised to the carboxy terminus of the Asx protein (see Fig. 4). All three antibodies either recognize a band the size of Asx on a western blot or can immunoprecipitate in vitro translated Asx (Sinclair et al. 1998b).

The mouse antibody was used to examine the stage specific expression pattern of Asx in wild type embryos (Fig. 5 A-G). From very early (1-2 hrs, Fig 5 A) to very late (~12 hrs, Fig 5 G) stages, Asx protein is distributed ubiquitously with a slightly stronger concentration in the CNS of later stages compared to other regions of the embryo. Staging details are given in the figure legend. To show that the mouse antibody was specific for the Asx protein, late stage homozygous *Df(2R)trix* embryos that lack Asx protein were stained with the mouse antibody and did not produce a signal (Fig. 5 H). For comparison, wild type mid to late stage embryos were stained with affinity purified sheep anti-Asx antibodies and produced an identical ubiquitous staining pattern (Fig. 5 I-L). Despite its expression in the CNS and its late stage head defects, *Asx* is not required for normal CNS or brain development as *Asx* mutant embryos have a morphologically wild type CNS and brain (see Fig.6).

The ubiquitous pattern of Asx protein expression in embryos is typical of all PcG genes studied so far (Paro and Zink 1992; Martin and Adler 1993; Lonie et al. 1994; DeCamillis and Brock 1994; Gutjahr et al. 1995). If Asx is expressed in all tissues at all stages of embryonic development, this raises the question of how Asx recognizes its target genes only in those tissues and those spatial domains where its activity is required.

Asx protein binds to unique target sites on polytene chromosomes

All PcG proteins tested so far are chromatin proteins that bind to discrete euchromatic sites. Polytene chromosomes are large cytologically visible chromosomes found in the salivary glands of late larval stages and are useful for examining the chromosome binding sites of specific proteins. Staining of polytene chromosomes with antibodies to specific PcG proteins has shown that many PcG proteins (Pc, ph, Pcl, and

Fig.5

Developmental expression of Asx protein in embryos. Embryos are oriented with anterior to the left, posterior to the right, dorsal up and ventral down. Asx is expressed ubiquitously at all stages and in all tissues. Embryos in A-H are stained with the mouse anti-Asx antibody. Embryos in I-L are stained with the affinity purified sheep anti-Asx antibody. **A** stage 3, ~1 hr. **B** stage 5, ~2 1/2 hrs., **C** stage 8, ~ 3 1/2 hrs., **D** stage 12, ~ 8 hrs., **E** stage 13, ~10 hrs., **F** stage 14 ~ 11 hrs., **G** stage 15 ~ 12 hrs. Embryos in **I-K** approximately match the staging of those in **E-G** respectively. **L** shows a stage 16 (~ 15 hrs) embryo. **H** is a stage 15 *Df(2R)trix* homozygous mutant embryo that lacks Asx protein. *Df(2R)trix* was balanced over a chromosome that contained a lac-Z reporter gene so that homozygous mutants could be identified with the absence of lac-Z staining.

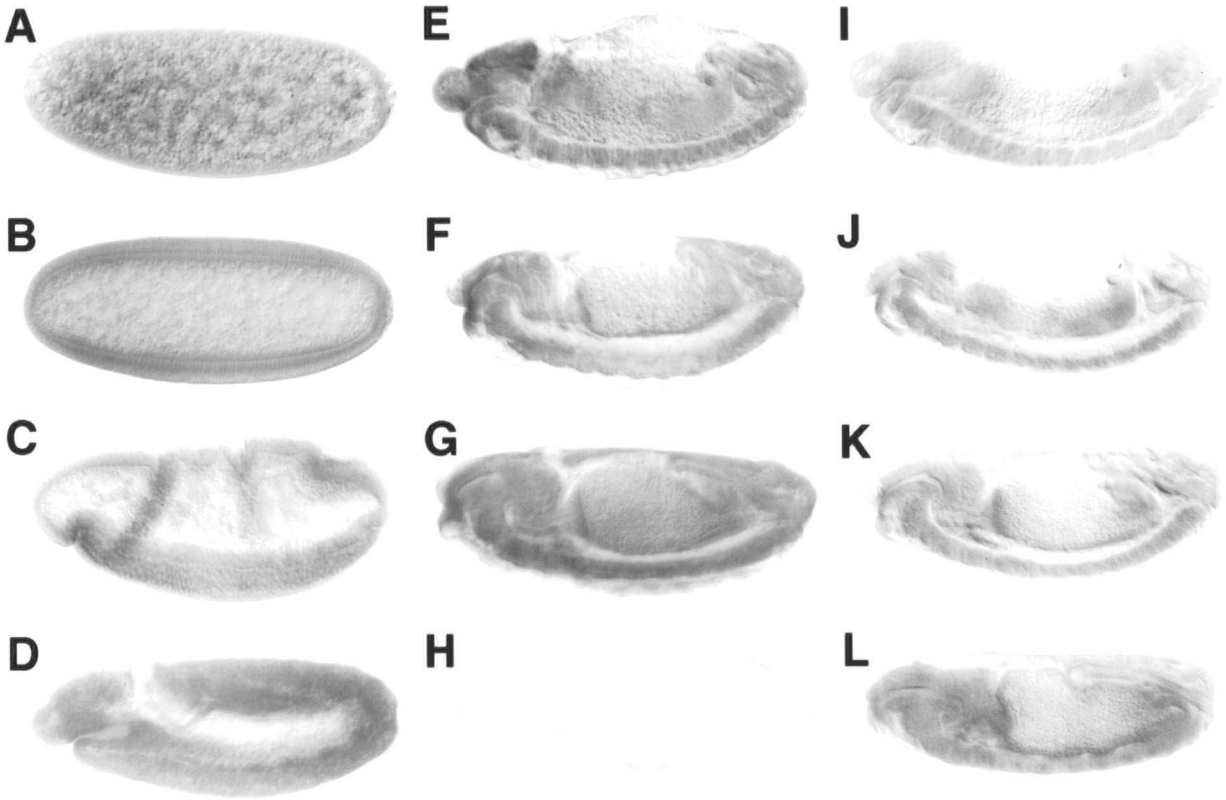
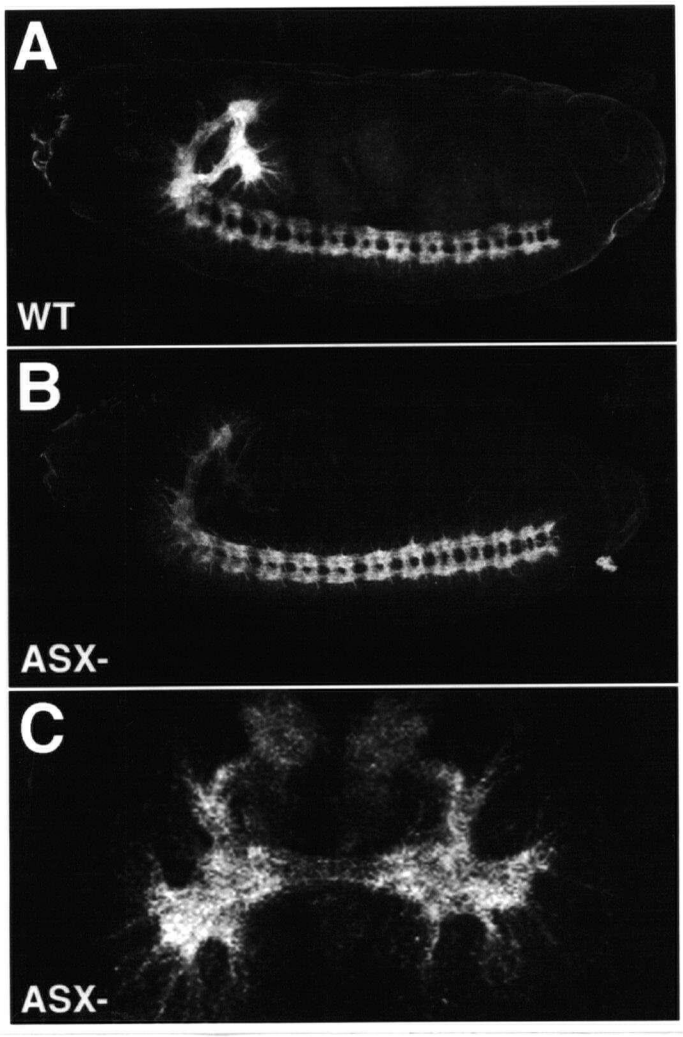

A
B
C
D
E
F
G
H
I
J
K
L

Fig.6

CNS and brain development in Asx mutant embryos. Embryos were stained with the axon specific monoclonal antibody BP102 and detected with a fluorescent conjugated secondary. Fluorescent images were collected with a confocal microscope. **A**, wild type CNS and brain in a stage 16 embryo. Embryo is oriented with anterior to the left. The image is collected at a slight angle so that the brain is facing towards the page while the ventral portion of the CNS is facing away. **B**, CNS in a *Df(2R)trix* homozygous mutant embryo. The image is collected so that the ventral portion of the CNS is facing towards the page. **C**, close up of a brain in a *Df(2R)trix* homozygous mutant embryo. Similar results were seen with the Asx^3 mutation.


A
WT
B
ASX-
C
ASX-

probably also Scm) bind to about 100 target loci in a completely overlapping pattern (Franke et al. 1992; Lonie et al. 1994; Peterson et al. 1997) and other PcG proteins (Psc, E(z), Su(z)2) overlap with a large number of those 100 sites but also bind at some additional unique sites (Rastelli et al. 1993; Carrington and Jones 1996). This large degree of overlap between PcG binding sites has been used to argue for at least one discrete PcG protein complex containing all the various PcG members. *Asx* has unique phenotypic 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 was different than other PcG proteins.

Polytene chromosomes were double labelled with the sheep anti-Asx antibody and a rabbit anti-Pc antibody to see if Asx protein would overlap with any PcG proteins at specific target sites (Fig. 7 A-C). Surprisingly, these double labelled chromosomes showed very little overlap between Asx and Pc (Fig. 7 C) and each antibody detected only about 40- 50 individual sites (Fig. 7A and B). However, when each antibody was tested alone, both the anti-Pc antibody and the anti-Asx antibody reliably detected between 70-80 sites (Fig. 7D and E respectively). This result suggests that one or both antibodies are interfering with each others ability to recognize their antigens at certain loci. This raises two possibilities: i) either Asx and Pc proteins completely overlap at all binding sites but the antibodies interfere with detecting this overlap, or ii) they only overlap at a subset of binding sites and Asx binds to some completely unique sites.

To distinguish between the above two possibilities, Asx sites were directly mapped to polytene chromosomes using HRP staining (Fig. 8). 90 sites were mapped to polytene chromosomes using the rabbit anti-Asx antibody. Similar results were seen with the sheep and mouse anti-Asx antibodies. To show that the signal on polytene chromosomes was specifically due to anti-Asx reactivity, rabbit anti-serum was depleted for anti-Asx reactivity by incubation with the carboxy terminus of Asx. When this immune-depleted serum was used to stain polytene chromosomes, no signal was apparent on the chromosomes (Fig.

Fig.7

Binding of Asx and Pc proteins to polytene chromosomes. Primary antibodies were detected with Texas Red (red) and FITC (green) fluorescent secondaries. **A-C**, chromosomes double labelled with sheep anti-Asx (red) and rabbit anti-Pc (green) antibodies. Regions of overlap are yellow. **D,** chromosomes singly labelled with anti-Pc only. **E,** chromosomes singly labelled with anti-Asx only. **F**, same nucleus as in E, stained with the fluorescent dye DAPI to show the banding pattern of the chromosomes.

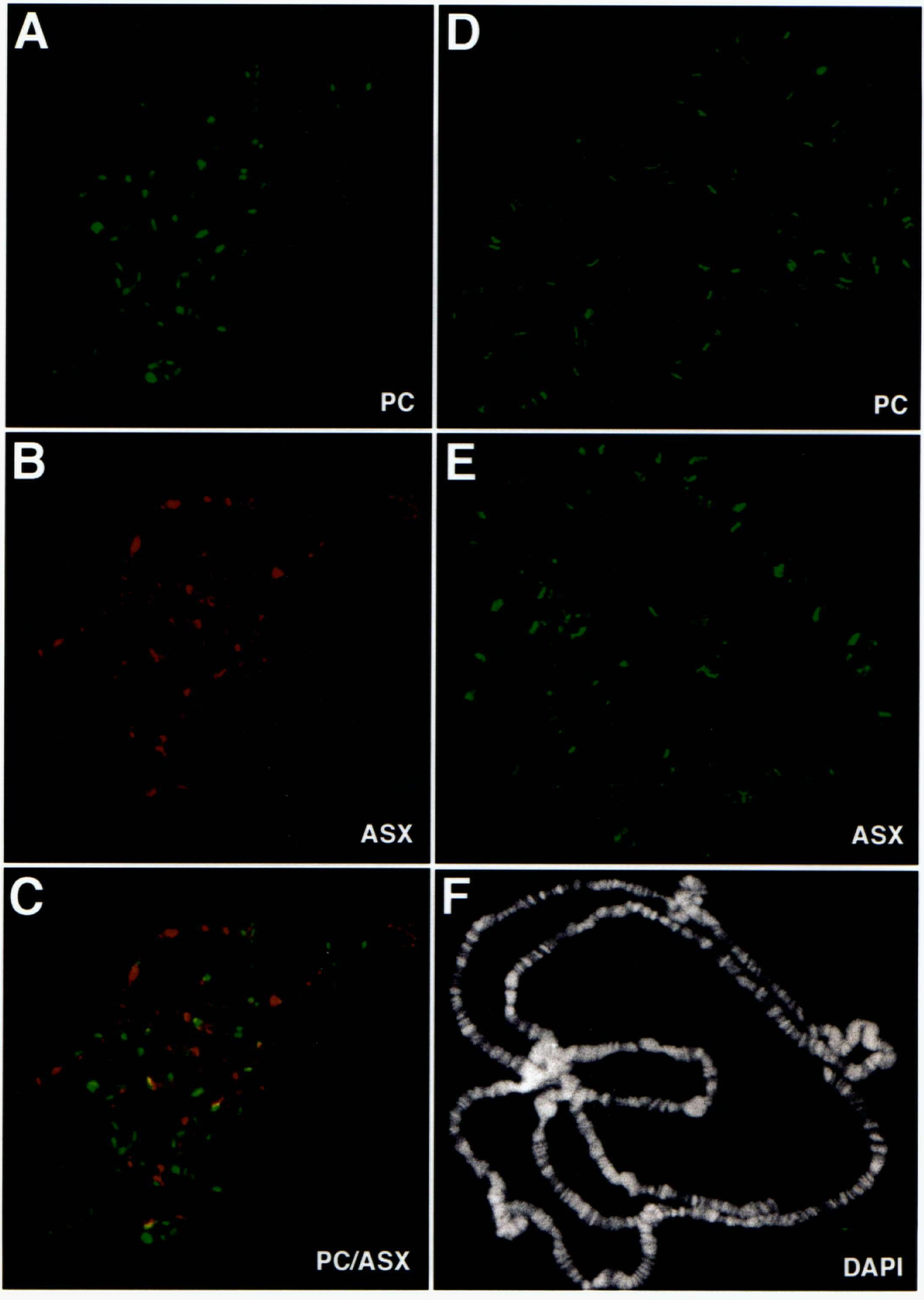

A
PC
D
PC
B
ASX
E
ASX
C
PC/ASX
F
DAPI

Fig.8

Rabbit anti-Asx HRP staining on polytene chromosomes. Chromosomes were counterstained with a blue-gray Giemsa stain to detect chromosome banding patterns and allow mapping of antibody binding sites. **A**, rabbit anti-Asx binding sites. The two homeotic complexes, the bithorax complex (BX-C) and the Antennapedia complex (ANT-C) are shown as well as a strong Asx binding site, 35AB. **B**, polytene chromosomes stained with immune depleted rabbit anti-Asx antibody. **C** and **D**, comparison of binding sites between Asx and ph, respectively, on the distal part of the X chromosome. Lines connect shared binding sites and arrowheads mark unique sites.

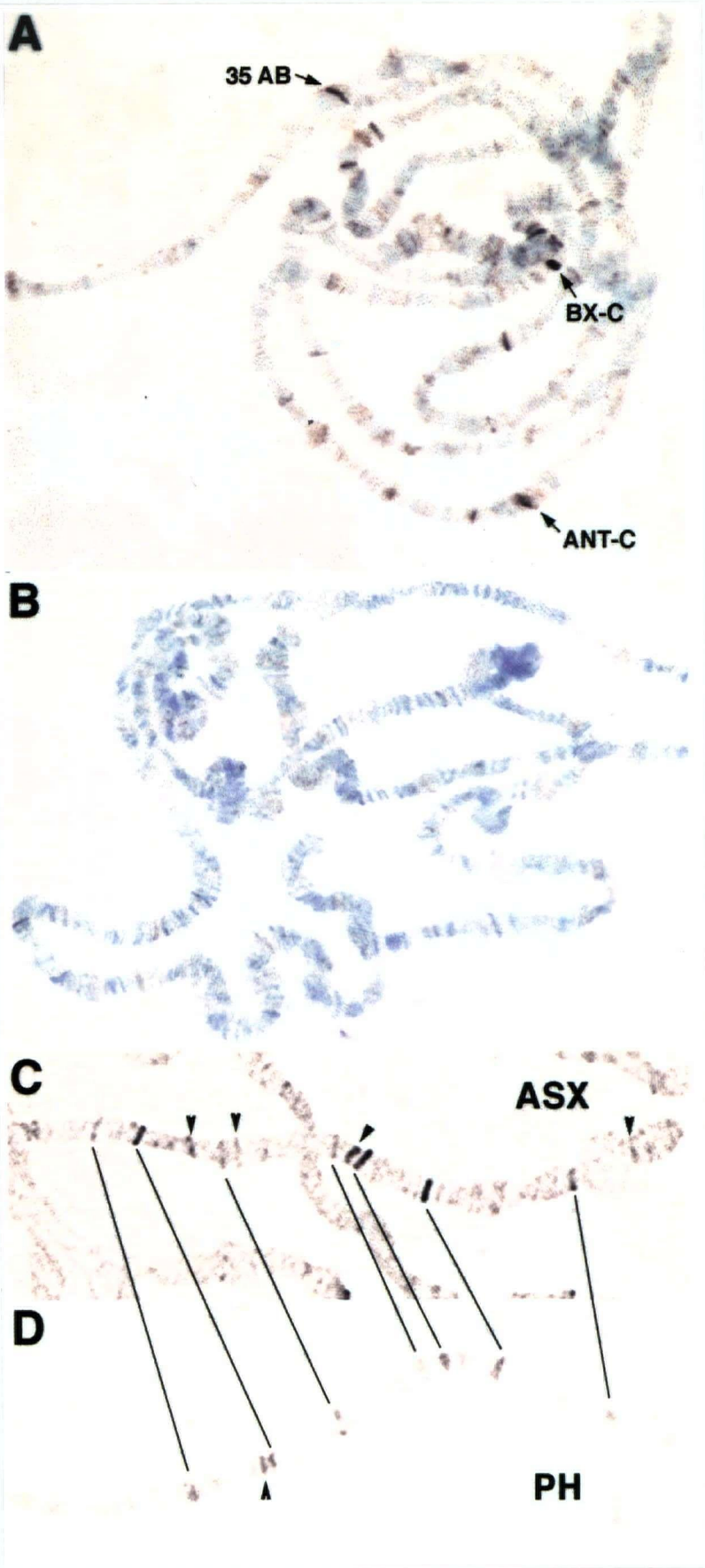

A
35 AB
BX-C
ANT-C
B
C
ASX
D
PH

Table 1: Comparison of Asx with Ph/Pc protein binding sites on polytene chromosomes

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 (**ph**)	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 (**E(z)**)	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
		-	38F		
		39F-40A	39EF		
		2R		*3R*	
		41C (**sxc**)	41CD	-	82E
		-	43C	-	83C
		44A	44A	84B(**ANT-C**)	84AB
		-	45C	84DE	84D
		46A	-	84F	84EF
		46CD	46C	85D	-
		47A	-	85EF (**Scm**)	85E
		48A (**en**)	48A	86C	86C
		49F (**Psc**)	49EF	87B	87BC
		50A	-	88A	88A
		51A (**Asx**)	51A	89B	89B
		-	51D	89C	89C
		56C	56C	89D	-
		-	57A	89E (**BX-C**)	89E
		57B	57B	90E	90E
		-	58CD	93E	93E
		-	58F	-	94DE
		-	59A	96A	-
		-	59C	96CD	96BC
		59F	59F	-	96F-97A
		60E	60E	97D	-
		-	60F	98BD	98CD
				99A	99AD
				99F	99F
				100AB	100A
				100F	100F

8B). Fig. 8A highlights examples of some Asx binding sites. Table 1 lists the cytological location of all 90 sites as well as highlighting the position of several genes and loci of interest. 63 of the 90 Asx binding sites overlap with Pc/ph/Pcl sites including the homeotic loci (Fig. 8A and table 1) and the *Asx* locus itself (table 1).Thus 37 Pc/ph/Pcl sites do not contain Asx protein. 27 of the Asx sites were completely unique to Asx and didn't overlap with any other PcG binding sites. To illustrate this, a comparison of Asx (Fig. 8C) and ph (Fig. 8D) binding sites is shown for part of the X chromosome.

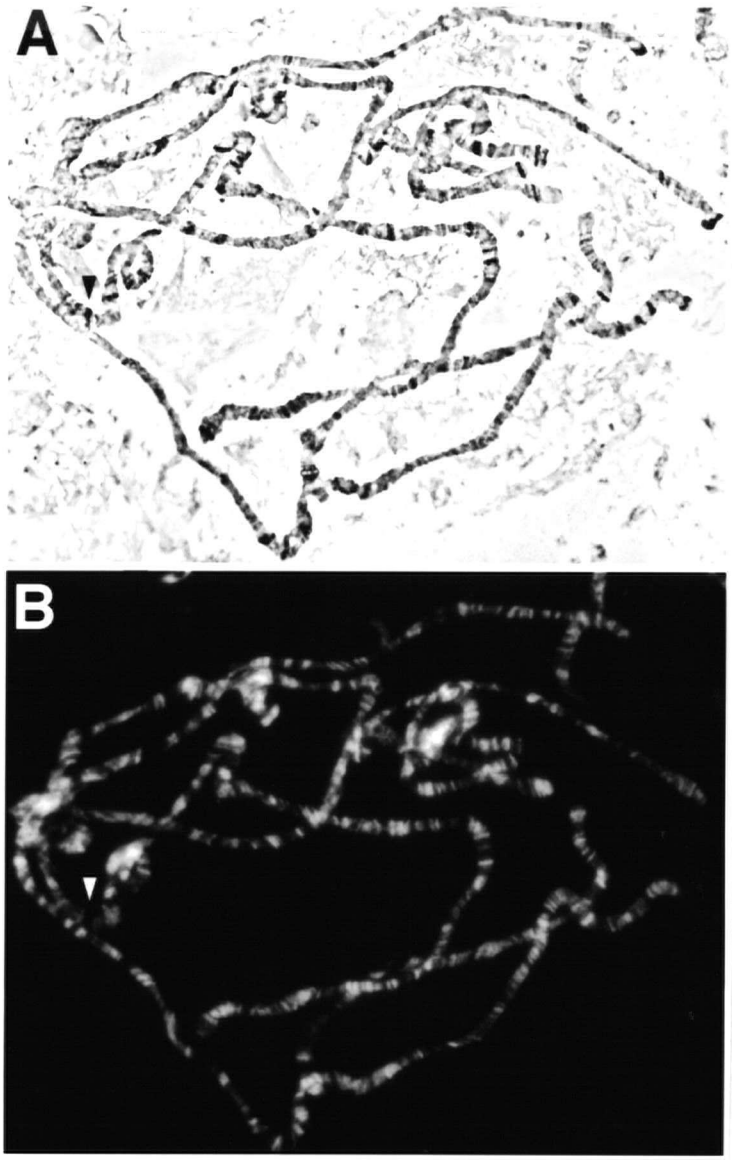
In addition to the differences in actual binding sites, there are also differences in staining intensity at specific sites. Sites 48A, 49EF and 100A all stain very intensely with antibodies to Ph or Pc but stain very weakly with Asx, whereas sites 35AB (see Fig. 8A), 56C and 93E stain very intensely for Asx but weakly for Ph or Pc. This argues that the accumulation of these proteins can vary at different target sites and this may relate to differences in activity or differences in complex formation at these target sites.

The Asx^{P1} mutation does not alter the distribution of Asx protein on polytene chromosomes

The Asx^{P1} mutation is caused by the insertion of a *P* transposable element in the 5' UTR of the *Asx* gene, thus the *P* mutation does not directly affect the coding sequence of *Asx* . Northern blots with Asx^{P1} mutants are essentially normal although the amount of transcript present looks slightly reduced compared to wild type (D. Sinclair, H. Brock unpublished data). To test the possibility that the molecular basis of theAsx^{P1} mutation may be to slightly reduce the dose of Asx protein or disrupt the ability of Asx to bind to its target sites, polytene chromosomes from Asx^{P1} homozygous mutants were stained with Asx antibody. An antibody to Asx still recognizes 90 sites on homozygous Asx^{P1} mutant polytene chromosomes (Fig.9). This indicates that the binding and accumulation of Asx protein at its polytene target sites is normal in Asx^{P1} mutants which strongly suggests that the molecular basis of the Asx^{P1} mutation does not involve reducing the dose of Asx

Fig.9

Binding of Asx to Asx^{P1} mutant chromosomes. **A**, 90 binding sites were detected with HRP staining. **B**, Chromosomes were counterstained with the fluorescent dye DAPI to help in identification of the number of binding sites. DAPI fluorescence is effectively quenched by HRP staining so every strong HRP binding site in A can be correlated with a quenched site in B (see arrowhead for an example).


A
B

protein or altering the binding capabilities of the Asx protein. However, it is still possible that Asx binding is slightly reduced at a level that is not detectable using HRP staining.

Discussion

Targeting PcG activity

PcG proteins are only required in specific regions and specific tissues of the embryo for regulation of individual homeotic genes. All the PcG proteins that have been examined are expressed ubiquitously and thus it is unknown how they recognize their targets only in those regions that require their activity. The activity of Asx is even more specific than other PcG proteins but despite its tissue and stage specific effects, Asx protein is expressed with no temporal or spatial specificity. One possibility is that the tissue specific activity of *Asx* is regulated by interactions between factors bound to tissue specific enhancers and Asx protein bound to PREs. No such factors have been identified but they could have only subtle mutant phenotypes that would make them refractory to traditional genetic screens and they may only be identified with the purification and identification of factors bound to PcG protein complexes.

Asx may have imaginal disc specific as well as tissue specific requirements for its activity

Although the Asx^{P1} allele has trxG as well as PcG phenotypes in the adult, it has no effect at all on regulation of the endogenous *Ubx* and *AbdB* loci in the embryo. The bxd14 reporter element creates a new trx binding site on polytene chromosomes and its expression is slightly reduced in a *trx* mutant background indicating that it is responsive to trxG activity. Asx^{P1} causes derepression of the bxd14 construct, indicating that it does have a mutant PcG phenotype in the embryo. However, it does not cause reduced expression of the construct and thus does not have a trxG embryonic phenotype. It could

be that the trxG effect is too subtle to be detected with this assay, or it may be that the Asx^{P1} mutation may have its effects at the level of imaginal disc development. This argues that there may be a different requirement for Asx activity during imaginal disc development than there is during embryogenesis.

The bxd14 maintenance element contains a functional PRE (Simon et al. 1990; Simon et al. 1993) that binds multiple high molecular weight PcG complexes (Hodgson and Brock 1998). However, a deletion, pbx^2, that removes the entire endogenous bxd14 element as well as some flanking sequences has no effect on the regulation of the endogenous *Ubx* gene in embryos (J. Hodgson, unpublished observation; Duncan 1987). This argues that the bxd14 regulatory sequence is not required for the maintenance of endogenous *Ubx* expression during embryogenesis. Since almost all PcG mutations have a very strong effect on the embryonic regulation of *Ubx*, this argues that the bxd14 is not the only PRE at the *Ubx* locus or that other regulatory sequences at the *Ubx* locus can maintain restricted expression throughout embryogenesis. Muller and Bienz (1991) have shown that some combinations of regulatory elements from the *Ubx* locus can act as PRE maintenance elements although these regulatory elements act as simple enhancers when present by themselves. Additionally, there is also evidence that there may be at least one other PRE at *Ubx*, the bx17 (Chiang et al. 1995), but it has not been as thoroughly characterized as the bxd 14 .

Although the embryonic phenotype of the pbx^2 deletion argues that the bxd14 is not required or is redundant for the embryonic regulation of *Ubx*, the pbx^2 deletion has an obvious adult homeotic phenotype (Bender et al. 1983; Duncan 1987) suggesting that the bxd14 element may be required for *Ubx* regulation in the imaginal discs. Next to the bxd14 and within the pbx^2 deletion, are a set of enhancers that are only expressed in the imaginal discs, but by themselves they are expressed with a non restricted pattern (Pirrotta et al. 1995). Pirrotta et al (1995) have argued that the bxd14 PRE functions by transferring

spatially restricted expression information from embryogenesis to the imaginal disc enhancers during larval growth and pupation.

Asx mutations have only a subtle effect on the CNS regulation of *Ubx* , but have a relatively strong effect on regulation of the bxd14 element. If the bxd14 element is crucial for the transfer of spatially restricted information from the embryo to the imaginal discs, then the central role of Asx in bxd14 regulation in the embryo may implicate Asx activity in this process. If the Asx^{P1} mutation alters imaginal disc regulation of homeotic genes, it may do this by disrupting *Asx* mediated transfer of spatially restricted information from the bxd14 to the imaginal disc enhancers.

Asx is a member of a PcG complex and binds close to Pc protein at multiple overlapping euchromatic sites

Asx binding overlaps with a large number of other PcG sites which is consistent with the possibility that Asx is part of a large PcG complex at these sites. The observation that chromosomes double labelled with antibodies to Asx and Pc do not show any overlap suggests that the two antibodies are sterically hindering each others ability to bind to specific sites. The fact that each antibody on its own can recognize many more loci than when they are present together supports this idea. If we assume that the Pc antibody can recognize up to 80 sites on its own and the Asx antibody should recognize about 27 sites that are completely unique to Asx, this gives a total of about 107 sites that should be recognized by both or one of the antibodies. On double labelled chromosomes, the total number of sites recognized by one or the other antibody is about 100 sites which is about the same as the 107 expected. However, the number of sites recognized by each antibody individually is only about 50 sites (compared to about 70 or 80 sites on individually labelled chromosomes) which indicates that at some sites of overlap, Asx antibody is preventing Pc from binding while at other sites of overlap, Pc antibody is preventing Asx from binding. There may be no significance as to which specific sites preferentially bind

which specific antibody as this could be a random event depending on particular local binding conditions.

Steric hindrance between the two antibodies argues that the respective antigens they each recognize are in close proximity to one another. This suggests the possibility that Asx is either within a PcG complex bound very close to Pc protein or Asx is contacting the complex very near the Pc protein. Using the yeast two hybrid system, Asx does not directly bind to Pc or any other PcG protein tested (M. Kyba and H. Brock, unpublished observation) but this does not rule out the possibility that Asx recognizes an intermediary protein that allows it to interact with Pc. However, the fact that Asx binds many sites where no Pc protein is present also argues that Asx does not require the presence of a Pc containing PcG complex in order to bind to chromosomal target sites.

In the salivary gland tissues, the homeotic genes are repressed and so the presence of all known PcG proteins bound to the homeotic loci strongly suggests that these proteins are in a functional complex together at these loci. However, PcG proteins also bind many loci that are obviously not repressed in salivary gland tissues such as the *ph* locus or the *Asx* locus itself. This suggests then that either i) PcG proteins are not global repressors and their specific function can vary at particular loci, perhaps by interacting with enhancer specific factors or ii) a bound PcG complex is not necessarily a functional complex. The fact that Asx and some other PcG proteins can bind to unique sites also suggests that there are different requirements for PcG activity at different sites at thus possibly different PcG complexes.

Chapter 2

Introduction

Asx was originally isolated as a lethal mutant that died at the end of embryogenesis and displayed mild posterior transformations of the cuticle (Nusslein-Volhard et al. 1984; Jurgens 1985). Posterior transformations are a hallmark of PcG mutations and are caused by the ectopic expression of homeotic genes. Most homeotic genes are expressed with restricted anterior boundaries so that when expression boundaries break down, homeotic gene expression expands in an anterior direction resulting in posterior transformations. An exception to this is the expression of the homeotic gene *Sex combs reduced* (*Scr*) that expands in both an anterior and a posterior direction (Glicksman and Brower 1988a), producing extra sex combs on the second and third legs of male flies. This dominant extra sex combs phenotype has been used to identify many members of the PcG as well as modifiers of PcG activity. Double mutant combinations between PcG genes tend to enhance the penetrance of this phenotype. The number of legs that display extra sex combs is a measure of the strength of the mutant interaction and has been used in the past in an attempt to categorize the PcG into specific functional subsets (Cheng et al. 1994; Campbell et al. 1995).

Mutations in members of the trithorax group (trxG) of genes reduce homeotic gene expression within their normal domains and cause posterior structures to be transformed into more anterior ones (Kennison 1993). An exception to this is the transformation of the first and second legs towards the third leg, suppressing the formation of sex combs on the first legs of male flies presumably due to a reduction in *Scr* expression (Ingham and Whittle 1980). Double mutant combinations between two trxG genes enhance the penetrance of trxG homeotic transformations (Kennison and Russell 1987; Kennison and Tamkun 1988). Double mutant combinations between a PcG gene and a trxG gene suppress each others homeotic mutations to produce an essentially wild type fly (Kennison and Russell 1987; Kennison and Tamkun 1988; Campbell et al. 1995). This mutual suppression of homeotic

phenotypes in PcG/trxG double mutants has led to the suggestion that the two groups of proteins either act antagonistically or have opposite and independent functions (Ingham 1983; Jones and Gelbart 1993).

The extra sex combs phenotype is rarely seen in adult heterozygous *Asx* mutant flies (Sinclair et al. 1992), but *Asx* can strongly enhance the extra sex combs phenotype of other PcG genes. In particular, the *Asx* allele *Df(2R)trix* strongly interacts with mutations in *Pc* to produce flies with multiple sex combs (Campbell et al. 1995). However, the fact that the Asx^{P1} allele has both trxG and PcG homeotic transformations in adult flies (Sinclair et al. 1992) raises the possibility that *Asx* may have a role in both repression and activation.

The major objective of this chapter was to determine if *Asx* could function as both a member of the PcG and the trxG. To test this possibility, Asx^{P1} homozygotes were crossed to both *Pc* and alleles of the trxG gene *trithorax* (*trx*). Asx^{P1} homozygotes showed strong enhancement of PcG phenotypes when combined with a *Pc* heterozygote and strong enhancement of trxG phenotypes when crossed to a *trx* heterozygotes. Lethal *Asx* alleles were crossed to *Pc* and *trx* alleles and found to enhance both PcG and trxG homeotic transformations. *Df(2R)trix*, the only known true null allele of *Asx*, showed an extra strong interaction with *Pc* not seen with other PcG mutations. This indicates that there may be a specific *in vivo* functional interaction between *Asx* and *Pc* . Although Asx^{P1} homozygotes strongly enhance *Pc* homeotic phenotypes, Pc binding is normal on Asx^{P1} homozygous polytene chromosomes. Thus the functional interaction between *Pc* and *Asx* may occur after the proteins are already bound to their target sites.

Results

Asx^{P1} homozygotes strongly enhance both PcG and trxG phenotypes

Unlike other Asx alleles, Asx^{P1} survives as a homozygote and occasionally displays trxG mutant phenotypes (abdominal segments 5 or 6 transformed towards a more anterior segment or transformation of the haltere towards the wing, see Fig.10C) as well as some typical PcG mutations (anterior abdominal segments transformed towards more posterior segments, see Fig.10D). The penetrance of these homeotic transformations is rather low and only occurs in about 10 - 20% of adult flies (Sinclair et al 1992, and Table 2).

Double mutant combinations between a PcG gene and a trxG gene usually suppress homeotic transformations to produce a nearly wild-type fly. However, homozygous Asx^{P1} mutations strongly enhance both the penetrance and expressivity of homeotic transformations seen with two heterozygous *trx* null alleles, *Df(3R)red* and trx^{B11} (Table 2 and Fig. 11). Heterozygous *trx* alleles almost never show transformations of the haltere towards the wing, and transformations in the abdomen are much weaker than when enhanced by Asx^{P1} (compare Fig.11C and D to E and F). As well, the transformation of the second thoracic segment (T2) towards the third (T3) is a very strong anterior transformation (see Fig. 11 E) that is never seen in Asx^{P1} or *trx* mutants alone (Table 2).

The *trx* allele trx^{Z11} is a point mutation in a specific protein motif termed the SET domain (Stassen et al. 1995) and is probably not a complete null mutant. Compared to *Df(3R)red* and trx^{B11}, trx^{Z11} is relatively weak and shows a low penetrance of abdominal homeotic transformations (see Table 2, row 3). Homozygous Asx^{P1} mutations enhance both the penetrance and the expressivity of homeotic transformations in trx^{Z11} mutants to the same level as with *Df(3R)red* and trx^{B11} (see Table 2). This indicates that the trx^{Z11} SET mutation alone is sufficient for the strong genetic interaction between Asx^{P1} and *trx*.

Double mutant combinations between PcG genes enhance PcG homeotic transformations such as the extra sex combs phenotype (Campbell et al. 1995). In particular, the *Asx* null allele *Df(2R)trix* strongly interacts with mutations in *Pc* to produce flies with multiple sex combs (Cambell *et al*, 1995). Asx^{P1} can behave like a typical PcG

Fig. 10

Example of anterior and posterior transformations seen in Asx^{P1} homozygotes. **A**, haltere on a wild type fly indicated by the black arrowhead. **B**, abdomen of a wild type male fly showing abdominal segments 3-6. Note that A5 and A6 are both heavily pigmented. **C**, an example of a haltere to wing transformation in an Asx^{P1} homozygote. The haltere (indicated by the black arrowhead) is much larger and has triple row margin bristles and the overall structure of a small wing. **D**, an example of both posterior and anterior transformations in the abdomen of an Asx^{P1} homozygote. Note the patches of extra pigmentation in A3 and A4 (white arrowheads, facing left) indicative of posterior transformations and the large unpigmented patch in A5 (white arrowhead, facing right) indicative of an anterior transformation.

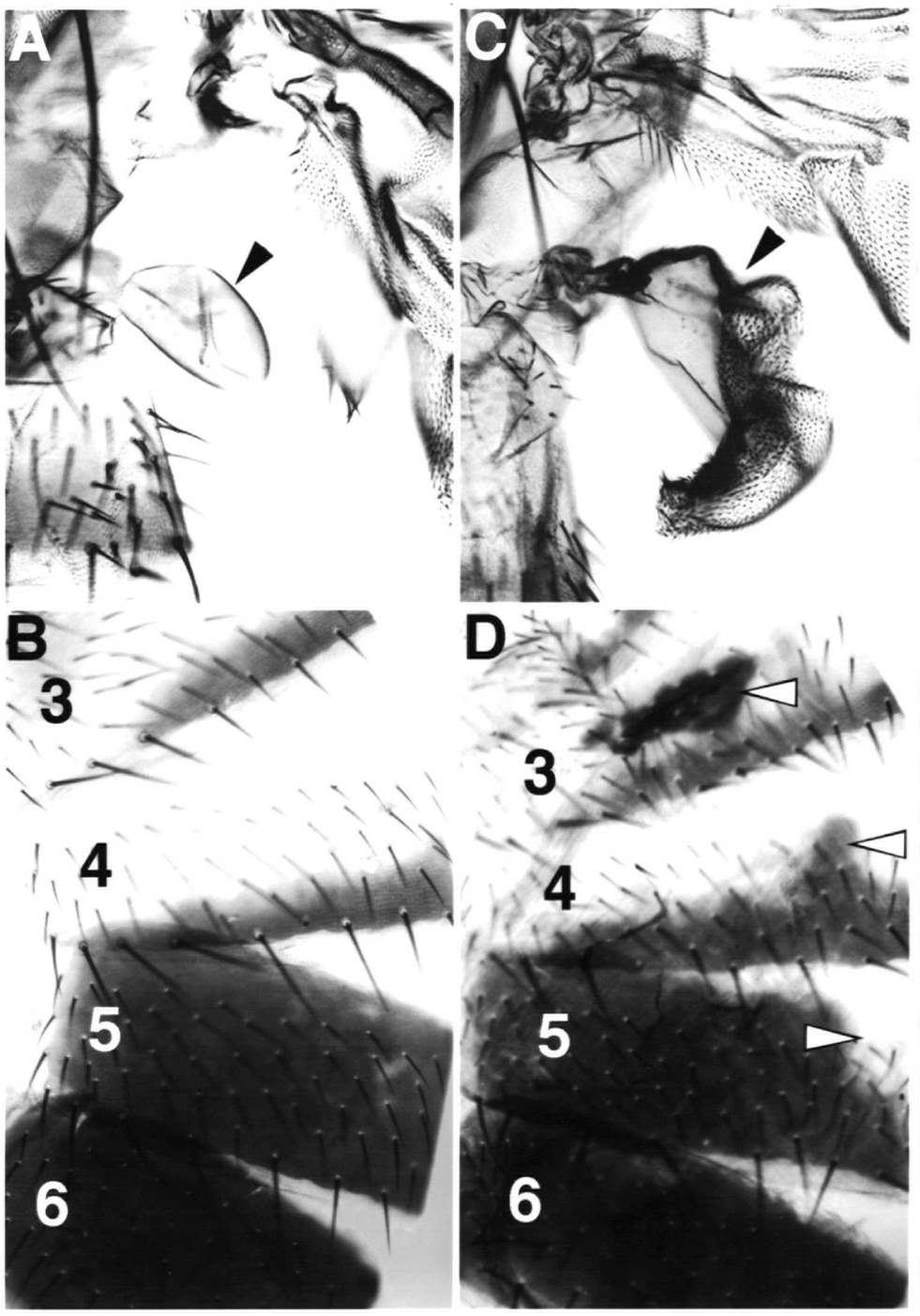

A
C
B
3
4
5
6
D
3
4
5
6

Fig. 11

Enhancement of *trx* phenotypes by Asx^{P1} homozygotes. **A**, thorax and haltere (arrowhead) of a wild type fly. **B** abdomen of a wild type fly. Note the pigmentation in abdominal segments 5 and 6. **C**, typical thorax and haltere of a heterozygous *trx* mutant. In this genetic background, single *trx* mutants have a wild type haltere (arrowhead) and thorax. **D**, example of a strong abdominal transformation in a single *trx* heterozygote. There are patches of light pigment in abdominal segment 5 (small arrow) which is indicative of the transformation of A5 towards a more anterior segment. **E**, thorax and haltere of a mutant that is homozygous for Asx^{P1} and heterozygous for *trx* . The haltere is much enlarged (arrowhead) indicating a partial transformation of the haltere towards the wing. Additionally, the posterior of the third thoracic segment, T3 (large arrow) is partially transformed towards the second thoracic (T2) segment, an anterior transformation that is not seen in Asx^{P1} or *trx* mutants alone. **F**, example of a strong abdominal transformation in a mutant that is homozygous for Asx^{P1} and heterozygous for *trx* . Abdominal segment 5 (small arrow) has very little dark pigmentation which is indicative of an almost complete transformation of A5 towards a more anterior segment.

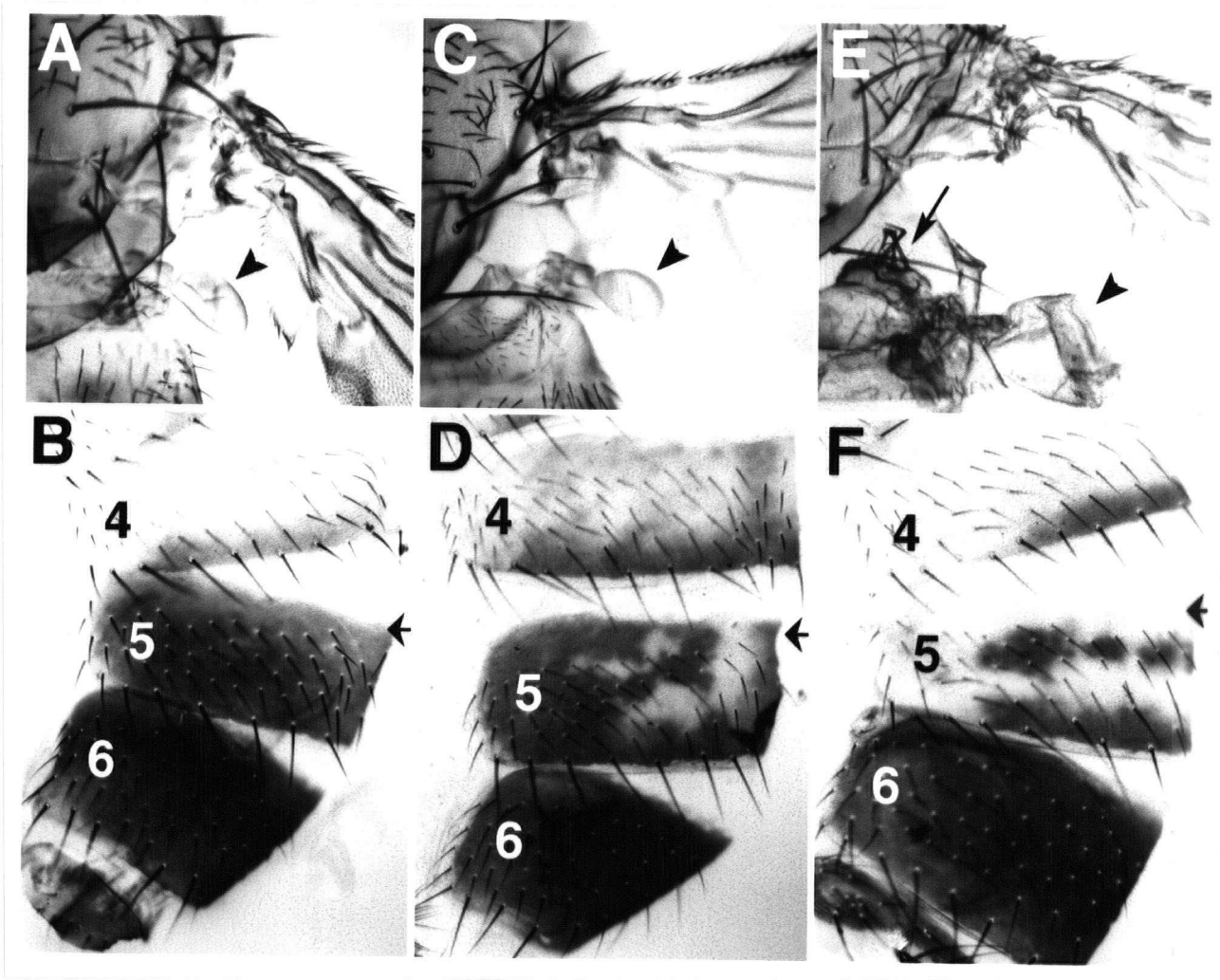

A
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Table 2: Enhancement of trxG and PcG phenotypes by Asx^{P1}

mutant allele	Asx^{P1}/Asx^{P1}; mutant / +	Asx^{P1}/Asx^{P1}; + / +	Asx^{P1}/ + ; mutant / +	Asx^{P1}/+ + / +
Df(3R)red	94.4 (54) †	19.1 (115) ‡	33.6 (110) §	0 (130)
trx^{B11}	100 (82) †	15.8 (82) ‡	14.3 (147) §	0 (151)
trx^{Z11}	97.1 (104) †	26.3 (76) ‡	3.0 (99) §	0 (95)
Pc^{4} *	40.0 (45) * 4.0 sex combs	4.2 (118) * 2.0 sex combs	12.2 (148) * 3.0 sex combs	0 (125) 2.0 sex combs

The first number is % penetrance of either anterior (trxG) transformations (†,‡,§) or posterior (PcG) transformations (*), the number in parentheses is the total number of flies scored for each genotype.

† scored for transformations of abdominal segment 5 (A5) or abdominal segment 6 (A6) towards more anterior segments, transformations of the posterior of the third thoracic segment (T3) towards the second (T2) or transformations of the haltere towards the wing

‡ only abdominal and haltere to wing transformations were seen

§ only abdominal transformations were seen

* abdominal transformations of A2-A4 towards A5 or A6 plus average number of legs with sex combs (wild type is 2.0) were scored

Table 3: Lack of enhancement of trxG and PxG phenotypes by the Asx^{P1} revertant Asx^{R11}

mutant allele	Asx^{R11}/Asx^{R11}; mutant / +	Asx^{R11}/Asx^{R11}; + / +	Asx^{R11}/ + ; mutant / +	Asx^{R11}/ + ; + / +
Df(3R)red	16.1 (62)§	0 (38)	15.9 (63)§	0 (40)
trx^{B11}	20.3 (79)§	0 (35)	17.4 (69)§	0 (61)
Pc^4 *	0 (80)* 3.5 sex combs	0 (85)* 2.0 sex combs	0 (98)* 3.5 sex combs	0 (110)* 2.0 sex combs

The first number is % penetrance of either anterior (trxG) transformations (§) or posterior (PcG) transformations (*), the number in parentheses is the total number of flies scored for each genotype.

§ only transformations of abdominal segment 5 (A5) or abdominal segment 6 (A6) towards more anterior segments were seen

* no abdominal transformations were seen, average number of legs with sex combs (wild type is 2.0) were scored

mutation in that homozygous Asx^{P1} mutations can also strongly enhance homeotic transformations of the PcG gene *Pc* (Table 2).

Asx^{P1} is homozygous viable but it is semi-lethal with strong *Asx* alleles. Asx^{R11} is a revertant line derived from Asx^{P1} in which loss of the P element insertion at the *Asx* locus is also associated with loss of semi-lethality with strong *Asx* alleles (Sinclair et al. 1992). Asx^{R11} homozygotes fail to enhance homeotic transformations of either *Pc* or *trx* (Table 3) indicating that the above described genetic interactions are specific for the Asx^{P1} insertion allele and are not due to a second site mutation on the Asx^{P1} chromosome.

Other *Asx* mutations can also enhance trxG phenotypes

PcG mutations enhance one anothers homeotic transformations. Consistent with this, all *Asx* alleles tested strongly enhance the extra sex combs phenotype of *Pc* and some also show enhanced penetrance of anterior to posterior abdominal transformations (Table 4). The genetic interaction between Asx^{P1} and *trx* could be due to neomorphic activity of the Asx^{P1} allele or it could reflect a more general interaction between *Asx* and *trx*. To distinguish between these possibilities, other *Asx* alleles were crossed to *trx* mutant alleles.

PcG and trxG mutations mutually suppress each others homeotic phenotypes. Double heterozygous combinations between Pc^4 and the *trx* null allele *Df(3R)red* completely suppresses *trx* abdominal homeotic transformations (Table 5b, cross 10). Double mutant combinations between two trxG genes generally show enhancement of trxG phenotypes. Surprisingly, when introduced from females, Asx^1, *Df(2R)trix*, Asx^9, and Asx^{13} heterozygotes all enhance the penetrance of trxG homeotic transformations in *Df(3R)red* heterozygotes (Table 5a, crosses 1-4), although not as dramatically as Asx^{P1} homozygotes do. Where tested, these *Asx* alleles can also enhance the penetrance of trxG homeotic transformations in trx^{B11} and trx^{Z11} heterozygotes (Table 5a, crosses 5-9).

Table 4: Enhancement of PcG phenotypes in *Asx;Pc*^{4} double heterozygotes

Asx allele	*Asx;Pc*^{4} DOUBLE HETEROZYGOTES
Df(2R)trix	2.2 (93) 3.4 sex combs
Asx^{1}	0 (34) 4.9 sex combs
Asx^{3}	69.0 (42) 4.7 sex combs
Asx^{8}	0 (30) 4.7 sex combs
Asx^{9}	0 (36) 5.8 sex combs
Asx^{13}	0 (55) 6.0 sex combs
Asx^{P1}	1.7 (60) 3.1 sex combs

The first number is % penetrance of abdominal posterior transformations.The number in parentheses is the total number of flies scored. Flies were also scored for the average number of legs with sex combs, the wild type number is 2.0. 6.0 is the maximum number of legs that can have sex combs, seen if all double heterozygotes show complete transformation towards the first leg. Only double heterozygotes are shown in the table because all other genotypic classes (including single *Pc* mutants alone) showed 0% abdominal transformations and the wild type average of 2.0 legs with sex combs.

Table 5a: *Asx* crosses that enhance the penetrance of *trx* phenotypes

CROSS FEMALES X MALES	*Asx;trx* DOUBLE HETEROZYGOTES	*trx* SINGLE HETEROZYGOTES
1) *Df(2R)trix* X *Df(3R)red*	6.1 (279)	2.5 (278)
2) Asx^{1} X *Df(3R)red*	32.0 (97)	14.2 (113)
3) Asx^{9} X *Df(3R)red*	49.2 (122)	28.3 (152)
4) Asx^{13} X *Df(3R)red*	41.3 (167)	26.2 (130)
5) *Df(2R)trix* X trx^{B11}	6.1 (359)	2.4 (338)
6) Asx^{9} X trx^{B11}	40.2 (117)	22.2 (81)
7) Asx^{13} X trx^{B11}	44.1 (102)	26.6 (94)
8) *Df(2R)trix* X trx^{Z11}	4.8 (230)	1.9 (210)
9) Asx^{9} X trx^{Z11}	25.5 (153)	10.6 (141)

Table 5b : Crosses that do not enhance the penetrance of trx phenotypes

CROSS FEMALES X MALES	DOUBLE HETEROZYGOTES	*trx* SINGLE HETEROZYGOTES
10) Pc^{4} X *Df(3R)red*	0 (114)	23.9 (67)
11) Asx^{8} X *Df(3R)red*	37.9 (153)	31.4 (169)
12) Asx^{3} X *Df(3R)red*	11.5 (156)	12.7 (165)
13) Asx^{P1} X *Df(3R)red*	21.0 (57)	18.4 (38)
14) *Df(3R)red* X *Df(2R)trix*	17.7 (237)	13.7 (117)
15) *Df(3R)red* X Asx^{1}	25.9 (139)	38.9 (113)
16) *Df(3R)red* X Asx^{9}	32.1 (53)	27.5 (40)
17) *Df(3R)red* X Asx^{8}	25.7 (101)	34.7 (101)
18) *Df(3R)red* X Asx^{P1}	20.6 (102)	19.7 (71)
19) *Df(3R)red* X Asx^{13}	13.9 (101)	27.2 (81)
20) *Df(3R)red* X Asx^{3}	31.1 (106)	36.9 (130)
21) trx^{B11} X *Df(2R)trix*	10.7 (187)	14.0 (157)
22) trx^{B11} X Asx^{9}	7.7 (39)	16.7 (36)
23) trx^{B11} X Asx^{13}	30.1 (103)	18.9 (53)
24) trx^{Z11} X *Df(2R)trix*	7.3 (218)	9.4 (245)
25) trx^{Z11} X Asx^{9}	29.8 (47)	15.9 (63)

Flies were scored for transformations of abdominal segment 5 (A5) towards more anterior segments. The first number is % penetrance of trxG transformations.The number in parentheses is the total number of flies scored for each genotype. Differences in penetrance between the double heterozygotes and the single *trx* heterozygotes were tested for significance using a Chi-square test (see Materials and Methods).

Asx^{3}, *Asx*^{8} and *Asx*^{P1} heterozygotes do not significantly enhance *Df(3R)red* homeotic transformations, but they also do not suppress them the way *Pc*^{4} does (Table 5b, crosses 11-13). For all crosses, when the *trx* allele is introduced from the females, the double heterozygotes fail to show significant enhancement of *trx* phenotypes (Table 5b, crosses 14-25). This indicates that the genetic interaction between *Asx* and *trx* has a maternal effect relative to the *Asx* mutant alleles.

The genetics shows that *Asx* behaves like a member of the PcG with respect to interactions with *Pc* , and can act as a member of the trxG with respect to interactions with *trx*, providing a different possible interpretation of the intermediate homeotic phenotype seen in the cuticle of *Asx* homozygous mutants. Double homozygous mutations between *Pc* and *trx* produce a weak PcG cuticle phenotype that looks very similar to homozygous single *Asx* mutants alone (Capdevila et al. 1986; Jurgens 1985; Breen and Duncan 1986). Thus, instead of viewing the weak cuticle phenotype of *Asx* mutants as resulting from weak PcG activity, this intermediate phenotype could be the result of abolishing both *Pc* and *trx* activity. *Asx* could therefore be a component of the system that integrates the activation signal of the trxG with the repression signal of the PcG.

The *Asx* mutation *Df(2R)trix* shows an extra strong PcG phenotype with *Pc*^{4}

All *Asx* mutations tested show strong enhancement of PcG phenotypes when crossed to *Pc*^{4}. The *Asx* null mutant *Df(2R)trix* enhances both the extra sex combs phenotype and the penetrance of abdominal posterior transformations. In addition, *Df(2R)trix;Pc*^{4} double heterozygotes also show an unusual darkly pigmented outgrowth in the first thoracic (T1) segment (Fig.12A and B). The penetrance of T1 outgrowths is quite variable and can range from 7% (from a total of 94 flies) to 24% (from a total of 116 flies) in individual crosses. The expressivity of the phenotype can range from a small, condensed outgrowth (Fig.12 A, B and Fig. 13 C) to producing an actual pseudo-wing on T1 (Fig.13

Fig.12

T1 mutant phenotype in *Pc^4;Df(2R)trix* double heterozygotes. **A-B**, darkly pigmented outgrowth on the first thoracic segment (T1) of *Pc^4;Df(2R)trix* double heterozygotes. **C**, darkly pigmented internal mass in the first thoracic segment of *Pc^4;Df(2R)trix* double heterozygotes. **D**, close up of the mass in (**C**). Upon close examination, the dark pigmentation is actually the result of condensed wing tissue and closely packed bristles.

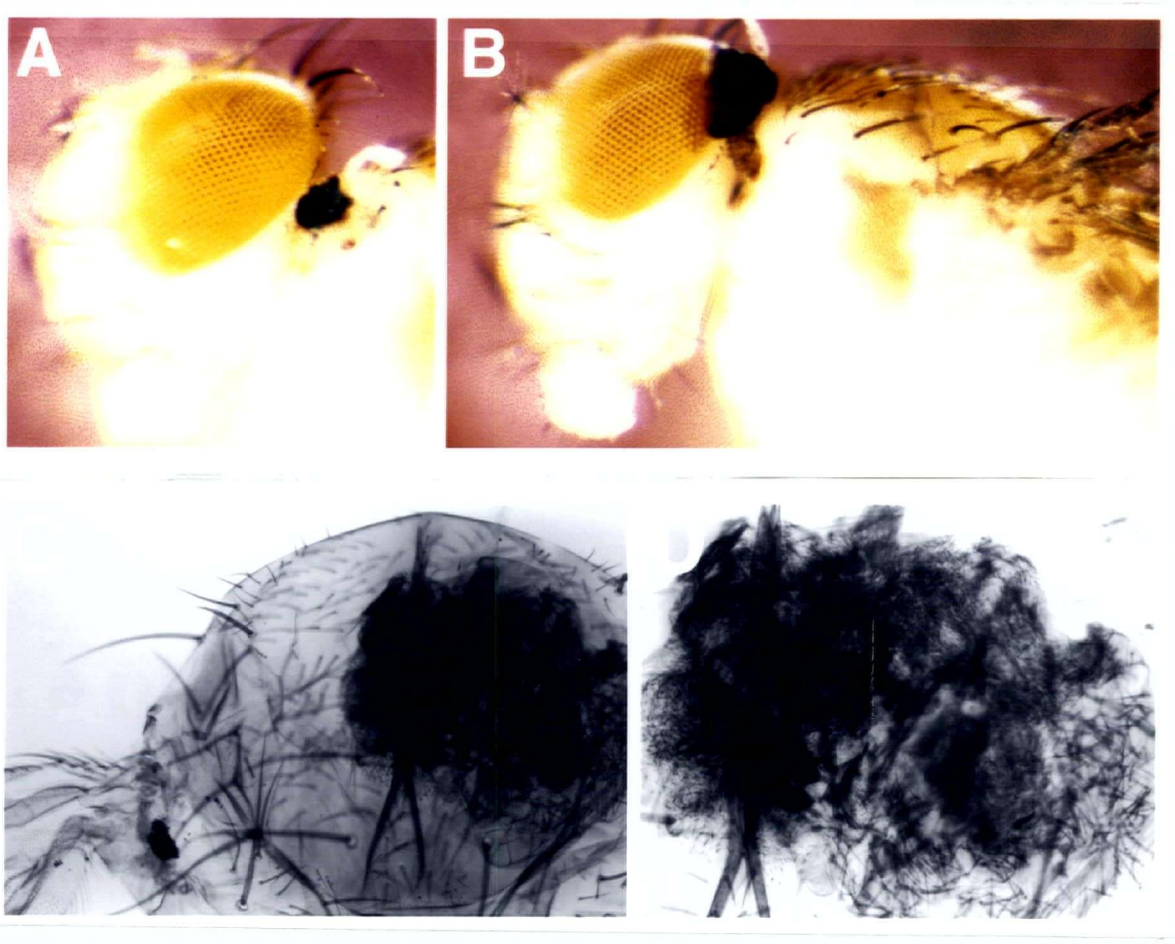
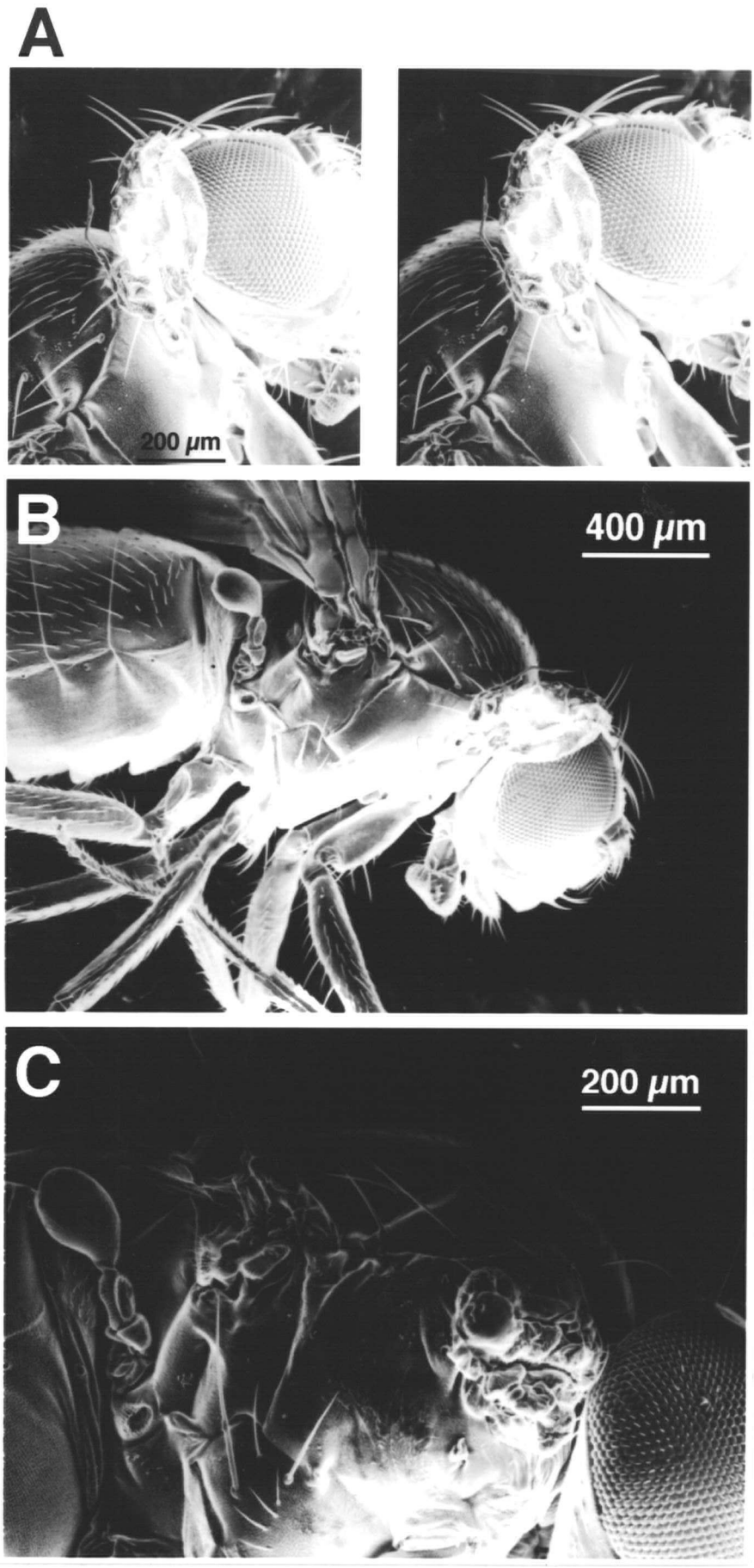

A
B

Fig.13

Scanning electron microscope (SEM) image of T1 mutant phenotype in *Pc*^{4}*;Df(2R)trix* double heterozygotes. **A**, stereo image of a scanning electron microscope (SEM) image of a pseudo-wing outgrowth on T1. **B**, SEM image of the same fly in (**A**) showing the size of the pseudo-wing relative to the rest of the fly. **C**, SEM image showing a small, condensed outgrowth on T1.


A
200 μm
B
400 μm
C
200 μm

A and B). In a few cases, instead of an actual outgrowth, there is a darkly pigmented internal mass (Fig. 12 C). Upon closer examination, the darkly pigmented internal and external growths are both made up of wing-like tissue (Fig.12 D). The internal growths are likely due to an unsuccessful imaginal disc evagination of a pseudo-wing from T1. Overall, this phenotype is interpreted to be a strong, but rarely seen, posterior transformation of T1 towards T2.

This T1 to T2 transformation is not seen when *Df(2R)trix* is crossed to mutations in the PcG genes *Sex combs on midleg* (*Scm*) or *Polycomblike* (*Pcl*) indicating that this interaction is probably not a generalized PcG-*Asx* interaction. The fact that crosses between *Df(2R)trix*, the only known *Asx* null allele, and the strong *Pc* allele Pc^4 can produce this unusually strong posterior transformation may indicate a specific, strong *in vivo* functional interaction between *Asx* and *Pc* .

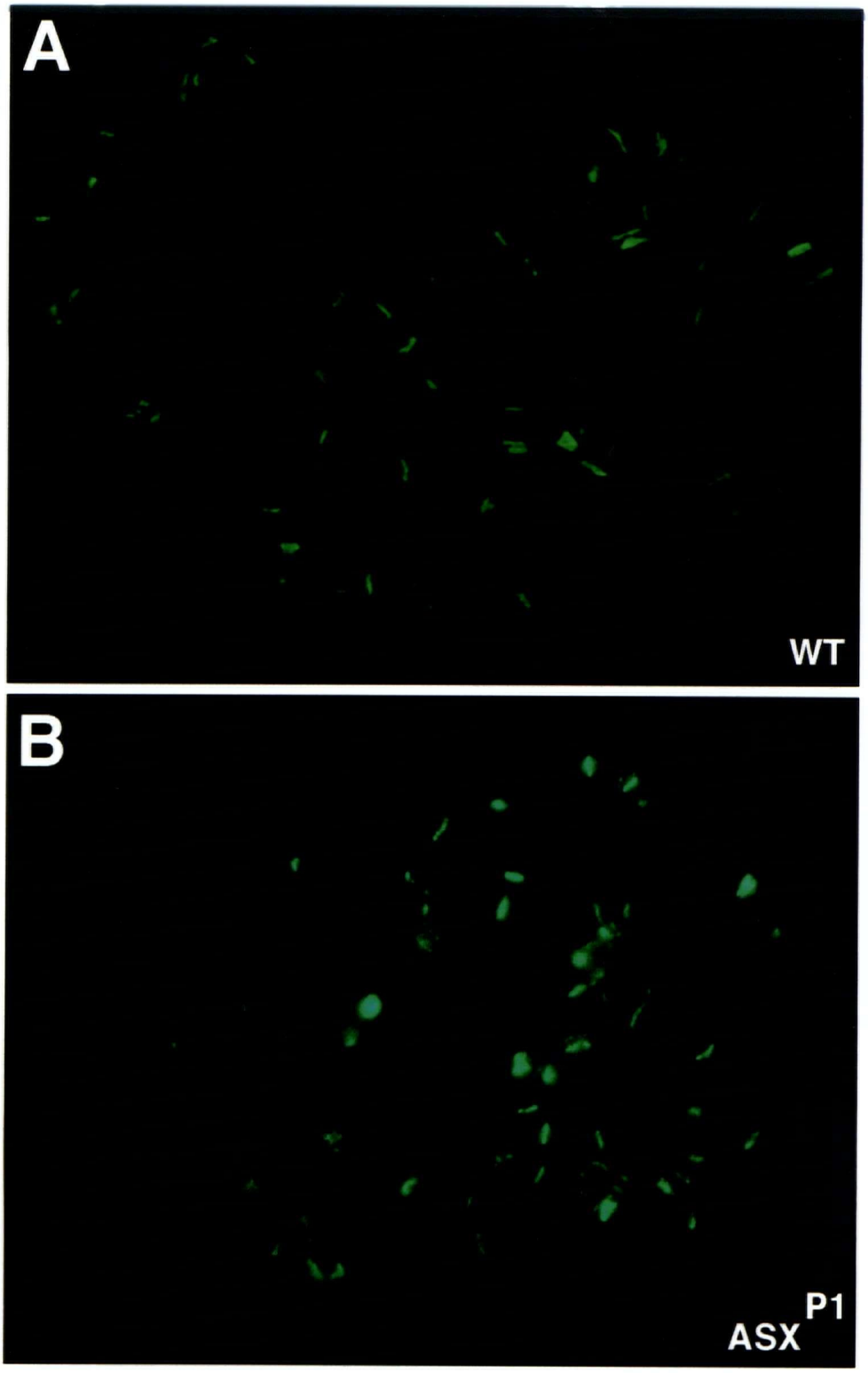
The binding of Pc is normal on Asx^{P1} mutant chromosomes

The Asx^{P1} mutation can strongly enhance *Pc* homeotic transformations both as a heterozygote and as a homozygote. PcG proteins are found bound to multiple loci on polytene chromosomes, including genes that are known to be active in the salivary glands. The trx protein is found bound along with Pc at multiple loci, including the homeotic loci, that are known to be repressed in the salivary glands. One possible interpretation of this data is that binding of PcG and trxG complexes is necessary but not sufficient for repression or activation respectively.

The enhancement of PcG phenotypes seen with Asx^{P1} mutants could be due to i) a reduction in the ability of PcG complexes to bind at target sites or ii) an alteration in functional activity of the complex once it is bound to its target site. To distinguish between these two possibilities, homozygous mutant Asx^{P1} polytene chromosomes were stained with an antibody to Pc to look for gross alterations in binding activity. Binding of Pc to homozygous Asx^{P1} polytene chromosomes was normal (Fig.14) indicating that the effect

Fig.14

Binding of Pc protein to Asx^{P1} mutant chromosomes. Pc protein was detected using a secondary conjugated to the fluorescent molecule FITC. **A**, WT chromosomes. **B**, homozygous Asx^{P1} mutant chromosomes. The image in (**A**) was collected using a confocal microscope and the image in (**B**) was photographed using a zeiss axiophot microscope.


A
WT
B
ASX P1

of the Asx^{P1} mutation probably occurs at the level of functional activity of the complex after it has bound to its target site.

Discussion

Asx mutations enhance both PcG and trxG homeotic phenotypes.

Asx^{P1} homozygous mutants strongly enhance both PcG and trxG mutations. All *Asx* alleles tested strongly enhance PcG phenotypes. Unexpectedly, some *Asx* alleles can also enhance trxG phenotypes. This enhancement shows a maternal effect with regards to the *Asx* mutant allele and could reflect an early developmental requirement for *Asx* activity in regards to *trx* function. By themselves, Asx^{P1} homozygotes have a low penetrance of both PcG and trxG adult phenotypes with a higher penetrance of trxG phenotypes compared to PcG phenotypes. This suggests that if *Asx* has both a trxG and a PcG function, the Asx^{P1} mutation has a stronger affect on the *trx*-dependent function of *Asx*. The molecular basis for the Asx^{P1} mutation is unknown. The *P* element does not interrupt the open reading frame and although northern blots show a slight decrease in the amount of transcript present (D. Sinclair, H. Brock unpublished), the level of Asx protein accumulation and binding to polytene target sites appears to be unaffected. The Asx^{P1} mutation may alter *Asx* expression in specific tissues such as the imaginal discs or it may somehow alter the translation of *Asx*.

The fact that some *Asx* alleles can enhance both PcG and trxG phenotypes implicates *Asx* in both PcG and the trxG activity. If *Asx* is required for the proper functioning of both the PcG and the trxG, *Asx* mutations would be expected to produce a phenotype similar to one seen in PcG/trxG double mutants. Consistent with this idea, *Pc;trx* and *esc;trx* homozygous double mutants suppress the strong trxG and strong PcG phenotypes seen with each gene individually to produce a more wild type embryo that has a slight PcG phenotype (Ingham 1983; Capdevila et al. 1986). The weak PcG phenotype

seen in these PcG;trxG double mutants is very similar to the weak PcG cuticle phenotype seen in *Asx* homozygous lethal mutants (Jurgens 1985; Breen and Duncan 1986).

The PcG gene *Enhancer of zeste* (*E(z)*) can also enhance the phenotypes of some trxG mutations. *E(z)* mutants have a strong PcG phenotype in the embryo but temperature sensitive studies have indicated that they have a strong trxG phenotype in the imaginal discs (LaJeunesse and Shearn 1996). Recently, it has also been shown that the GAGA protein, the product of the *trithoraxlike* (*trl*) locus (a trxG gene), functions at PREs and is required for the binding of PcG complexes to target sites (Hagstrom et al. 1997; Hodgson and Brock 1998). This suggests that there may be a subset of genes that either are required for the function of both groups or are involved in mediating the establishment of repression versus activation at target loci. It also suggests that the relationship between PcG mediated repression and trxG mediated activation is not entirely antagonistic and that there is a functional interdependance.

Asx may interact specifically with both trx and Pc proteins

The trx^{Z11} allele is a point mutation in the SET domain of *trx* (Stassen et al. 1995). The SET domain has an unknown function but it is found in other regulatory proteins such as the trxG protein ash-1, the PcG protein E(z), and in Su(var)3-9, a chromatin protein. Interestingly, a specific *E(z)* SET domain point mutation alters the *E(z)* SET domain and makes it more similar to the *trx* SET domain (L. Sipus, R. Jones, H. Gyurkovics, unpublished data). This *E(z)* point mutant has strong trxG phenotypes, suggesting that the SET domain itself plays an important role in mediating activation and repression of the homeotic loci. The trx^{Z11} allele is enhanced by Asx^{P1} homozygotes to the same extent as null alleles of *trx* , suggesting that the SET domain mutation alone is sufficient for the strong interaction seen between Asx^{P1} and *trx*. *In vitro* binding assays show that the SET domain of trx can bind to a carboxy terminal region of Asx (Kyba et al. 1998). Polytene staining shows that these two proteins overlap at multiple sites (Kyba et al.

1998), suggesting that the two proteins may also interact *in vivo*. These data suggest that Asx and trx proteins directly interact with one another and that Asx probably modifies trx activity.

Asx genetically interacts much more strongly with *Pc* than with most other PcG genes suggesting that the two proteins may have a specific *in vivo* functional interaction. Antibodies to Pc protein and an antibody to the amino terminus of Asx sterically hinder one another from binding to specific sites on polytene chromosomes suggesting that the antigens are close together. Although they have not been shown to directly physically interact *in vitro*, Asx and Pc protein are probably bound quite close to one another at specific target sites. Antibodies to trx have indicated that trx protein can be found bound to multiple sites overlapping with Pc protein. It is possible that trx binds to the carboxy terminus of Asx while the amino terminus of Asx binds to a small intermediary protein that binds to Pc. Asx could therefore mediate activation versus repression of the homeotic loci by directly regulating the activity of both trx and Pc proteins.

Chapter 3

Introduction

PcG genes were originally categorized as members of the PcG based on two criteria: i) they either display homeotic phenotypes which result from the ectopic expression of homeotic genes, or ii) they enhance the homeotic phenotypes of other PcG mutations. Ideally, a PcG gene should do both but this has not always been the case. Some PcG genes (such as *Asx* and *Psc*) were originally identified by their failure to undergo head involution and had only very weak homeotic mutant phenotypes (Nusslein-Volhard et al. 1984; Jurgens 1985). Their role in homeotic gene regulation was not as apparent until they were combined with other PcG mutations (Jurgens 1985). Double and triple mutant combinations between weak PcG mutations can produce an overall strong mutant phenotype (Jurgens 1985). As well, some PcG genes show strong enhancement of homeotic phenotypes in other PcG mutants but by themselves have no obvious homeotic phenotypes (Sato et al. 1983; Adler et al. 1989). *Enhancer of Polycomb* (*E(Pc)*) and *Suppressor of zeste 2* (*Su(z)2*) are examples of two PcG mutations that can strongly enhance the homeotic phenotypes of some other PcG mutations, but on their own do not show adult homeotic transformations or homeotic misexpression phenotypes in the embryo (Sato et al. 1983; Adler et al. 1989; McKeon and Brock 1991a; Soto et al. 1995). Only when the maternal component of the *E(Pc)* gene is completely removed do mutant embryos exhibit a very slight ectopic expression of the homeotic gene *AbdB* in the embryo (Soto et al. 1995).

Using the above criteria, most strong members of the PcG (ie. those that display dose sensitive dominant adult homeotic transformations) have probably already been identified. However, using genomic deletions to enhance the extra sex combs phenotype of other PcG mutations, it has been estimated that the genome may contain up to 25 unidentified PcG genes (Jurgens 1985; Landecker et al. 1994). There are two major problems with identifying new PcG mutations using genetic means.

First of all, not all mutant PcG combinations show enhancement of PcG homeotic phenotypes. Some double or triple mutant PcG combinations produce very strong homeotic mutant phenotypes and some do not show any genetic interaction at all (Campbell et al. 1995; Cheng et al. 1994). This has been used to argue for specific functional subsets within the PcG that may reflect the existence of distinct functional complexes (Campbell et al. 1995; Cheng et al. 1994). Most of the traditional genetic screens have looked for PcG interactors using *Pc* (and in one case *Pcl*) alleles so these screens may have missed mutants that only interact with other PcG genes (Kennison and Tamkun 1988; Landecker et al. 1994). Screening the entire genome looking for genetic interactors with the entire panel of known PcG mutants would be tedious.

The second major problem with a genetic approach is that not all genes that contribute to PcG function may be dose sensitive. The most straight forward method of screening for new mutations is to look for enhancement of adult homeotic transformations in double heterozygous mutants. However, this is only effective if a new mutation is dose sensitive and can enhance other PcG mutations as a heterozygote.

A more direct way of identifying new PcG genes is to use the yeast 2 hybrid system to screen for proteins that interact with a target PcG protein of interest and then to try and establish a possible *in vivo* functional role for the new gene. This has two major advantages over a genetic approach; 1) it can be used to identify proteins that contribute to the function of only one PcG gene product and may be relatively dose insensitive and 2) it provides an immediate cDNA clone, avoiding the difficult process of cloning a gene of interest after a genetic interaction has been identified.

Asx protein can be found bound to multiple chromosome sites containing other PcG proteins. At some of these sites, it may bind extremely close to the PcG protein Pc, suggesting that Asx is part of a PcG complex at these sites. Asx does not interact directly with any PcG proteins in the yeast 2 hybrid system (M. Kyba and H. Brock, unpublished)

so if it is a member of a PcG complex, it must interact with the complex through proteins other than those tested.

Unlike other PcG genes, *Asx* has tissue-specific effects on homeotic gene regulation, suggesting that its activity is required differentially in different tissues. *Asx* itself is expressed ubiqitously in all tissues, so it is unclear how the protein is targeted to its sites of activity only in those tissues where it is required. One possibility is that binding to its target sites is mediated by other proteins that are themselves expressed in a tissue specific pattern.

The main objective in this chapter was to take four new proteins retrieved from a yeast 2 hybrid cDNA library screen that interact with Asx, and to try to determine if they could contribute to the *in vivo* function of *Asx*.

To test the possibility that there may be genes that contribute to the function of *Asx* but do not themselves have any obvious homeotic phenotypes, a yeast-2 hybrid screen was initiated in our lab using the carboxy terminus of the Asx protein (M. Kyba and H. Brock, unpublished data). Several interacting genes were recovered (M. Kyba and H. Brock, unpublished data) and four of them were chosen for further analysis. Genomic deletions that uncover PcG loci tend to enhance the homeotic transformations of other PcG mutations, so genomic deletions that contain a dose-sensitive gene suspected of interacting with *Asx* should enhance the severity of homeotic transformations in double mutant combinations. To test this possibility, the chromosomal location of each interactor was mapped and large genomic deletions that remove each endogenous locus were tested for genetic interactions with *Asx*. A large genomic deletion (*Df(3L)ZN47*) that removes the locus of the interactor termed *z40* shows weak enhancement of trxG phenotypes in an *Asx* mutant background indicating that this protein may contribute to one specific aspect of *Asx* function. The observed genetic interaction may be weak because *z40* is not a very dose-sensitive gene, so homozygous mutant *Df(3L)ZN47* embryos were examined for the regulation of several homeotic genes. Surprisingly, *Df(3L)ZN47* mutant embryos have

highly target specific embryonic homeotic misexpression phenotypes, including extensive derepression of the homeotic gene *Sex combs reduced* (*Scr*). This particular pattern of target specific homeotic gene regulation has not been seen with any other PcG mutations. It is unknown what the relationship is between these embryonic phenotypes and *Asx* function in particular, but since *Df(3L)ZN47* interacts with *Asx* mutations in the adult, one possible interpretation is that the *z40* gene product mediates *Asx* target specificity.

Results

Mapping the interactors

The genomic location of each interactor was mapped by hybridizing the labeled cDNA's to polytene chromosomes (Fig. 15). The *z3*, *z34* and *z40* genes are all on the third chromosome while the *z11* locus maps to the X chromosome. The *z3* interactor maps to 85E (Fig. 15 A), *z11* to 14C (Fig. 15 B), *z34* to 100E (Fig. 15 C) and *z40* to 65A (Fig. 15 D).

A genomic deletion that uncovers the *z40* locus displays enhanced trxG abdominal transformations in an *Asx* mutant background

Large genomic deletions that remove each of the above loci were tested for their ability to enhance adult homeotic transformations of *Asx* mutations. Deletions of *z11* and *z34* failed to have any noticeable effect. The *z3* interactor maps to the same location as the PcG gene *Sex combs on midleg* (*Scm*) but it does not have a sequence that matches *Scm* and thus is a unique gene (Bornemann et al. 1996). Any deletions that remove *z3* would also remove *Scm* and so this locus could not be genetically tested.

Southern blotting was used to confirm that the large genomic deletion *Df(3L)ZN47* completely removes the *z40* locus (Fig. 16, A-D). *Df(3L)ZN47* shows variable penetrance of a weak trxG homeotic transformation of abdominal segment 5 (A5) towards more anterior abdominal segments. In a heterozygous Asx^3 mutant background, the penetrance

Fig. 15

Mapping of interactor cDNA's to polytene chromosomes. **A**, *z3* at 85E. **B**, *z11* at 14C. **C**, *z34* at 100E. **D**, *z40* at 65A.

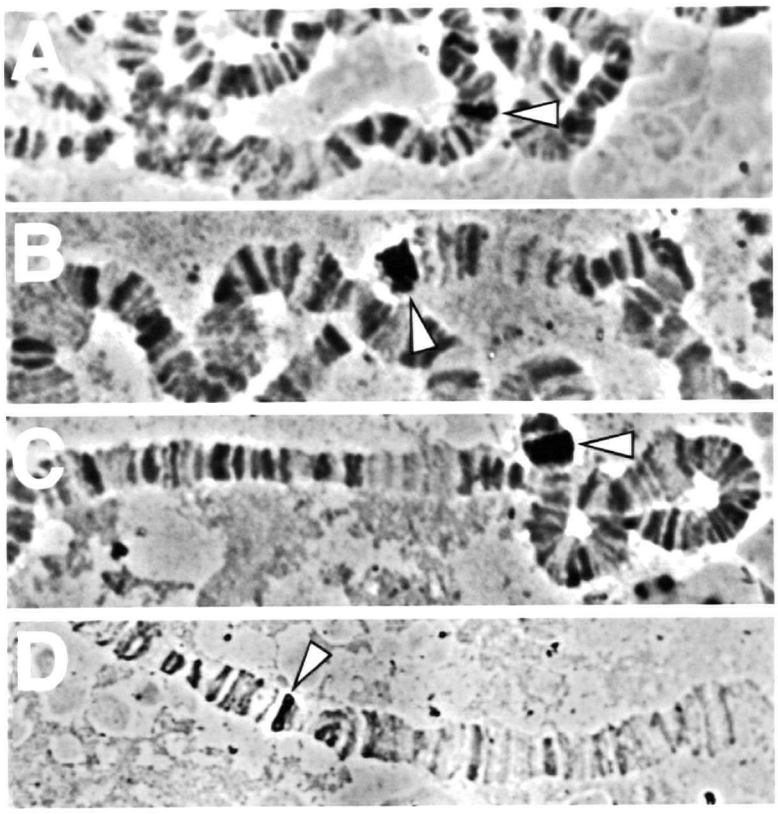
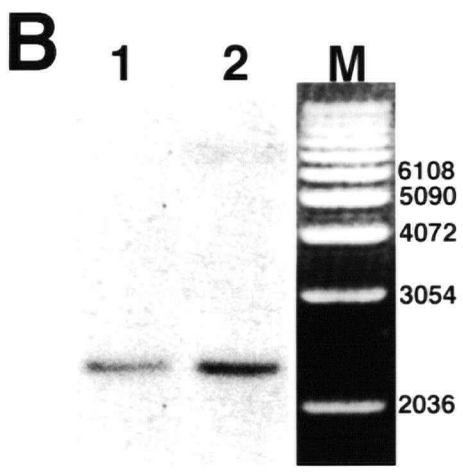
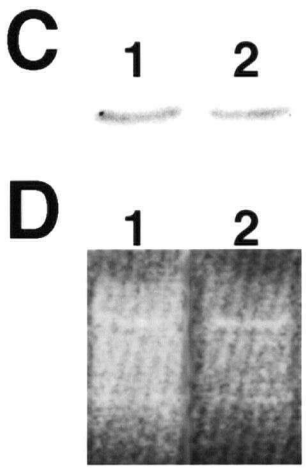

A
B
C
D

Fig. 16

The large genomic deletion *Df(3L)ZN47* removes the *z40* locus which produces a transcript of 1.8 kb in length. **A**, polytene chromosome showing the *z40* 1.0 kb cDNA fragment signal at 65A and the extent of the *Df(3L)ZN47* deletion. The deletion extends from cytological position 64C to 65C. **B**, Genomic Southern blot with DNA prepared from *Df(3L)ZN47* heterozygous flies (lane 1) and wild type flies (lane 2) probed with the 1.0 kb *z40* cDNA fragment. There should be half as much DNA from region 64C;65C in the *Df(3L)ZN47* flies. The signal in lane 1 is about half as intense as in lane 2, indicating that the 1.0 kb *z40* cDNA fragment is derived from the 64C;65C region. The M lane contains 1 kb ladder molecular weight marker DNA, sizes are given in bp. **C**, same lanes as in (**B**) probed with a DNA fragment from the 48A region, outside the boundaries of the *Df(3L)ZN47* deletion. Signal intensity is about equal in both lanes indicating the amount of genomic DNA loaded in each lane is about equal. **D**, gel of blot from (**B**) also showing the amount of genomic DNA loaded in lanes 1 and 2 is about equal. **E**, Northern blot probed with the 1.0 kb *z40* cDNA fragment giving a signal at about 1.8 kb. Sizes are marked in bp.


A
Df(3L)ZN47


B
1
2
M
6108
5090
4072
3054
2036


C
1
2
D
1
2

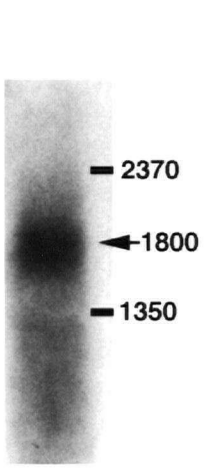

E
2370
1800
1350

Table 6a : Enhancement of *Df(3L)ZN47* trxG phenotypes by heterozygous Asx^3 mutations

Females X males	*Df(3L)ZN47/Asx*	*Df(3L)ZN47/+*	*Asx/+*
Asx^3 X *Df(3L)ZN47*	100 (36)	90.5 (42)	15.9 (44)
Df(3L)ZN47 X Asx^3	100 (25)	93.8 (32)	0 (28)

Table 6b : Enhancement of *Df(3L)ZN47* trxG phenotypes by Asx^{P1}

mutant allele	Asx^{P1}/Asx^{P1}; *Df(3L)ZN47 / +*	Asx^{P1}/Asx^{P1}; + / +	Asx^{P1}/ + ; *Df(3L)ZN47 / +*	Asx^{P1}/+ + / +
Df(3L)ZN47	72.9 (59)	18.1 (238)	28.1 (235)	0 (385)

The first number in each column represents the percentage of flies of each genotype that had a transformation of abdominal segment 5 (A5) towards the anterior. The number in brackets represents the total number of flies scored for each genotype. Average number of sex combs per fly was also examined and found to be wild type.

of these weak trxG abdominal transformations is slightly increased (Table 6a). No genetic interaction was seen between *Df(3L)ZN47* and the *Polycomb* allele Pc^4.

Flies that are homozygous for the Asx^{P1} mutation and heterozygous for *Df(3L)ZN47* are semi-lethal as observed by the relative absence of this genotype compared to others in the table 6b cross. All the surviving Asx^{P1} *;Df(3L)ZN47/+* flies showed a significant increase in the penetrance of strong transformations of A5 towards the anterior, similar to the phenotypes seen when *Asx* alleles are crossed to *trx* mutations.

The full length *z40* transcript is 1.8 kb

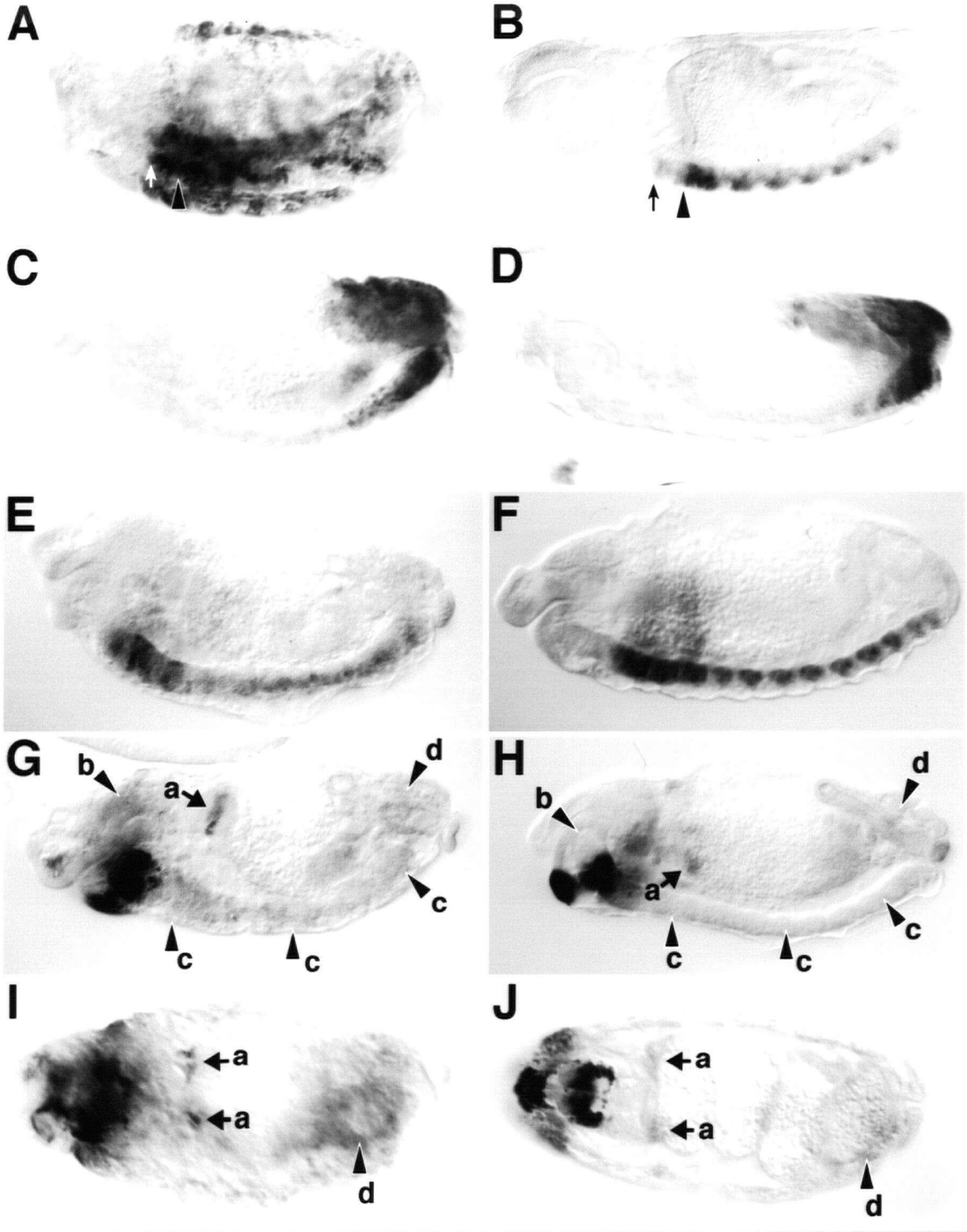
The partial *z40* cDNA retrieved from the yeast 2 hybrid interactor screen is only about 1.0 kb in length. A Northern blot was probed with this cDNA to determine the size of the transcript produced by the *z40* locus and the full length size of the *z40* transcript was determined to be 1.8 kb (Fig. 16, E). By examining a *Drosophila* EST database, a further 360 nucleotides of *z40* were recovered and the resulting cDNA now includes the full *z40* open reading frame (M. Kyba, unpublished result).

The genomic deletion *Df(3L)ZN47* that uncovers the *z40* locus has embryonic homeotic misexpression phenotypes

The interactions of *Df(3L)ZN47* with different *Asx* mutations is relatively weak. One reason could be that the *z40* gene product is relatively dose-insensitive so that one wild type copy can fulfill most functions of the *z40* gene. To examine the effect of complete removal of the *z40* locus on homeotic gene regulation, *Df(3L)ZN47* homozygous mutant embryos were stained with antibodies to the homeotic proteins Ultrabithorax (Ubx), Abdominal B (AbdB), Antennapedia (Antp), and Sex combs reduced (Scr) (Fig. 17). The deletion that removes the *z34* locus was also examined but no alterations in homeotic gene regulation were seen.

Fig. 17

Expression of various homeotic genes in *Df(3L)ZN47* homozygous mutants (**A,C,E,G,I**) compared to wild type embryos (**B,D,F,H,J**). The embryo in **A** is viewed from a ventral-lateral position to better highlight the *Ubx* expression in the ventral nerve cord, anterior is to the right and posterior to the left. Embryos in **B-H** are laterally viewed with anterior to the left, posterior to the right, dorsal up and ventral down. Embryos in **I** and **J** are ventrally viewed with anterior to the left and posterior to the right. **A** and **B**, expression of *Ubx* is increased in PS5 of *Df(3L)ZN47* mutants (**A**) compared to wild type (**B**) but the PS5 boundary is maintained (white arrow). The parasegment 6 (PS6) boundary is marked with an arrowhead and the PS5 boundary is marked with a small white arrow in (**A**) and a small black arrow in (**B**). **C** and **D**, *AbdB* expression is normal in *Df(3L)ZN47* mutants (**C**) compared to wild type (**D**). **E** and **F**, *AntP* expression is slightly reduced in *Df(3L)ZN47* mutants (**E**) compared to wild type (**F**). **G** to **J**, *Scr* expression is ectopically expressed in *Df(3L)ZN47* mutants (**G** and **I**) compared to wild type (**H** and **J**). **G**, Mutant *Df(3L)ZN47* embryos lack many of the typical morphological landmarks of wt embryos, but the patch of *Scr* expression in the head appears to be in the brain (arrowhead,*b*). *Scr* expression is also seen in the central nervous system (arrowheads,*c*), and in the posterior of the embryo (arrowhead,*d*). **H**, wild type *Scr* expression can be seen in PS2 and 3, in the foregut (small arrow,*a*), and very slightly in the hindgut (arrowhead,*d*). There is no *Scr* expression in the brain (arrowhead,*b*) or the CNS (arrowheads,*c*). **I**, the misexpression of *Scr* in the posterior of the embryo (arrowhead,*d*) is more obvious in this ventral view where *Scr* is expressed in a large patch. *Df(3L)ZN47* mutants show essentially wild type *Scr* expression in the foregut (small arrows, *a*). **J**, expression of *Scr* in the posterior of wild type embryos is limited to a small region in the hindgut (arrowhead,*d*).


A
B
C
D
E
F
G
b
a
d
c
c
c
H
b
d
a
c
c
c
I
a
a
d
J
a
a
d

Df(3L)ZN47 homozygous mutant embryos show extensive morphological defects (Fig. 17, A,C,E,G,I). The midgut is bloated and undefined and the embryos are shorter and have an overall stunted appearance. The CNS lacks definition and axon formation is disorganized. Anterior embryonic development appears to be slowed compared to posterior development. For example, in the embryo shown in Fig.17 G, the embryonic head and brain (arrowhead,*b*) has the features of a stage 11 embryo while the hindgut (arrowhead,*d*) has the features of a stage 13-14 embryo.

Despite the lack of defining morphology, the PS5 expression boundary of the *Ubx* gene appears to be maintained in *Df(3L)ZN47* homozygous mutants (compare Fig.17 A to B, small arrows mark the PS 5 expression boundary, arrowheads mark the PS 6 expression boundary). However, in wild type embryos, *Ubx* is expressed at much lower levels in PS5 relative to PS6 and only in the anterior compartment of PS5 (see Fig.17 B). In *Df(3L)ZN47* homozygous mutants, *Ubx* expression is increased so that PS5 expression is now equal to that of PS6 and *Ubx* is also expressed ubiquitously throughout the PS5 compartment (see Fig. 17 A).

There is no alteration in the expression of *AbdB* in *Df(3L)ZN47* homozygous mutants (Fig. 17 C) compared to wild type embryos (Fig. 17 D).

Regulation of the *Antp* gene is altered in that expression of *Antp* is slightly reduced in *Df(3L)ZN47* homozygous mutants (Fig. 17 E) compared to wild type embryos (Fig. 17 F). Reduction of homeotic gene expression is usually associated with mutations in members of the trxG. Since homeotic proteins expressed in posterior regions repress the expression of homeotic genes with more anterior expression boundaries, this reduction could be indirect and due to the slight increase of *Ubx* expression. However, *Antp* expression is reduced in regions where there is no *Ubx* expression which indicates that the reduction of *Antp* expression cannot be explained entirely by an increase in *Ubx* expression.

Mutations in most PcG genes normally cause suppression of *Scr* expression in the gut due to repression from expanded posterior homeotic gene expression boundaries. Surprisingly, *Scr* expression is widely derepressed in multiple tissues of *Df(3L)ZN47* homozygous mutants (Fig. 17 G and I). In wild type embryos, *Scr* expression is normally restricted to PS2 and PS3 (Fig. 17 H and J) and the foregut (Fig. 17 H and J, short arrows, *a*) with a slight expression in the hindgut (Fig. 17 H and J, arrowhead,*d*). Because *Df(3L)ZN47* homozygous mutants lack morphological landmarks in the head, it is difficult to compare *Scr* expression in the head region with wild-type embryos. However, *Scr* appears to be ectopically expressed in the brain of *Df(3L)ZN47* homozygous mutants compared to wild-type (compare Fig. 17 G, *b* arrowhead with Fig. 17 H, *b* arrowhead). In *Df(3L)ZN47* homozygous mutants *Scr* is also ectopically expressed throughout the CNS (Fig. 17 G compare to 17 H, *c* arrowheads), and in the posterior of the embryo (Fig. 17 G and I compare to 17 H and J, *d* arrowheads). Ectopic staining of Scr protein in these regions is relatively weak, probably due to repression from other homeotic genes preventing full expression of the *Scr* gene in these regions. Despite its lack of morphology, expression of *Scr* in the foregut appears normal (Fig. 17, G and I compare to H and J, short arrows, *a*).

In typical PcG mutants, regulation of all the homeotic loci are affected resulting in derepression of the homeotic genes. *Df(3L)ZN47* is unusual because it displays a range of homeotic gene misexpression phenotypes. In *Df(3L)ZN47* homozygous mutants, *Scr* expression is widely derepressed in many tissues. *Ubx* is only weakly derepressed in PS5, while *Antp* expression is actually suppressed. *AbdB* expression is unaltered and appears normal. This indicates that the *z40* locus could regulate only some homeotic loci and possibly have a different specific role at each locus.

An attempt was made to make double homozygous *Asx;Df(3L)ZN47* mutant embryos but no double mutant combinations were recovered suggesting that embryos of this genotype all died early in embryogenesis.

Df(3L)ZN47 does not affect the regulation of two PcG dependent *Scr* pairing sensitive regulatory elements

Two putative PREs have been identified in the regulatory region of the *Scr* gene, a 10.0 kb*Xba*I sequence and an 8.2 kb *Xba*I sequence. They were identified because they show PcG dependent variable repression (or variegation) of a *miniwhite* reporter gene. The *miniwhite* reporter gene is responsible for depositing pigments into the eye in a cell autonomous manner so if the gene is on in some cells and off in others, a red and white mosaic or variegated pigment pattern results. Normally, when additional copies of the *miniwhite* reporter gene are introduced into the genome, eye pigment is increased. However, expression of the *miniwhite* reporter gene is reduced when a second copy of either of the above constructs is introduced on to the homologous chromosome. This has been interpreted to mean that the two elements are somehow pairing with one another in *trans* and increasing the repression of the *miniwhite* reporter gene. This *trans* enhancement of repression is termed pairing sensitivity. Although not all PREs that show pairing sensitive repression are necessarily maintenance elements, most PcG maintenance elements have a pairing sensitive effect.

The variegation of the 10.0 kb and the 8.2 kb *Xba*I sequences is decreased in some, but not all, PcG mutant backgrounds, indicating that the repression is at least in some cases PcG dependent. *Asx* mutations do not affect variegation of either construct. Since it has such a strong effect on regulating the endogenous *Scr* gene, *Df(3L)ZN47* was tested with lines containing these response elements to see if it would alter the regulation of these transgenes.

Df(3L)ZN47 was tested with one line containing the 8.2 kb *Xba*I construct and three separate lines containing the 10.0 kb *Xba*I construct. In all cases, *Df(3L)ZN47* had no detectable effect on eye variegation. Thus, *Df(3L)ZN47* probably does not regulate the

Scr gene through either of these regulatory sequences, indicating that there may be other important regulatory elements at the *Scr* locus that have not yet been identified.

Discussion

Df(3L)ZN47 genetically interacts with Asx mutations

Df(3L)ZN47 is a large genomic deletion that removes the *z40* locus as well as many other genetic loci. Thus, the results with this deletion are suspect at least to the extent that they do not rule out the possibility that another gene in the deletion other than *z40* is responsible for the observed genetic interaction. However, the fact that this deletion removes the *z40* locus and *z40* interacts with *Asx* in the yeast 2 hybrid system favors the possibility that these results are due to the *z40* locus itself.

Df(3L)ZN47 heterozygous mutants have a very weak trxG homeotic mutation in the abdomen. In an Asx^3 mutant background, the double mutants show complete penetrance of this phenotype. However, since the penetrance of the phenotype in the single *Df(3L)ZN47* heterozygous mutants was high to start with, the significance of this increase is difficult to gauge. *Df(3L)ZN47* interacts much more strongly with Asx^{P1} in that it appears to be semi-lethal with it. This semi-lethality could be indirect and simply due to the combined mutant effects of the Asx^{P1} mutation and the *Df(3L)ZN47* deletion, but the fact that all the survivors show a significant increase in the penetrance of strong trxG abdominal transformations argues for a specific genetic interaction between Asx^{P1} and the *Df(3L)ZN47* deletion.

This enhancement of trxG phenotypes suggests that the *Df(3L)ZN47* deletion (and therefore possibly the *z40* gene itself) contributes to *Asx* activity in trxG mediated homeotic gene regulation. Anterior homeotic transformations in the abdomen are generally caused by reduced expression of the *abdA* gene. Unfortunately, no antibodies were available to look for possible alterations of *abdA* regulation in *Df(3L)ZN47* homozygous mutant embryos. However, the fact that *Df(3L)ZN47* homozygotes could suppress the

expression of the homeotic gene *AntP* (which is a trxG phenotype) at least argues for the possibility that *z40* could also be required for activation of the *abdA* homeotic locus.

Df(3L)ZN47 removes the *z40* locus and has target specific effects on homeotic gene regulation

Df(3L)ZN47 specifically causes the extensive derepression of the *Scr* gene. This is unique for two reasons. No PcG mutation examined to date causes extensive derepression of only one specific homeotic locus. Also, PcG mutations tend not to show ectopic *Scr* expression in the embryo. This second observation is due to the fact that the proteins of the BX-C (Ubx, abdA and AbdB), all repress expression of *Scr*. When these genes are ectopically expressed in PcG mutants, the result is the downregulation of *Scr* expression throughout most of the embryo. However, even in PcG mutants, *Scr* expression is still maintained at relatively normal levels in its normal domain of expression, parasegments 2 and 3. This is probably due to the fact that moderate levels of *Antp* expression in these two parasegments maintains the expression of *Scr*. Both extra low levels and extra high levels of *Antp* expression tend to suppress the expression of *Scr*. In the *Df(3L)ZN47* homozygous mutants, although ectopic *Scr* expression is spatially extensive, the levels of ectopic expression are relatively low. This is probably due both to repression from the presence of *Ubx*, *abdA* and *AbdB* expression, and to suppression of activity from the reduced levels of *Antp* expression observed (see below).

In addition to its effect on the regulation of *Scr*, *Df(3L)ZN47* also has a weak effect on the regulation of both *Antp* and *Ubx*. Ubx is not ectopically expressed outside its normal domain but parasegment 5 (PS5) expression is increased to levels normally only seen in parasegment 6 (PS6). This indicates that the product from *Df(3L)ZN47* is somehow required for proper *Ubx* expression in PS5, but not in other parasegments. *Antp* expression is reduced in *Df(3L)ZN47* mutants. Reduction of homeotic gene expression is normally considered to be a trxG phenotype. The only observable adult phenotype in

Df(3L)ZN47 heterozygotes is a weak trxG transformation in the abdomen, which raises the possibility that expression of the homeotic gene *abdA* is also reduced in *Df(3L)ZN47* mutants.

In vivo functional role for *z40*

One thing that might be expected from a screen designed to identify new PcG genes that are not easily identified using a traditional genetic approach is that these new genes might have non standard (in terms of PcG function) effects on the regulation of the homeotic loci. The *z40* gene seems to fit this idea as it is relatively dose insensitive in terms of adult homeotic phenotypes and it displays an unusual array of target specific effects on homeotic gene regulation. Since double homozygous combinations between *Asx* and *Df(3L)ZN47* apparently did not produce viable embryos, it is difficult to determine the extent to which the embryonic phenotypes of *Df(3L)ZN47* contribute to the *in vivo* function of *Asx.*

However, one unknown aspect of PcG function is how PcG proteins act on specific target sites only in domains where their activity is required. All PcG proteins are expressed ubiquitously throughout the embryo. Thus one possibility is that PcG proteins interact with other proteins that themselves have target specific activity. The z40 protein could be such a target specific factor and may contribute to *Asx* function by targeting Asx activity towards different specific loci. The combination of both PcG and trxG homeotic mutant phenotypes is also consistent with a possible *in vivo* interaction with *Asx* that itself has been implicated in both PcG and trxG activity. Thus *z40* could be required for the *Asx* mediated activation of some target loci and the *Asx* mediated repression of others.

Asx protein binds to some chromosomal target sites very near the Pc protein but it does not bind to Pc directly (M. Kyba, unpublished). However, the z40 protein has been tested and it interacts directly with the Pc protein both in the yeast-2 hybrid system and in an *in vitro* GST fusion assay (M. Kyba, unpublished). This result provides a possible role

for z40 protein in the function of Asx. Perhaps z40 binds to Pc only at specific target loci and then the Asx protein binds to z40 and is able to interact with Pc. *Asx;Pc* heterozygous mutants show strong enhancement of the extra sex combs phenotype that results from the ectopic expression of *Scr*. The fact that *Df(3L)ZN47* mutants show strong derepression of the *Scr* locus is consistent with the possibility that it interacts with *Pc* and *Asx* in such a manner. The fact that *Df(3L)ZN47* heterozygous mutants fail to enhance the extra sex combs phenotype of *Asx* mutations may again reflect the relative dose-insensitivity of the *z40* locus.

Chapter 4

Introduction

Not all double heterozygous combinations between different PcG mutations show the same level of enhancement of dominant homeotic phenotypes. Some double heterozygous combinations produce flies with very strong PcG homeotic phenotypes while some mutant combinations only produce weak , or in some cases, no PcG homeotic phenotypes at all (Campbell et al. 1995). One of the reasons for this variability in the degree of enhancement seen is at least partly due to the fact that some PcG genes have a strong maternal component that can provide partial rescue of mutant phenotypes. However, the variations in phenotypic strength seen with different mutant combinations cannot be wholly explained by variations in maternal contribution since one would then expect that all mutant combinations between genes with a strong maternal component should interact only weakly. To some extent, strong genetic interactions probably reflect important *in vivo* functional interactions.

Asx shows strong genetic interactions with only a subset of PcG genes which include *Polycomb* (*Pc*), *Polycomblike,*(*Pcl*), *Sex combs extra* (*Sce*) and *super sex combs* (*sxc*) (Campbell et al. 1995). The strongest interaction is seen between *Asx* and *sxc* (Campbell et al. 1995). The sxc^3 mutation is lethal or semi-lethal as a double heterozygote with all *Asx* alleles tested (D. Sinclair and H. Brock, unpublished data). In cases where a small proportion of double heterozygotes survive, they show multiple PcG homeotic phenotypes including extra sex combs, transformations of abdominal segment 4 towards more posterior segments and transformations of the wing towards the haltere (D. Sinclair and H. Brock, unpublished data). Other *sxc* alleles are not lethal with *Asx* which argues that the sxc^3 mutation is a gain of function mutant that is directly interfering with the function of Asx protein. This could indicate that there is an interaction between the Asx and sxc proteins *in vivo*.

There are only five alleles of *sxc*, all isolated in the original screen in which *sxc* was identified (Ingham 1984). The *sxc* gene has not been cloned, perhaps because it is situated at the base of 2R in the 41C region, very close to centric heterochromatin. Alleles of *sxc* display segmentation defects, a phenotype rarely seen in PcG mutants but one that is also seen with several *Asx* alleles. Homozygous *sxc* mutations survive through embryogenesis all the way through pupation and die as pharate adults just before eclosure (Ingham 1984). These dead pharate adults display typical PcG homeotic mutations such as extra sex combs and wing to haltere transformations as well as occasionally displaying the rare antenna to leg transformation (Ingham 1984) that is only seen in mutants of one other PcG gene, *Pc*.

The lethal interaction between sxc^3 and *Asx* and the fact that both *Asx* and *sxc* have segmentation defects suggests that Asx and sxc proteins may interact directly in vivo and that *sxc* may contribute to one or several aspects of *Asx* function. To start examining the possibility that the two proteins may interact, an attempt was made to clone the *sxc* gene using *P* element insertional mutagenesis.

P element insertion mutagenesis has long been used as a method for cloning genes of interest in *Drosophila*. *P* elements are transposable elements that can exise and reinsert into different locations throughout the genome when they are in the presence of a source of transposase. By screening for specific mutations, an insertion in a particular gene of interest can be selected for. A *P* insert line can then be used to make a library of genomic DNA from which DNA flanking the site of insertion can be recovered by screening with *P* element sequences. Alternatively, flanking DNA can be recovered by using inverse PCR methods or by using a *P* element construct that can be rescued as a plasmid. A rescuable *P* element is modified to contain an antibiotic resistance gene as a selectable marker and a plasmid origin of replication (see Fig. 18A). When cut with *Xba*I and religated, the selectable marker plus origin of replication are combined with flanking DNA sequences and can be selected for as a plasmid that confers antibiotic resistance on competent cells.

The problem with *P* insertional mutagenesis is that the chances of getting an insertion event in a target gene of interest is quite low. *P* elements do not insert randomly into DNA but the exact sequence preferences for *P* element insertions are unknown. For any particular locus, the chance of an getting an insertion can range from 1/1000 (or 0.1%) to greater than 1/100,000 (or 0.001%). In practical terms, it is usually too labour intensive to screen for more than 10,000 possible events, so multiple *P* elements are often mobilized to increase the likelihood of a single *P* inserting into the target locus. This approach creates the problem of cleaning up multiple *P* elements left over throughout the genome after the screen is completed. A cleaner approach uses a "local hop" (Zhang and Spradling 1993; Tower et al. 1993). Almost 30% of all mobilization events will result in the *P* element preferentially reinserting into the genome within 100 kb of its starting point (Tower et al. 1993). A particular *P* element only mobilizes about 7-10% of the time so if you start within 100 kb of your target gene, the chances of getting a *P* element reinsertion event within this 100 kb can be as high as 2% (Tower et al. 1993; Zhang and Spradling 1993). Thus local hops require two things; i) the approximate location of the gene of interest, ii) a *P* element that is within 100-200 kb nearby. One problem with local hops is that *P* elements will often make small deletions when they excise from the DNA (Tower et al. 1993) so any local *P* screen must differentiate between small deletions that remove the locus of interest and genuine *P* insertion events.

A local *P* screen was attempted for the *sxc* locus using a *P* element construct (a *P*[*lacZ,ry*$^{+}$] element, also called PZ) that is homozygous lethal and inserted in the 41C region, but not in the *sxc* gene itself. Two separate *P* screens resulted in the generation of multiple small deletions in the region including five new *sxc* alleles but no insertion events were recovered. To enrich for insertion events and select against deletions, the original screen was redesigned using a small deletion that was lethal with the original insertion but was viable with *sxc* mutations. This screen was attempted by two undergraduate students and they successfully recovered two potential inserts in the *sxc* gene.

Results

A local *P* screen for *sxc* resulted in the generation of new *sxc* alleles

P insertional mutagenesis was attempted on the *sxc* locus using a *P*[*lacZ,ry*$^{+}$] (or PZ) element (shown in Fig. 18A) that was inserted within the 41C region. The PZ element is inserted in an uncharacterized lethal site (*l(2)02047*) and is therefore homozygous lethal, but it is viable with *sxc* null alleles indicating that the uncharacterized lethal site is not *sxc* itself. For ease, the PZ element inserted at *l(2)02047* in 41C will be referred to simply as P41C.

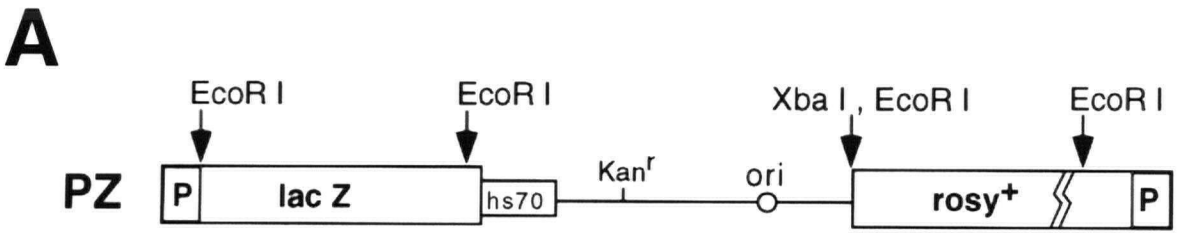
Details of the screen are given in Fig. 19. The screen was initially done with the presumed *sxc* null allele *sxc*^{4} and the phenotype screened for was lethality with *sxc*^{4}. Genuine *P* element insertion events can be distinguished from deletions because lethality due to a *P* element insertion is revertable by remobilizing the *P* element and causing it to excise out of the gene. As well, if the *P* element has inserted into a new location, it should produce a new band on a genomic Southern blot so this can be used as quick way of checking for possible insertion events.

Out of 2,856 chromosomes (ie. individual pair matings), 14 mutants lethal with *sxc*^{4} were recovered. When these 14 mutants were retested by crossing them to the *sxc* null alleles *sxc*^{1} and *sxc*^{5} and the *sxc* semi-lethal mutant *sxc*^{2}, only two of the putative *sxc* mutants were lethal or semi-lethal with these other *sxc* alleles, although all 14 mutants continued to be lethal with *sxc*^{4}. The most likely explanation for this is that the *sxc*^{4} mutant has a second lethal mutation between the *sxc* mutation and the lethal insertion site of the P41C element. Thus, out of these 14 lethals, 12 affected this second lethal site but not *sxc* itself and only therefore 2 were genuine *sxc* alleles (see Table 7).

The two new *sxc* alleles recovered from the screen were originally named *sxc*$^{III\text{-}48}$ and *sxc*$^{IX\text{-}1}$. The exact nature of these mutations is unknown, they could be deletions extending from P41C to *sxc* , they could be insertions in *sxc* that immediately remobilized

Fig. 18

Diagram and restriction digest of the PZ construct. **A**, diagram of the PZ construct showing the approximate positions of *EcoR*I and *Xba*I restriction enzyme cut sites. There is only one internal *Xba*I site. The construct contains a copy of the wild type *Drosophila rosy* gene (rosy$^+$) and a heat shock promoter (hs 70) linked to a *lacZ* gene. Cutting an inserted PZ element with *Xba*I and religating it produces a smaller functional plasmid containing flanking DNA and a Kanamycin resistance gene (Kan^r) and origin of replication (ori). The *P* element ends that are required for transposition are indicated by boxed Ps. Each is approximately 500 bp's in size. **B**, A restriction digest of the entire circularized element shown in (**A**). Lane 1, 1 kb ladder DNA, sizes are marked in bp's. Lane 2, the PZ plasmid cut with *Xba*I produces a single ~18 kb band. Lane 3, cutting with *EcoR*I produces a distinctive set of 3 internal bands (indicated by white arrows) and one flanking band. Starting from the smallest to the largest, the *EcoR*I fragments are 3.0 kb, 3.5 kb, 4.5 kb and 7.0 kb. The 3.0 kb fragment contains the lacZ gene, the 3.5 kb fragment contains the Kan^r and the ori and the 4.5 kb band contains the bulk of the *rosy* gene. The 7.0 kb fragment contains the rest of the *rosy* gene, the P element ends, plus some extra sequences not shown in the diagram.


A
EcoR I
EcoR I
Xba I , EcoR I
EcoR I
PZ
P
lac Z
hs70
Kan^r
ori
rosy+
P

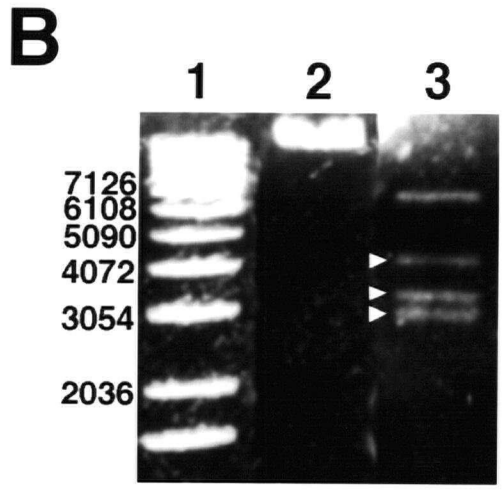

B
1
2
3
7126
6108
5090
4072
3054
2036

Fig. 19

Local *P* screen for *sxc* insertions. The PZ element in its original position (labelled P41C) was mobilized by crossing it to a source of transposase (P[ry^+ Δ 2-3]). Individual putative transposition events (labelled P41C*) were then captured by balancing them over a CyRoi marked balancer chromosome. Individual events were screened by crossing single males to 5-10 females carrying an *sxc* mutation. The F3 was examined for the phenotypes described in the figure.

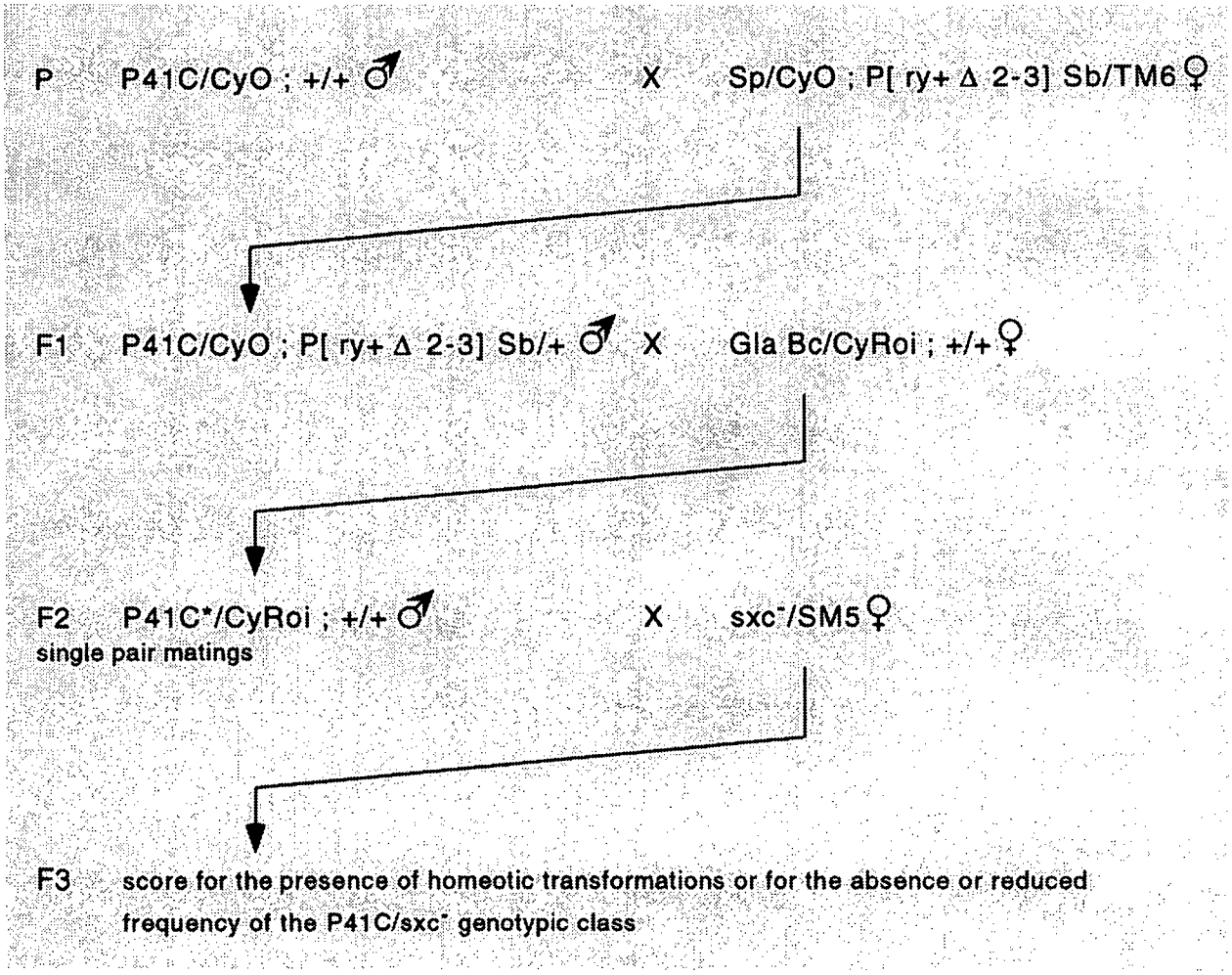

P P41C/CyO ; +/+ ♂ X Sp/CyO ; P[ry+ Δ 2-3] Sb/TM6 ♀
F1 P41C/CyO ; P[ry+ Δ 2-3] Sb/+ ♂ X Gla Bc/CyRoi ; +/+ ♀
F2 P41C*/CyRoi ; +/+ ♂ X sxc⁻/SM5 ♀
single pair matings
F3 score for the presence of homeotic transformations or for the absence or reduced frequency of the P41C/sxc⁻ genotypic class

Fig. 20

Southern blots of individual putative lines. **A**, genomic DNA was digested with *Xba*I and probed with a radioactively labelled lacZ probe. Lane 1, genomic DNA from the original P41C line produces a large ~10 kb band. Lane 2, genomic DNA from sxc^{7}. Lane 3, genomic DNA from the *II-78* line. Lane 4, genomic DNA from sxc^{6}. **B**, genomic DNA was digested with *EcoR*I and probed with the full length PZ element shown in Fig. 18. Lane 1, genomic DNA from the original P41C line produces a specific pattern of bands. Three internal bands from the PZ element itself are produced (marked with black arrowheads, compare with *EcoR*I digestion shown in Fig. 18). One very large band that contains flanking DNA from the *rosy* side of the PZ construct is also produced (white arrowhead). Lane 2, sxc^{10}. Lane 3, *II-78*. Lane 4, sxc^{6}. Lane 5, sxc^{8}. Lane 6, *6-3* line, a derivative of P41C in which the *P* element has failed to move. Lane 7, sxc^{9}. All of the genuine *sxc* alleles are missing the internal PZ bands, indicating that the PZ element has undergone an internal deletion in these lines. No new bands are present in the *sxc* lanes that are not present in either the P41C or *6-3* lanes.

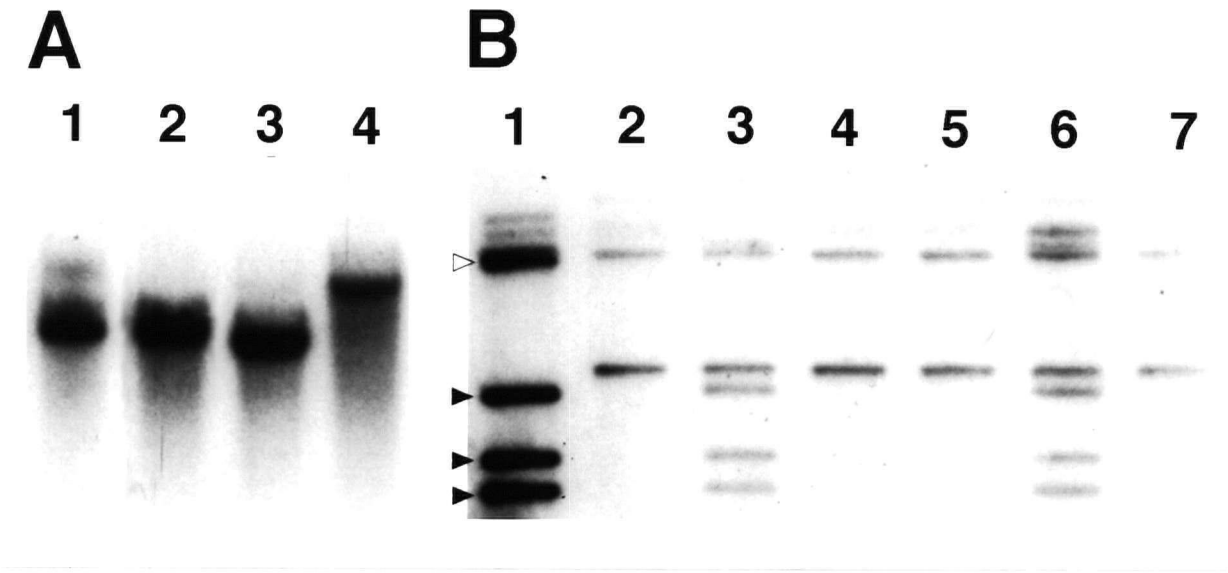

A
1 2 3 4
B
1 2 3 4 5 6 7

Table 7: Results of two separate *sxc* local *P* screens

Total number of chromosomes screened	Total number of putatives recovered	Total number of new *sxc* lethal alleles recovered	Rate at which new *sxc* alleles were generated
2,856	14	2	0.07 %
4,757	6	3	0.06 %
Totals 7,613	20	5	0.07 %

causing an internal *sxc* deletion, or they could be *P* element insertions. Because of these variables, these and all other *sxc* alleles that were recovered were named simply using standard genetic notation. Following standard genetic notation, these alleles will now be known as sxc^6 and sxc^7, respectively. Genomic DNA was prepared from sxc^6 and sxc^7 stocks, blotted and probed with DNA from the lacZ gene. Compared to the original P41C line, the *P* element in sxc^7 did not produce a band of a different size indicating that the *P* element had not re-inserted into a new location (see Fig 20A). The sxc^6 line did produce a new band when probed with lacZ, a larger 20 kb band. A reversion of lethality was attempted on sxc^6 by remobilizing the *P* element. Usually, when a *P* element transposes to a new location, it leaves a copy of itself behind or it causes a small deletion in its original location. Occasionally, when it moves to a new location it excises completely from the DNA and leaves no trace of itself behind. This is called a clean excision and in order to revert a mutant phenotype associated with a *P* element insertion a clean excision is required. The P41C element in its original position reverts its lethality at a rate of 20/832 or 2.4%. Reversion of lethality with *sxc* was attempted on the sxc^6 line but out of 1091 flies, no revertants were found. It was concluded that the sxc^6 and sxc^7 lines were not P element insertions and were probably deletions based on the fact that they either; i) did not produce a new band on a Southern blot or ii) the lethality of the line was not revertable.

The same screen was attempted again with two modifications. First, instead of sxc^4, the null allele sxc^1, which should contain no second site mutations, was used to screen for *P* insertions. Second, *P* element insertions often cause more subtle mutations rather than lethality, so every single cross was examined for semi-lethals and homeotic mutations as well as lethal interactions. With these two modifications, the same screen as outlined in Fig. 19 was attempted. 4,757 chromosomes were screened and three lethal lines were recovered and established (see Table 7). No semi-lethal lines were recovered and no crosses showed any homeotic transformations. The lethal lines recovered continued to be lethal with strong *sxc* alleles so they were named sxc^8, sxc^9 and sxc^{10}. None of these

lethals produced new bands on a southern (Fig. 20B) and none of them were revertable for lethality with sxc^1. Again, none of these lethal lines appeared to be inserts and all were probably deletions.

Out of a total of 7,613 chromosomes, five new *sxc* alleles, all probably deletions, were recovered giving a recovery rate of *sxc* alleles of about 0.07 % (see Table 7). No insertions were recovered indicating that *P* element insertion at the *sxc* locus occurs at a rate lower than 1/7,613.

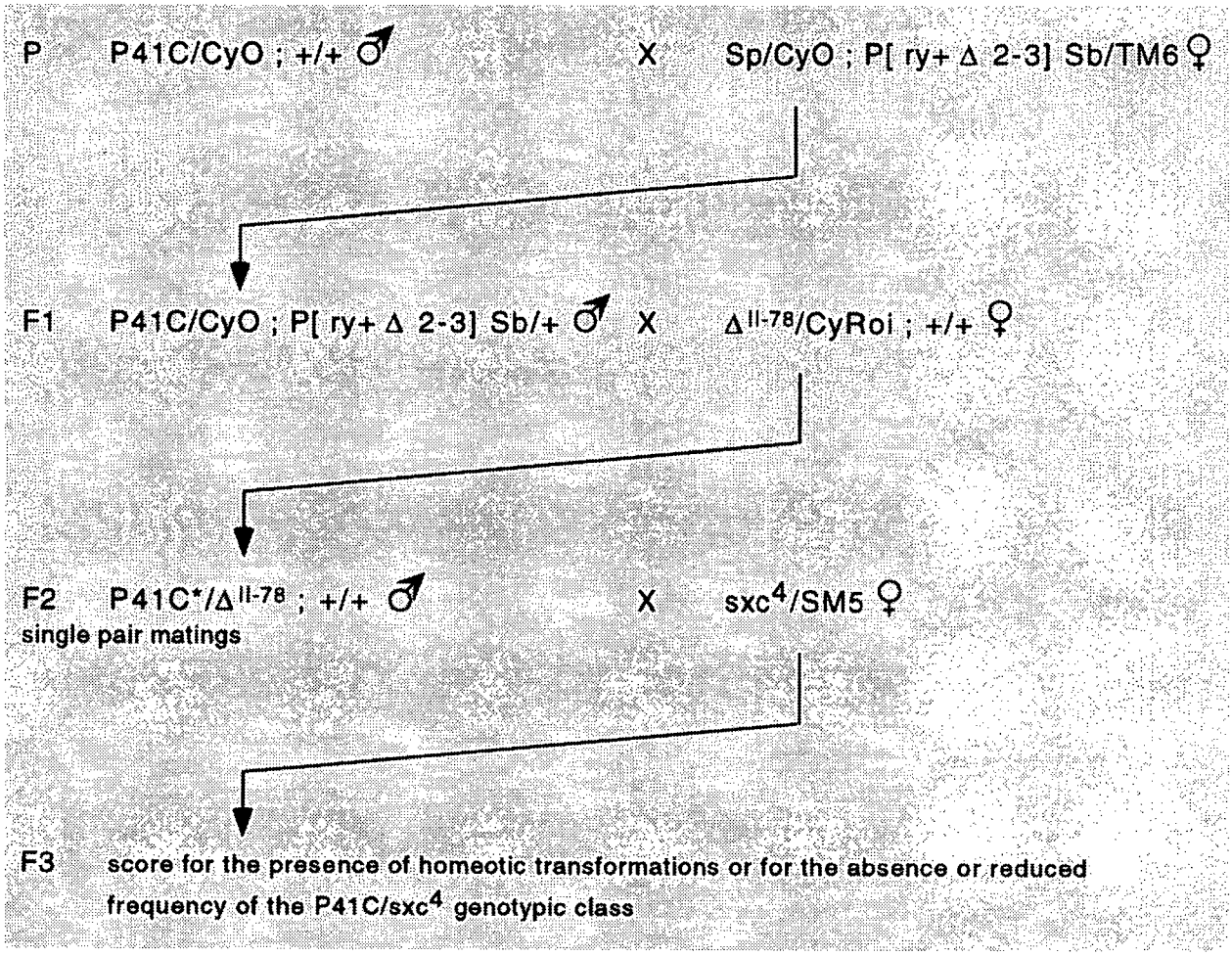
A *P* element insertion enrichment screen produced putative *sxc* insertions

From the above screens, it is obvious that local *P* screens in the region of *sxc* generate deletions much more readily than insertions. One other problem with the local *P* screen is that most of the time, when a *P* element is exposed to a source of transposase, it doesn't move at all. To try and enrich for insertion events, the original screen was modified to select for mobilizations of the *P* element and to select against the generation of deletions (see Fig.21).

The P41C element was mobilized as before by crossing it to a transposase source. P41C excision events were selected for in the germline of the F1 flies by crossing them to $\Delta^{II\text{-}78}/CyRoi$ flies. $\Delta^{II\text{-}78}$ is a double mutant recovered from the very first screen. It is lethal with sxc^4 and with the original P41C insert but not with other *sxc* alleles. It is presumed to be a deletion based on the fact that it does not produce a new band on a Southern (see Fig.20) and its lethality with sxc^4 is not revertable. When crossed together as stable stocks, heterozygous $P41C^*/\Delta^{II\text{-}78}$ flies die. Selecting for heterozygous $P41C^*/\Delta^{II\text{-}78}$ survivors in the F2 selects for P41C elements that have hopped out of their original location (and are no longer lethal with $\Delta^{II\text{-}78}$) and it also selects against deletions that would affect the P41C lethal site or the sxc^4 second lethal site. Thus, for any $P41C^*/\Delta^{II\text{-}78}$ survivors we know that the *P* element has been mobilized and we also know that it did not generate a deletion in the process. The $P41C^*/\Delta^{II\text{-}78}$ flies can then be

Fig. 21

P enrichment screen for *sxc* insertions. Details are given in the text.


P P41C/CyO ; +/+ ♂ X Sp/CyO ; P[ry+ Δ 2-3] Sb/TM6 ♀
F1 P41C/CyO ; P[ry+ Δ 2-3] Sb/+ ♂ X Δ II-78/CyRoi ; +/+ ♀
F2 P41C*/Δ II-78 ; +/+ ♂ X sxc4/SM5 ♀
single pair matings
F3 score for the presence of homeotic transformations or for the absence or reduced frequency of the P41C/sxc4 genotypic class

crossed to sxc^4 to select for mutations in the *sxc* locus itself. The selection works as follows. Δ^{II-78}/sxc^4 heterozygotes will die (Δ^{II-78} is lethal with sxc^4). Both P41C and Δ^{II-78} chromosomes will be viable over *SM5* which is marked with a dominant *Curly* marker. P41C should be viable over sxc^4 and produce straight winged flies. If the *P* element has inserted in the *sxc* locus the $P41C/sxc^4$ genotypic class should either be absent, reduced in frequency or exhibit homeotic transformations.

The modified screen was attempted by two undergraduate students, Susan Leong-Sit and Ester O'Dor under my direction. Out of 261 Δ^{II-78}/sxc^4 individual pair matings, 17 putatives were found based on the fact that these flies showed tergite defects and weak homeotic transformations in the abdomen. Lines were established by individually crossing curly winged males to $\Delta^{II-78}/CyRoi$ females. Crosses that produced straight winged survivors contained the P41C* putative. *CyRoi* siblings were collected and used to establish stocks of the putative insertion lines.

Discussion

The *P* element insertion enrichment screen

The insertion enrichment screen provides a modification of the local P hop, allowing for selection against deletions. Unfortunately, the enrichment screen was not possible until the original screen had been done. Thus the enrichment screen is probably not generally applicable to other loci. A modification of the enrichment screen could have provided further enrichment for insertion events. The main problem with the screen as it was used is that it selects for the *P* element hopping out of the P41C locus, but it does not select for *P* element reinsertion. In many cases, the P41C element probably hopped out and then was lost. One way to ensure that you are testing only reinsertion events would be to select for the presence of ry^+. If the entire screen was done in a *ry* mutant background, then only those flies that had the *P* element would be ry^+. Thus at the F2 stage, you would be selecting for flies that were $P41C^*/\Delta^{II-78}$ and were also ry^+ so that you would know

that they all contained an insert. The problem with this approach is that it would have been too time consuming to make all the needed stocks ry^-, so the screen was attempted without selecting for re-insertions at the F2 stage.

Future work

Now that we have acquired putative inserts in the *sxc* locus, work has continued on cloning the *sxc* gene. Once the gene is cloned, it would be interesting to see if the sxc protein can interact with Asx. One of the odd things about Asx is that in the yeast two hybrid system, Asx does not interact with any PcG proteins, although it does interact with trx, a member of the trxG. Other genetic and molecular data are consistent with the likelihood that Asx is, however, somehow part of a PcG complex. Mutations in *Asx* show strong enhancement of the mutant phenotypes of other PcG genes and Asx protein appears to be bound close to Pc protein at many chromosomal target sites. If Asx protein actually does interact directly with sxc protein, perhaps this is how Asx participates in a PcG complex. Alternatively, Asx binds to many target sites where there are no other PcG proteins bound. It would be interesting to see if antibodies to sxc overlapped with Asx at these unique target sites. The fact that both *sxc* and *Asx* mutations can show segmentation defects, a phenotype not seen in most other PcG mutations, may indicate a specialized functional role for these two proteins that would be reflected in binding to unique target sites.

General Discussion

Asx may be a component of the system that mediates activation versus repression at target loci

Asx mutations can enhance both trxG and PcG phenotypes. If a gene is important for the activity of both the PcG and the trxG, we would expect that a mutation in such a gene should have the same phenotype as double mutations between PcG and trxG genes. *Pc;trx* and *esc;trx* double homozygous mutations to a large degree cancel out each others PcG and trxG homeotic mutations and produce an embryo that is more wild type than with either mutation individually, but still has a slight overall PcG phenotype (Ingham 1983; Capdevila et al. 1986). Homozygous *Asx* mutants have a weak PcG phenotype in the cuticle that is very similar in appearance to the phenotype of these PcG;trxG double mutants (Jurgens et al. 1984; Breen and Duncan 1986). This supports the possibility that a mutation in *Asx* is disrupting the activity of both the PcG and the trxG, or at least the activity of the *Pc* and *trx* genes. The PcG gene*E(z)* also displays both PcG and trxG phenotypes (LaJeunesse and Shearn 1996). The trxG protein GAGA is required for transcriptional activation from chromatin templates *in vitro* (Tsukiyama et al. 1994) but it has also recently been found associated with PcG complexes and PREs (Strutt et al. 1997; Hodgson and Brock 1998). Taken together, all of this suggests that the traditional view of two distinct, separate and antagonistic groups of genes with one set of activators and one set of repressors is probably inaccurate and there is much more functional interdependance between the trxG and the PcG than previously thought.

Pc and trx proteins overlap at a large number of target sites on polytene chromosomes (Chinwalla et al. 1995). This is surprising because one would not necessesarily expect both an activating complex and a repressive complex to be bound to the same targets. Some of these target loci are active in the salivary glands (such as the PcG genes themselves) and some of them are repressed (such as the homeotic loci). This

suggests that binding of either a PcG or a trxG complex is not itself sufficient for either repression or activation and that there is some other step downstream from binding that determines if a particular locus is repressed or activated. *Asx* may be a component of such downstream activity.

Asx shows strong genetic interactions with both *Pc* and *trx* and is thus required for the activity of both genes. Asx binds directly to the trx SET domain *in vitro* (Kyba et al. 1998) and it binds very near the Pc protein *in vivo.* Pc and Asx do not bind to each other directly *in vitro*, but they both bind to z40 protein and thus may interact with each other through this z40 interaction. The binding sites for trx protein overlap with about 66 of the total of 90 Asx binding sites (Kyba et al. 1998), suggesting that the two proteins may also interact *in vivo.* An overlap between trx and Asx of 66 sites is very close to the 63 sites of overlap between Asx and Pc. Although it has not been shown directly, it is possible that Asx, Pc and trx protein all overlap at the same set of sites. It would be interesting to see if there are two separate complexes, one with Asx-trx the other with Asx-Pc, or if there is just one specific complex containing all three proteins. One possibility is that Asx protein binds to both Pc and trx at the same time and mediates crosstalk between two separate complexes, although it is also possible that Asx is a member of two separate and distinct complexes; a PcG complex and a trxG complex.

Asx has tissue specific effects and binds to unique target sites

The fact that*Asx* may be important for the activity of both the PcG and the trxG complicates the interpretation of homeotic derepression phenotypes seen in *Asx* mutant embryos. To some extent, if the weak PcG cuticle phenotype of *Asx* mutants results from disrupting both PcG and trxG activity, weak PcG derepression phenotypes in *Asx* mutant embryos probably results from the same thing. However, this does not explain tissue specific differences in regulation and the observation that although *Asx* mutants produce a weak *Ubx* derepression phenotype in the CNS, there is no effect on the regulation of *Abd*

B in the CNS at all. This suggests that *Asx* activity is not uniform and it does not regulate all targets in an equal manner.

Most of the genetic and molecular data favors the idea that there are one or more PcG complexes involved in regulating target loci. The fact that Psc, Su(z)2, and E(z) proteins as well as Asx can bind to some polytene sites in the absence of other PcG proteins (Rastelli et al. 1993; Carrington and Jones 1996) suggests that these proteins participate in some non-PcG complexes or that they are able to bind and be active at some target sites on their own.

Some *Asx* mutations have segmentation defects, mutant phenotypes that are usually associated with mutations in the early acting segmentation genes. Asx protein is present in early embryos so it is possible that it has a role in these early regulation events. Of the PcG, only *sxc* and *pleiohomeotic* (*pho*) also have segmentation defects (Breen and Duncan 1986; Ingham 1984). *Asx* and *sxc* show an unusually strong, GOF genetic interaction with each other (D. Sinclair, unpublished) suggesting the possibility that the two proteins may interact *in vivo.* One possibility is that Asx and sxc proteins interact and are required for the regulation of some early acting segmentation genes. It would be interesting to see if sxc protein overlaps with Asx at all of its unique binding sites.

Future work

There are several important questions that remain: 1) what is/are the mechanism(s) of PcG/trxG requirements for *Asx* activity?, 2) what is the basis of the tissue specific requirements for *Asx* activity?, and 3) is Asx a member of multiple functional complexes at different target sites?

First of all, to determine if Asx is a member of both Pc and trx containing complexes *in vivo,* antibodies to Asx, trx and Pc, could be used for three separate co-immunoprecipitation experiments. Co-IP experiments have been done with ph and Pc antibodies (Franke et al. 1992) and an immunoprecipitation with a ph antibody purifies a

soluble complex with a band the size of Asx (J. Hodgson, H, Brock unpublished). This suggest that PcG proteins can interact as soluble complexes. If Asx is a member of two separate complexes, then one would expect that Asx would copurify with both Pc and trx but that Pc would not be present in the trx co-IP and vice versa. If Pc, trx and Asx were to all co-purify together this would be strong evidence that these three proteins interact *in vivo* and that Pc and trx are members of complexes that interact directly with each other. However, if Asx protein interacts with both Pc and trx containing complexes only after they have bound to DNA, it may not copurify with soluble complexes.

To deal with this possibility, one could map the position of Asx containing complexes at a regulatory target and compare the position of Asx containing complexes with other PcG and trxG proteins. An antibody to ph has been used in a supershift assay to identify ph containing complexes bound to the bxd5.1 minimal PRE (Hodgson and Brock 1998). It seems likely that Pc will be present in most of these ph containing complexes, but it would be interesting to see if Asx and trx proteins overlap with any or all of these protein complexes. If Asx containing complexes are completely separate from Pc containing complexes but are found present in trx containing complexes (or vice versa), this might suggest that *in vivo* , the bound complexes directly interact with each other and thus are able to alter chromatin structure at a target locus.

The fact that Asx can bind to multiple target sites without other PcG proteins present suggests that it may be a member of different functional complexes. The supershift assay could be used to screen for Asx complexes bound to different PREs and then specific complexes could be purified using DNA affinity chromatography. It would be interesting to purify separate Asx complexes from the PREs of different homeotic gene targets and determine if there are different specific constituents at each target. This would be one way to identify possible factors involved in target specific activity. The purified proteins could be used to raise antibodies and then screen an expression library to identify new genes

involved in the regulation of specific targets. It would be interesting to see if z40 was present only at specific targets.

The tissue specific requirements for *Asx* activity suggests that Asx may interact with tissue specific enhancer elements at target loci. An enhancer element that has a CNS specific *Ubx* expression pattern was identified (Christen and Bienz 1992) but no other tissue specific enhancers are known. Thus another important use of mapping Asx containing complexes would be to see if they map to any specific regulatory sites that do not contain other PcG protein complexes. This may identify tissue specific elements and purification of any bound constituents may reveal what factors account for the tissue specific activity of *Asx*.

Conclusion

Traditional models of PcG function have depended largely on the fact that PcG proteins were distinct components of repressive complexes and that trxG proteins were distinct components of activation complexes. Both genetic and molecular data now suggest that this simple view is inaccurate and that repression and activation may require some level of cooperative activity between the two sets of proteins.

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