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Related chromosome binding sites for *zeste*, suppressors of *zeste* and *Polycomb* group proteins in *Drosophila* and their dependence on *Enhancer of zeste* function

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Polycomb **group genes are necessary for maintaining homeotic genes repressed in appropriate parts of the body plan. Some of these genes, e.g.** ***Psc, Su(z)2*** **and** ***E(z)*****, are also modifiers of the** ***zeste-white*** **interaction. The products of** ***Psc*** **and** ***Su(z)2*** **were immunohistochemically detected at 80-90 sites on polytene chromosomes. The chromosomal binding sites of these two proteins were compared with those of** ***zeste*** **protein and two other** ***Polycomb*** **group proteins,** ***Polycomb*** **and** ***polyhomeotic*****. The five proteins co-localize at a large number of sites, suggesting that they frequently act together on target genes. In larvae carrying a temperature sensitive mutation in another** ***Polycomb*** **group gene,** ***E(z)*****, the** ***Su(z)2*** **and** ***Psc*** **products become dissociated from chromatin at non-permissive temperatures from most but not all sites, while the binding of the** ***zeste*** **protein is unaffected. The polytene chromosomes in these mutant larvae acquire a decondensed appearance, frequently losing characteristic constrictions. These results suggest that the binding of at least some** ***Polycomb*** **group proteins requires interactions with other members of the group and, although** ***zeste*** **can bind independently, its repressive effect on** ***white*** **involves the presence of at least some of the** ***Polycomb*** **group proteins.**

Key words: chromatin assembly/homeotic genes/maintenance of expression/repression

Introduction

Many developmentally important genes have complex and extensive regulatory regions through which they receive multiple inputs and regulate their expression in different regions, tissues or structures of the developing organism. Typical examples may be found among the segmentation genes which establish the metameric organization by responding to maternal cues and to the rapidly changing expression pattern of the other segmentation genes. Once established, the metameric pattern is maintained and affects the expression of many other genes throughout later development even when the initial cues that originally informed the pattern have faded away. Homeotic genes, such as the members of the Antennapedia (ANT-C) or Bithorax (BX-C) complexes, although initially activated in response to the transient expression pattern of the segmentation genes, must maintain their expression through later development with the appropriate distribution both among and within segments. Genetic and molecular evidence has shown that the products of the gap genes *hunchback* and *tailless* repress the activation of *Ubx*, thus setting the anterior and posterior boundaries, respectively, of its domain of expression (White and Lehmann, 1986; Qian *et al.*, 1991). However, after gastrulation, gap gene function fades off but the homeotic genes continue to be required and to be expressed in their appropriate domains throughout development. Therefore, a new mechanism must take over to maintain the homeotic domains of expression along the anterior-posterior axis.

A set of genes, of which *Polycomb* (*Pc*) is the prototypical member, appears to be responsible for this maintenance (Lewis, 1978; Denell and Frederick, 1983; Jürgens, 1985) as well as for the maintenance of the expression pattern of *engrailed* and other segmentation genes (Dura and Ingham, 1988; Heemskerk *et al.*, 1991). The *Pc* group of genes has probably many other targets since mutations in the group can also alter dorso-ventral patterning and the development of the central and peripheral nervous system (Smouse *et al.*, 1988; Adler *et al.*, 1991). Genes of the *Pc* group are not required to establish the initial pattern of *Antp* or *Ubx* expression, but in the absence or insufficiency of one or more member of the group, the homeotic genes begin to be expressed outside their normal domain, as embryonic development proceeds (Struhl and Akam, 1985; Wedeen *et al.*, 1986; Dura and Ingham, 1988; Simon *et al.*, 1992). Although individual members of the *Pc* group may also have specific effects not shared by the other members, they appear to act together in regulating homeotic genes. In several cases loss of function or decreased function of one member enhances loss-of-function phenotypes of another while duplications of one member may compensate for the loss of activity of another (Jürgens, 1985; Kennison and Russell, 1987). The number of genes involved, their mutual genetic interactions and the dosage dependence have suggested that they might assemble in multimeric complexes to fold the chromatin of target genes in a condensed and transcriptionally inactive form, much as the suppressor of variegation genes are thought to condense heterochromatin (Locke *et al.*, 1988). Such a complex would form only at target genes that are not actively transcribed, thus locking them in an inert form in those cells in which they had originally been repressed. In consequence, the *Pc* group genes would serve to maintain a transcriptional pattern of activity long after the activity of the initial regulators has subsided, after gastrulation. Paro (1990) has proposed that they would thus imprint a determined state of the chromatin that can be inherited by the cellular progeny.

The *zeste-white* interaction is another genetic phenomenon, which at first sight has little to do with the regulation of homeotic genes. Here, a particular allele of the *zeste* gene, z^1, suppresses the expression of the *white* gene in the eye of the fly in a way dependent on the proximity of two copies of the *white* gene such as is produced by the pairing of two homologous chromosomes (Gans, 1953; Jack and Judd, 1979; reviewed by Pirrotta, 1991). The function

of the *zeste* gene is associated with transvection effects at the *Ubx* gene and other loci (Kaufman *et al.*, 1973; Gelbart and Wu, 1982), but its interaction with *white* seems to be a phenomenon distinct from its role in transvection. The product of the *zeste* gene binds *in vitro* to the regulatory regions of many genes such as *white*, *Ubx*, *decapentaplegic*, *Antp* (Benson and Pirrotta, 1988). In salivary gland chromosomes it is found associated with more than 60 sites, including the two major homeotic complexes (Pirrotta *et al.*, 1988). Its role in normal gene expression is not fully clear although, when bound near a promoter, it stimulates its activity both *in vitro* and *in vivo* (Biggin *et al.*, 1988; Laney and Biggin, 1992; P.Miller and V.Pirrotta, unpublished) and it has been proposed that, by binding at distant regulatory elements, it facilitates their looping to contact the promoter complex. Its ability to assemble into large multimeric complexes is essential for its interaction with the *white* gene and for transvection promoting activity (Bickel and Pirrotta, 1990; Chen *et al.*, 1992). Mutations in a number of genes can either suppress or enhance the *zeste*-mediated repression of *white*, with the same locus frequently able to generate both suppressing and enhancing alleles. Surprisingly, at least three of these *modifer of zeste* loci, *Psc*, *E(z)/pco* and *Su(z)302/Scm*, have been found also to be members of the *Pc* group. A fourth locus, *Su(z)2*, although not strictly a *Pc* group gene, seems functionally related to at least some members of the group because it interacts with *Psc* and *Scm* mutations enhancing or failing to complement their phenotypes (Adler *et al.*, 1989; Wu *et al.*, 1989). These observations suggest that the repression of *white* by the z^1 product is molecularly related to the maintenance of repression mediated by the *Pc* genes and probably requires some of the *Pc* gene products.

Three members of the *Pc* group have been cloned and sequenced. *Pc* itself is a relatively small protein of 390 amino acids that bears sequence similarity to the heterochromatin protein HP1, the product of the *Su(var)205* gene (Eissenberg *et al.*, 1990; Paro and Hogness, 1991). The *polyhomeotic* (*ph*) product is a large protein (169 kDa) containing runs of repeated amino acids and a zinc finger motif, suggesting that it might interact directly with DNA (DeCamillus *et al.*, 1992). Immunolocalization of the *Pc* and *ph* proteins on the salivary gland polytenic chromosomes has shown that they are found at approximately 100 cytological locations with a complete coincidence between the two proteins (Franke *et al.*, 1992). Among the sites with which these proteins are associated are the cytological locations of the two major homeotic complexes, ANT-C and BX-C, as well as the sites of several members of the *Pc* group themselves (Zink and Paro, 1989; DeCamillis *et al.*, 1992). In the work presented here we have used the same approach to map the chromosomal sites at which the *Su(z)2* and *Psc* proteins are found in the salivary gland chromosomes. The *Psc* and *Su(z)2* genes map adjacently to one another and have also been cloned recently (Brunk *et al.*, 1991a; Å.Rasmuson and V.Pirrotta, unpublished). Both encode large proteins (170 and 149 kDa respectively) with sequence resemblance to one another and to a class of vertebrate nuclear factors such as the *bmi-1* proto-oncogene (van Lohuizen *et al.*, 1991; Brunk *et al.*, 1991a). The region of homology contains a cysteine-rich motif that resembles the zinc fingers of other DNA-binding proteins. We show that the *Su(z)2* and *Psc* products are nuclear proteins associated with more than 80 sites in salivary gland polytene chromosomes. We have compared these sites with those of the *Pc* and *ph* proteins and with sites at which the *zeste* protein is found. We have also shown that the association of the *Su(z)2* and *Psc* proteins with the chromosomes is dependent on the presence of active *E(z)* protein.

Results

Immunodetection of Su(z)2 and Psc products

We cloned the genomic region containing the *Su(z)2* and *Psc* genes starting with a genomic clone from the *vestigial* region (Williams and Bell, 1988). This clone, which contains the proximal breakpoint of the $Su(z)2^5$ deficiency, allowed us to jump into the *Su(z)2–Psc* region (Å.Rasmuson, C.-S.Chan and V.Pirrotta, unpublished results). However, for the *Su(z)2* and *Psc* cDNA clones used in this work, we are indebted to P.Adler in whose laboratory they were isolated. We used fragments from the protein coding region of the corresponding cDNAs to generate β-galactosidase fusion proteins with which to raise antibodies. Since the two

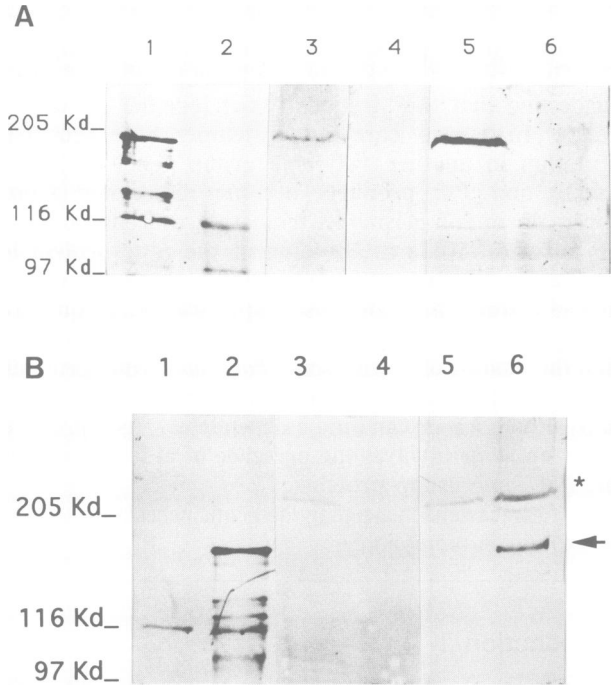

Fig. 1. Western blot detection of *Psc* and *Su(z)2* products. (**A**) A composite of a filter stained with anti-*Psc* antibody. Lane 1: extract of bacteria expressing *Psc*; lane 2: extract of bacteria expressing *Su(z)2*; both blotted and stained with anti-*Psc* antibody. The antibody detects the *Psc* band and a multitude of lower bands corresponding to degradation products and β-galactosidase (lane 1). The only band detected in extracts expressing *Su(z)2* protein is β-galactosidase (lane 2). Lanes 3 and 4 contained extracts from wild type embryos and embryos carrying a deletion of the *Psc* gene ($Psc^{Arp\text{-}1}$), respectively. Lanes 5 and 5 contained extracts from heat shocked flies carrying respectively the *hsp70–Psc* and the *hsp70–Su(z)2* constructs. Both lanes were blotted and stained for *Psc*. (**B**) A similar set of lanes containing similar extracts except that the embryonic extracts in lane 4 were from a stock carrying the $Su(z)2^{Arp\text{-}1}$ deletion and the blotted filters were stained with anti-*Su(z)2* antibody. The arrow indicates the heat shock-induced band of *Su(z)2* protein and the asterisk shows the endogenous band detected by the antibody. The gels were run in parallel for equal distances and aligned using molecular weight standards.

proteins contain a region of homology (Brunk *et al.*, 1991a; van Lohuizen *et al.*, 1991), we selected fragments from non-homologous regions of the coding sequence to avoid the possibility of cross-reactivity. Figure 1 shows that the affinity-purified antisera detect proteins of the expected molecular weights [180 kDa for *Psc* and 150 kDa for *Su(z)2*] in extracts from bacteria expressing full length *Su(z)2* or *Psc* cDNAs. The numerous faster migrating bands are most probably degradation products since they are absent in extracts from uninduced bacteria or bacterial hosts lacking the *Su(z)2* or *Psc* genes. To compare the bacterial products with those made in the fly, we analyzed extracts from wild type flies containing the *Su(z)2* or *Psc* cDNAs expressed under the control of the *hsp70* promoter. After heat induction, both transformed fly lines produce a protein detected in Western blots by the corresponding antibody (Figure 1). The band seen in hs-Psc flies after induction corresponds in molecular weight to the *Psc* protein expressed in bacteria and is consistent with the molecular weight predicted by the sequence. The antibody does not detect a corresponding band in flies expressing the *hs-Su(z)* transposon, suggesting that the endogenous *Psc* product is too weak or not expressed in adult flies. In wild type embryos, on the other hand, the anti-*Psc* antibody detects a band of the same mobility as the hs-Psc or the bacterially expressed *Psc*, which is absent in extracts from embryos carrying a deficiency of the *Psc* gene. The anti-*Su(z)* antibody also detects a new band in induced flies containing the *hs-Su(z)* transposon but in this case another, higher molecular weight band is also detected, which is also present in flies lacking the transposon. This band is also seen in embryonic extracts but is absent in extracts of embryos carrying a deficiency of the *Su(z)2* gene, indicating that it is most probably the product of the endogenous gene. We conclude from these results that the antibodies recognize their respective proteins without cross-reaction between *Su(z)2* and *Psc* and that they detect no other important component (although faint additional bands are visible in the adult fly extracts). The molecular weight discrepancy in the case of *Su(z)2* suggests that the protein produced by the endogenous gene is 30–40 kDa larger than that encoded by the cloned gene. This is quite possible, since the *Su(z)2* cDNA, isolated by Brunk *et al.* (1991a), is not full length and may lack as much as 700–1000 nucleotides from the 5′ end, when compared with the size of the mRNA detected in Northern blots.

Immunolocalization of Su(z)2 and Psc proteins

Both anti-*Su(z)2* and anti-*Psc* antibodies stain the nuclei of a wide variety of tissues in embryos and larvae. In particular, the polytenic nuclei of salivary glands stain prominently in larvae at the wandering stage. The staining begins to fade and one nucleus after another turns off as the larvae approach pupariation. By the time the spiracles are everted, only sporadic nuclei in the gland body still stain in a characteristic all-or-none fashion but staining persists in the neck of the glands where virtually all the nuclei stain strongly (see Figure 6). Heat shock induction of the *hsp70–Psc* or *hsp70–Su(z)2* constructs results in intense and widespread staining irrespective of stage (not shown).

To determine if the *Su(z)2* and *Psc* proteins are associated with specific chromosome sites, we used the affinity purified antibodies to stain salivary gland polytene chromosomes. Figure 2 shows that both proteins are found at a large number of specific cytological sites, between 80 and 90 strong sites, of which 51 are shared by the two proteins. A number of more weakly staining sites are also sporadically detected. As expected from the staining of the whole glands, not all nuclei stain and the intensity of the staining varies from one nucleus to the next, however, among the better stained nuclei, the pattern of the major bands was very consistent and reproducible. For comparison we also stained chromosomes with antibodies directed against *zeste* product. All three antibodies stain primarily sites in the euchromatic arms with little detectable staining in the chromocenter and only two or three bands staining on the partly heterochromatic fourth chromosome. This preference for euchromatin persists even when the proteins are overproduced by heat shock induction of larvae carrying the *hsp70* promoter constructs. Overproduction of the *Su(z)2* and *Psc* proteins,

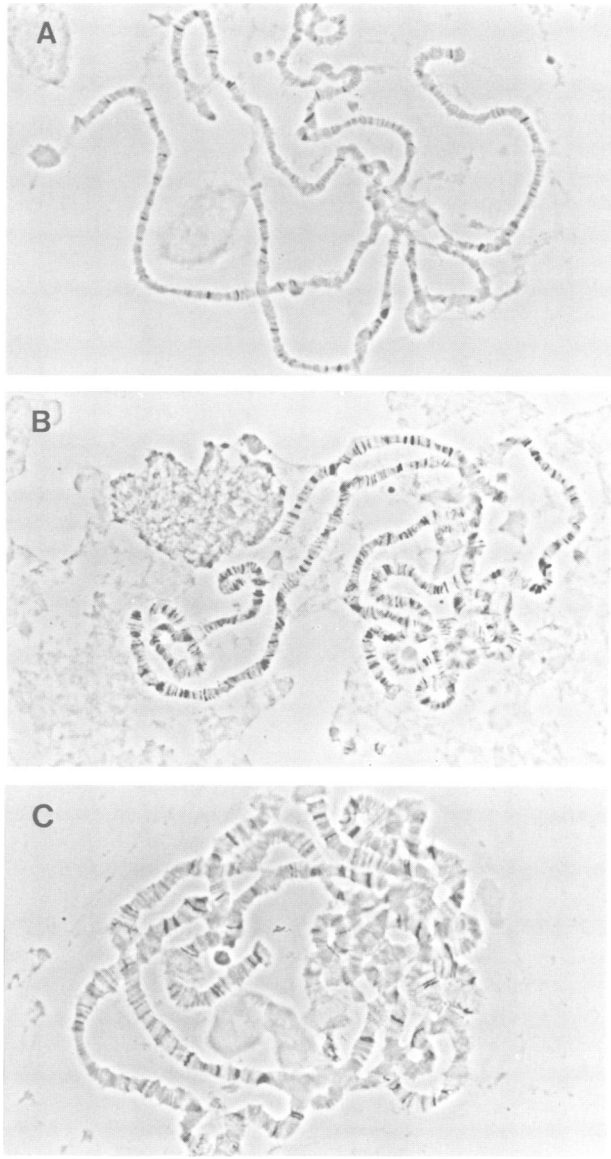

Fig. 2. Immunohistochemical localization of *Psc*, *Su(z)2* and *zeste* proteins on polytene chromosomes. Chromosome spreads from wild type larvae were stained with the appropriate affinity-purified antibody, using nickel–cobalt enhanced peroxidase staining. (**A**) anti-*Psc* antibody; (**B**) anti-*Su(z)2* antibody; (**C**) anti-*zeste* antibody.

Table I. Polytene chromosome binding sites for *Psc*, *Su(z)2*, *ph*, *Pc* and *zeste* proteins

Chromosome 1		Chromosome 2		Chromosome 3	
1A	*Su, Psc, ph, Pc*	21AB	*Su, Psc, ph, Pc*	61A	*Psc (ph,Pc)*
1C/D	*z*	21C	*Su z*	61C	*Su, ph, Pc*
1F-2A	*Su*	22A	*Su, Psc, ph, Pc*	61D	*Su (ph,Pc)*
2D	*Psc, ph, Pc*	22B	*Su, Psc, ph, Pc, z*	61F	*Su, Psc, ph, Pc*
3A	*Su z*	22C	*ph, Pc*	62B	*Su z*
3CD	*Su, z*	22F/23A	*Su, Psc z*	62F	*Su, Psc, ph, Pc*
4C	*Su, Psc, ph, Pc, z*	24A	*Su, Psc, ph, Pc, z*	63F-64A	*Su, ph, Pc, z*
5A	*Su, Psc, ph, Pc, z*	25D	*Su*	64BC	*Psc*
5D	*ph, Pc*	25E/F	*Su, Psc, ph, Pc, z*	64E	*Su*
7B	*Su, Psc, ph, Pc, z*	26A	*Su, Psc, z*	65D	*ph, Pc*
8A	*Su, Psc, ph, Pc, z*	26F/27A	*Su, Psc, ph, Pc, z*	65E	*Su*
8B	*Su ph, Pc*	27B	*z*	66A	*Su, Psc*
8F/9A	*Psc, ph, Pc*	28A	*ph, Pc*	66C	*Su*
9E	*Su, Psc z*	28D	*Su*	66EF	*Psc, ph, Pc*
11AB	*Su, Psc*	28F/29A	*Su z*	67C	*Su*
12D	*Su ph, Pc z*	29E	*Su, Psc, ph, Pc*	67D	*Psc, ph, Pc, z*
13E1	*(ph, Pc)*	30B	*Su, (ph,Pc)*	67EF	*Su, Psc z*
14B	*Su, Psc, ph, Pc*	30C	*ph, Pc*	68A	*Su, Psc, ph, Pc*
16D	*Psc, ph, Pc*	32EF	*Psc, ph, Pc*	68C	*Su, z*
17A	*Su, Psc, ph, Pc, z*	33B	*ph, Pc*	69C	*Su, Psc, ph, Pc*
17E1,2	*ph, Pc*	33F/34A	*Su, Psc, ph, Pc*	69D	*Su, Psc, ph, Pc, z*
17F	*ph, Pc*	34C	*(ph,Pc)*	70A/B	*Psc, ph, Pc z*
18A	*Su*	34D	*(ph,Pc)*	70C	*z*
18D	*Su, Psc z*	35AB	*Su, Psc, ph, Pc*	70DE	*Su, Psc, ph, Pc*
19D	*ph, Pc*	35D	*ph, Pc*	71F	*Su, Psc*
		36A	*Su, ph, Pc*	72F	*Su, Psc z*
Chromosome 4		36B	*Su, Psc, ph, Pc, z*	73C	*z*
		37A	*Psc*	73F-74A	*Psc*
102B	*Su*	37B	*ph, Pc*	75C	*Su*
102C	*Psc, ph, Pc*	38F	*Su, Psc, ph, Pc*	76C	*Psc, ph, Pc*
102D	*Psc, ph, Pc*	39E/F	*ph, PC*	77E	*Psc (ph,Pc)*
		41CD	*ph, Pc*	78A	*Su*
		42A	*Su, Psc z*	78E/F	*Psc, ph, Pc*
		43A	*Su z*	79B	*Psc (ph,Pc) z*
		43BC	*Psc, ph, Pc*	81F-82A	*Su (Psc)*
		44A	*Su, Psc, ph, Pc*	82D/E	*Psc, ph, Pc, z*
		45C	*Psc, ph, Pc, z*	83C	*ph, Pc*
		46C	*Psc, ph, Pc z*	84A/B	*Su, Psc, ph, Pc, z**
		47AB	*Su, Psc z*	84D	*Su, Psc, ph, Pc, z*
		47E	*z*	84EF	*Su, Psc, ph, Pc*
		48A	*Psc, ph, Pc, z*	85A	*z*
		48C	*Su*	85D	*Su z*
		49EF	*Su, Psc, ph, Pc, z**	85E	*ph, Pc*
		50C	*Su, Psc z*	86A	*z*
		51A	*Psc, ph, Pc*	86C	*Su, Psc, ph, Pc*
		51D	*ph, Pc*	87B	*Su, Psc, (ph,Pc) z*
		53C	*z*	87B/C	*Su ph, Pc, z*
		54A	*z*	87F-88A	*Su, Psc, ph, Pc*
		55A	*z*	89B	*Psc, ph, Pc*
		55D/E	*Su z*	89C	*Su, Psc, ph, Pc, z*
		56C	*Su, Psc, ph, Pc, z**	89E	*Su, Psc, ph, Pc, z*
		56E/F	*z*	90D	*Su,*
		57A	*ph, Pc*	90E	*Psc, ph, Pc*
		57B	*Su ph, Pc*	92A	*(Psc) z*
		58C/D	*Su ph, Pc, z**	92F	*Su, z*
		58F	*Psc*	93E	*Su ph, Pc z*
		59A	*Su ph, Pc*	94D/E	*Psc, ph, Pc z*
		59C	*ph, Pc*	96A	*Su z*
		59F	*Psc, ph, Pc*	96B/C	*Su, Psc, ph, Pc, z*
		60C/D	*Su, Psc*	96F-97A1 3 bands for	*ph, Pc*
		60E	*Su, Psc, ph, Pc, z*	98C/D	*ph, Pc*
		60F	*(ph,Pc)*	99AD	*Su, Psc, ph, Pc, z**
				99f	*Su, z*
				100A	*Psc, ph, Pc*
				100F	*(Psc) ph, Pc*

like overproduction of the *zeste* protein (Pirrotta *et al.*, 1988), results in a massive increase in the number and intensity of bands detected compared with uninduced controls or to chromosomes from wild type larvae with or without heat shock treatment. Overproduction of one protein did not alter the pattern of bands staining for another protein, indicating that the heat treatment does not disrupt the binding and that ectopic binding of one protein does not induce new binding sites for another. These results show that the antibodies detect the *Su(z)2* and *Psc* proteins respectively, but we cannot be sure that they detect no other proteins. No other significant component was detected in Western blots of embryonic extracts and, at least in the case of *Psc*, the same results were obtained with two different preparations of antibodies, one raised in rabbit and one in rat.

It is apparent from a cursory examination that there is considerable overlap in the distribution of endogenous proteins detected by the three antibodies. The known genetic interactions of *Su(z)2* and *Psc* with *zeste* on the one hand and with the *Pc* group genes on the other hand raised the possibility that these proteins might be acting together at least at some of their target loci. We therefore tabulated the sites stained by each of our three antibodies and compared them with the sites reported for *Pc* and *ph* antibodies (Zink and Paro, 1989; DeCamillis *et al.*, 1992; Franke *et al.*, 1992). Table I summarizes our results, collated for each protein from many nuclei both within one gland and from different glands. Each band was identified on the basis of a large number of observations, dozens for the easily recognized ones but at least five for those harder to characterize. To improve detection and to distinguish chromosomal bands from staining bands, we counterstained the chromosomes with DAPI, which labels the chromosomes and whose fluorescence is strongly quenched by even light staining with peroxidase (Karr and Kornberg, 1989). Overall, the five proteins have a very similar, though not identical distribution. Franke *et al.* (1992) have reported a complete correspondence between the *Pc* and *ph* sites. Of the 83 *Psc* sites, 63 coincide in appearance with the *Pc–ph* sites. *Su(z)2* shares 52 sites out of 89 with *Psc* and 48 with *Pc–ph*. The *zeste* distribution resembles more that of *Su(z)2* (47 sites in common) but *zeste* also has 38 sites in common with *Psc* and 32 with *Pc–ph*. The comparison of the *zeste*, *Psc* and *Su(z)2* binding sites with those of the *Pc* and *ph* proteins would gain value if chromosomes could be stained in parallel for the different proteins. Unfortunately, at the time we were not able to obtain antibodies against *Pc* or *ph* proteins. Direct comparison of the distribution of our three proteins revealed that in some cases the stained sites, although very close, may be distinguishable. For example, at 84A/B, the site of the *Antennapedia* homeotic complex, staining for *Psc* produces two very close bands at 84A4,5 and 84B1,2, *Su(z)2* gives a band at 84B1,2 and *zeste* gives one band at 84B3-6 (Figure 3 and data not shown).

The precision of localization varies with the chromosomal site. Although in Table I we have specified the position only

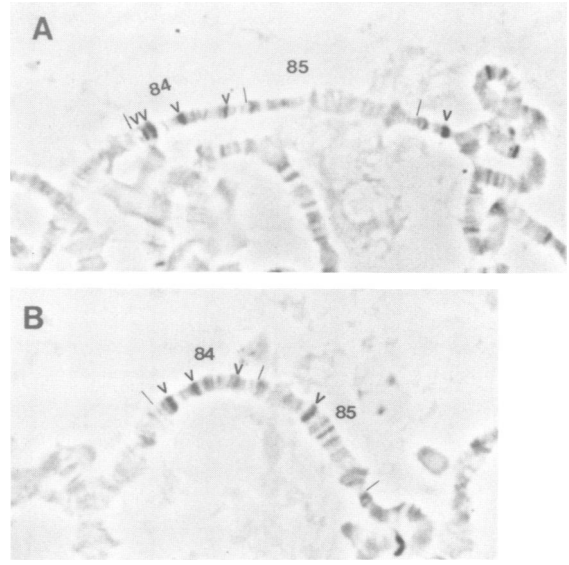

Fig. 3. Binding sites for *Psc* and *Su(z)2* in the proximal region of chromosome 3R. (**A**) A chromosome stained for *Psc* and (**B**) a chromosome stained for *Su(z)2*. The binding sites for the two proteins are very similar except for an extra *Psc* band at 84A in the Antennapedia complex and an extra *Su(z)2* band at 85D.

to a letter division, the resolution is usually considerably better than this. In immunostained preparations we estimate that it is on the average ~100 kb, in agreement with the experience of others (Spierer *et al.*, 1983; De Camillis *et al.*, 1992). What is the significance of the overlap in distribution of the five different proteins? Conservatively, we can distinguish at least one site per letter division of the polytene chromosomes or 600 total sites. If a given protein occupies 80 sites, any chromosomal site has an average probability of 80/600 of being occupied. The probability of a given site being occupied by two different proteins by chance alone would be the product of the two or ~10/600. We observe instead overlaps of 40 or more for any two proteins. Furthermore, the fact that a large number of overlaps are common to all five proteins puts the coincidence well beyond the realm of chance. However, two apparently coincident cytological binding sites might be separated by as much as 100–200 kb, depending on the cytology of the region.

Inactivation of E(z) product

That *zeste*, *Su(z)2* and *Psc* proteins should share at least some of the chromosomal binding sites is not surprising since certain *Su(z)2* and *Psc* mutations suppress or enhance the *zeste–white* interaction. In principle this interaction could be achieved in different ways. *Su(z)2* and *Psc* might, for example, regulate *zeste* or some other gene whose product is necessary for *zeste*-mediated interactions. The fact that many chromosomal sites reveal apparently coincident *zeste*, *Su(z)2* and *Psc* bands suggests that, more likely, these three proteins may act together. Current models for the action of

The data for *Psc*, *Su(z)2* and *zeste* were derived from more than 10 identifications for most of the sites and at least five for some of the more difficult regions. The *Pc* and *ph* data are derived from Zink and Paro (1989) and from DeCamillis *et al.* (1992), who provided a revised list of sites for the two proteins. In addition, we have used an unpublished list of sites from Zink and Paro (personal communication). The asterisk marking some *zeste* binding sites indicates that although the stained bands are in the cytological division listed, they clearly do not co-localize precisely with the *Su(z)2* and *Psc* bands at that site. Sites listed in parentheses are weaker staining sites.

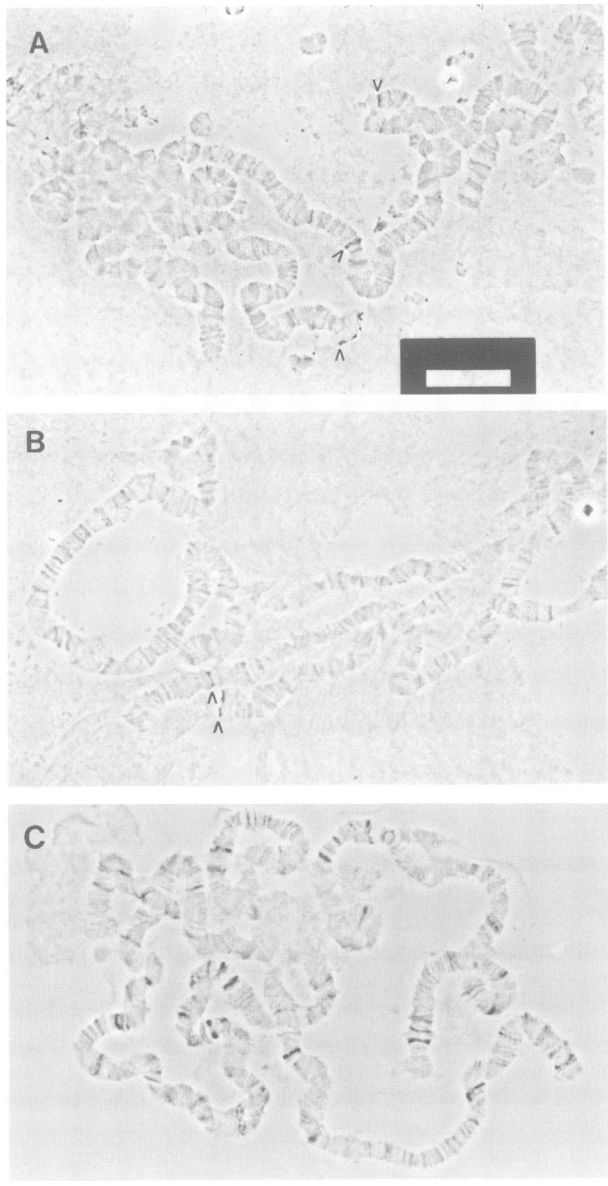

Fig. 4. Effect of $E(z)^{S2}$ on polytene chromosomes. The *Psc*, *Su(z)2* and *zeste* proteins were localized on polytene chromosomes prepared from homozygous $E(z)^{S2}$ larvae raised at 29°C. Only a few *Psc* bands (**A**) or *Su(z)2* bands (**B**) remain (indicated by arrowheads) while the *zeste* band pattern (**C**) is not appreciably altered. Note the characteristic flat, low contrast and swollen appearance of the chromosomes compared to those in Figure 2. The white bar in the black frame represents 2 μm.

the *Pc* group genes also envision the assembly of a chromatin structure involving many gene products and Franke *et al.* (1992) have already shown that the *Pc* and *ph* products complex together before binding to the chromatin. The absence of one gene product might then prevent the correct assembly of the entire structure. The *Enhancer of zeste* or *E(z)* locus is another modifier of *zeste* which, like *Psc*, is also a member of the *Pc* group. We have looked at chromosomes from larvae heterozygous for *Pc* or *E(z)* mutations to see if insufficiency of these gene products had a visible effect on the banding pattern for our three proteins. Although flies heterozygous for these mutations show phenotypic effects, we could not detect substantial alterations in the number or intensity of immunostaining bands (not shown). Complete loss of *Pc* or *E(z)* function is generally

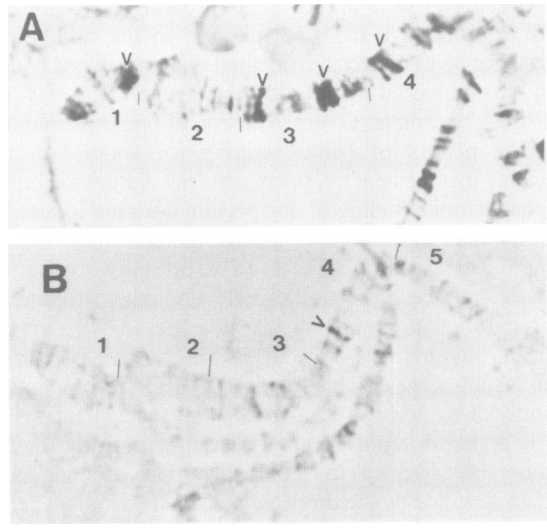

Fig. 5. Comparison of *Su(z)2* bands in wild type and $E(z)^{S2}$ chromosomes. The tip of the X chromosome from wild type larvae (**A**) shows four staining sites for *Su(z)2* protein only one of which remains in the same region from $E(z)^{S2}$ larvae raised at 29°C (**B**). Note that the characteristic constrictions at 3C and after the 2B puff are absent from the $E(z)^{S2}$ chromosome.

lethal, although in the case of *E(z)* the maternally supplied product is apparently sufficient to permit survival of homozygous mutants until early pupal stages (Jones and Gelbart, 1990). A simple way to remove both zygotic and maternal *E(z)* product at any time during development is to use $E(z)^{S2}$, a temperature-sensitive loss-of-function allele (Jones and Gelbart, 1990). Embryos homozygous for this mutation were collected at 23°C for 48 h, then raised at 29°C until the third instar stage. Under these conditions, the polytene chromosomes have a strikingly altered appearance (Figure 4). They are larger, thicker, puffier and with very low contrast, as if affected by a general decondensation; the cytology is abnormal, the Bridges bands become fainter and more difficult to identify and many of the characteristic constrictions are absent, including that typically found at the Bithorax locus.

Immunostaining of these chromosomes for the three different proteins leads to different results: while the *zeste* bands are present and apparently normal, both the number and intensity of the *Su(z)2* and *Psc* bands are greatly reduced (Figure 4). This is not a non-specific effect of elevated temperature since wild type larvae treated in parallel or $E(z)^{S2}$ larvae raised at room temperature stain normally for all three proteins and their chromosomes have a normal appearance. Moreover, the differential effect on heat treated $E(z)^{S2}$ glands was shown by mixing them with heat treated glands from wild type larvae on the same microscope slide. The $E(z)^{S2}$ chromosomes are always easily identified by their appearance and they always show loss of *Su(z)2* and *Psc* staining but not of *zeste* staining. The loss of staining is not due to a general weakening of all signals. If this were the case, we would expect that the weaker signals would be preferentially affected. Instead, diluting the anti-*zeste* antibody causes a parallel weakening and loss of staining in both wild type and $E(z)^{S2}$ chromosomes. The specificity of the effect is also indicated by the fact that some of the bands that disappear are normally among the strong ones, while some of those that remain are normally among the

weaker ones (Figure 5). About 10 sites for each protein retain staining in the $E(z)^{S2}$ chromosomes. Although the altered appearance of the chromosomes makes the cytological identification difficult, we were able to determine that many of the remaining sites are common to both proteins, for example 4C, 8A, 21A, 49EF (the locus of the *Psc* and *Su(z)2* genes), 69CD and 84AB (the site of the ANT-C). In at least some cases, however, one of the two proteins remained but not the other. Most of the identifiable sites that persist in $E(z)^{S2}$ chromosomes seem to be sites common to all five of our proteins. Thanks to a small amount of anti-*ph* antibody provided to us by Dr H.Brock we were able to determine that *ph* protein is also lost from the majority of sites in $E(z)^{S2}$ chromosomes but persists in a few cases. We were not able, however, to carry out a sufficient number of experiments to identify these sites.

Lack of functional *E(z)* protein does not cause the loss of *Su(z)2* and *Psc* proteins but only their dissociation from the chromatin. Figure 6 shows that when whole salivary glands from heat treated $E(z)^{S2}$ larvae are stained for *Su(z)2* and *Psc* the proteins are still detectable and localized in the nuclei but are not associated with the chromatin. At this level the only visible consequence of the heat treatment is to eliminate the difference in the distribution of *Psc* staining between early and late third instar larve. Instead of fading off as the larvae begin to pupate, remaining only in sporadic nuclei, *Psc* stain continues to be found in all nuclei, suggesting that the lack of *E(z)* function results in deregulation of the *Psc* gene or perhaps in increased stability of the *Psc* protein. In contrast, *Su(z)2* expression continues to be turned off at later stages even in the absence of *E(z)* function.

The loss of *Psc* and *Su(z)2* proteins from the chromosomes occurs progressively within a relatively short time of inactivation of *E(z)*. We raised $E(z)^{S2}$ larvae at 22°C, then raised the temperature and stained their chromosomes after different lengths of heat treatment. After 1 h at 34°C the appearance of the chromosomes is still fairly normal but the number and intensity of the *Su(z)2* and *Psc* bands is noticeably decreased. The disappearance of the bands progresses with longer treatment and by 2.5 h the staining pattern becomes similar to that of larvae raised at non-permissive temperature. The appearance of the chromosomes becomes puffier but even after 2.5 h does not reach the degree of decondensation visible in most chromosomes of larvae raised at high temperature (data not shown). These results suggest that the loss of *Su(z)2* and *Psc* proteins from the chromosomes begins very soon after inactivation of *E(z)* product but is gradual rather than sudden and complete. Though we cannot rule out the possibility that the loss of *E(z)* function induces the expression of a protein that causes the dissociation of *Psc* and *Su(z)2* products from the chromatin, we interpret these results to mean that a multiprotein complex depends on *E(z)* function for assembly or stability. The slow loss of bands indicates either that the complex, once formed, retains some stability when *E(z)* is removed or that, as Jones and Gelbart (1990) concluded, the $E(z)^{S2}$ product is not completely inactivated at high temperature.

Discussion

The phenotypic effects of *Su(z)2* and *Psc* mutations and the amino acid sequence homology between the two proteins and a class of DNA-binding nuclear factors led us to expect that they would be found associated with chromatin. Our results show that the *Su(z)2* and *Psc* products are in fact localized in a discrete number of specific sites on the polytene chromosomes. The sites are generally reproducible but different nuclei in the same preparation show great variability in staining, consistent with the observation that in whole salivary glands some isolated nuclei stain strongly and are surrounded by nuclei that do not stain appreciably. Since in earlier third instar larvae the salivary gland nuclei are uniformly stained, we suppose instead that the expression of the two genes is in a state of transition more advanced in some nuclei than in others and may be subject to a feedback mechanism that results in a sharp on−off threshold. The fact that the two proteins disappear from salivary gland nuclei after pupation also raises the question of whether *Pc* group proteins are continuously needed to maintain homeotic genes repressed. It is possible that regulation no longer requires the *Psc* product. During pupation, the body of the gland is histolysed and the adult organ is regenerated from a ring of imaginal tissue in the neck of the gland. It could be argued that expression of homeotic genes in the cells of the gland body becomes irrelevant but there is no indication that these genes become derepressed in the salivary glands at this stage.

The list in Table I accounts for the stronger and more reproducible sites staining for *zeste*, *Su(z)2* and *Psc*. A multitude of more weakly binding sites undoubtedly exist and may be responsible for the myriad of bands revealed when the proteins are overproduced (see also Pirrotta *et al.*, 1988). In addition, it is important to note that some chromosomal sites may not be accessible in salivary glands, as suggested by the fact that the *white* locus, a biological target of *zeste* protein, is not one of the sites stained by anti-*zeste* antibody in salivary glands (Pirrotta *et al.*, 1988). The sites listed in Table I represent therefore a minimum number rather than an exhaustive compilation of potential target sites. The genes known to map at the cytological sites to which these proteins are bound are a rather heterogeneous group. Prominent among them are homeotic complexes, both because they are sites of broad and intense staining and because of the known genetic interactions with members of the *Pc* group. Their loci are binding sites for all five proteins, although in the case of the ANT-C the *zeste* binding site is clearly distinguishable from the binding sites of *Su(z)2* and *Psc*. A number of other homeobox-containing genes are also represented: *cut* at 7B1-2, and *Distalless/Brista* at 60E5-6 stain for all five proteins while *even-skipped* at 46C3-11 and *engrailed* at 48A3-4 are associated with *Psc*, *ph* and *Pc* only. In addition, *decapentaplegic* at 22F1-2, which interacts genetically with *zeste*, also corresponds to staining sites for all five proteins. While we have no proof that immunochemical staining at these cytological sites is due to binding to these genes, these loci are good candidates for targets of the *Pc* group genes. Genetic and molecular data show that, like the homeotic loci, they have complex and extensive regulatory regions that direct expression in intricate patterns at different stages of development and are, in some cases, dependent on the *Pc* genes (Dura and Ingham, 1988; Cohen *et al.*, 1989; St Johnston *et al.*, 1990; Heemskerk *et al.*, 1991; Jack *et al.*, 1991).

As observed originally by Zink and Paro (1989), some of the binding sites correspond to the genetically mapped

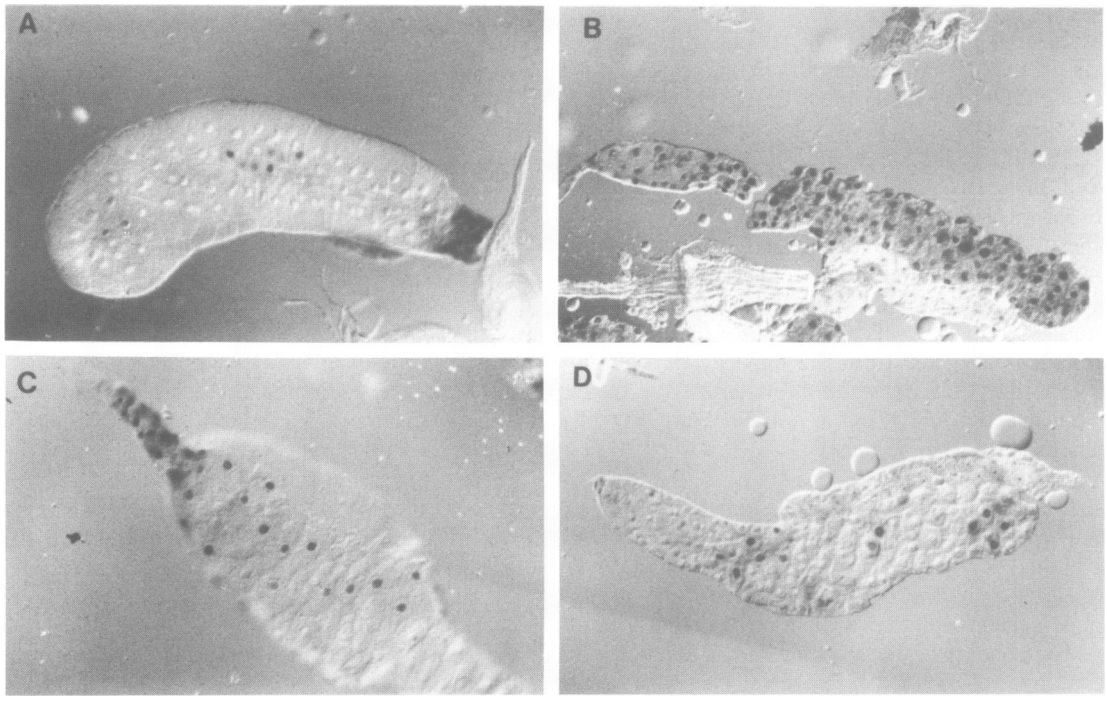

Fig. 6. Antibody staining of whole salivary glands from wild type and $E(z)^{S2}$ larvae. The top two photographs show staining with anti-*Psc* antibody; (**A**) wild type glands and (**B**) glands from $E(z)^{S2}$ larvae raised at 29°C. The lower two photographs show staining with anti-*Su(z)2* antibody; (**C**) wild type and (**D**) $E(z)^{S2}$ glands at 29°C. In all cases the glands were dissected from larvae at the stage of spiracle eversion and puparium formation. Note that while the staining for both *Psc* and *Su(z)2* becomes characteristically sporadic in wild type larvae at this stage, in $E(z)^{S2}$ larvae *Psc* staining remains at the earlier uniform stage.

loci of *Pc* group genes, suggesting the possibility that some members of the group may control one another, forming a regulatory network. Our results and those of DeCamillis *et al.* (1992) confirm that the cytological regions corresponding to *ph, exd, Asx, E(z)* and *E(Pc)* are also binding sites for *Pc, ph* and *Psc* and, in some cases, for *Su(z)2* and *zeste* proteins. The known effects of *Pc* group genes are generally negative: at homeotic loci they are needed to maintain the repressed state. In the salivary glands we would expect the BXC genes and at least some of the ANT-C genes to be repressed, consistent with the binding of *Pc* group proteins at these loci. However, binding cannot be always associated with inactivation since the *Pc* group genes themselves are presumed to be active in salivary glands. In particular, if the presence of *Pc, ph, Psc, Su(z)2* and *zeste* at 49EF represents binding to the *Psc* and *Su(z)2* loci, the control they exercise is not necessarily repressive since we have evidence that these genes are expressed in salivary gland cells. It is interesting to note, however, that the sudden all-or-none mechanism by which both *Psc* and *Su(z)* are turned off in salivary gland nuclei is suggestive of a regulatory mechanism in which other *Pc* group genes might participate. Consistent with this hypothesis is the observation that in the absence of *E(z)* function this all-or-none mechanism is relaxed in the case of *Psc*, though not in the case of *Su(z)2* (see Figure 6).

As noted by DeCamillis *et al.* (1992), some of the chromosomal sites listed in Table I correspond to the cytological locations of modifiers of position effect variegation (PEV). Although, given the large number of *modifier of PEV* this correspondence may be accidental, several arguments strengthen its validity. A relationship between *Pc* group genes and *modifiers of PEV* is suggested by the sequence similarity between *Pc* protein and the product of *Su(var)205* (Paro and Hogness, 1991). Furthermore, it has been proposed that both *Pc* group genes and *modifiers of PEV* are involved in the assembly of condensed forms of chromatin (Locke *et al.*, 1988) and observations cited by DeCamillis *et al.* (1992) indicate that many *Pc* group genes are also *modifiers of PEV*. It is unlikely that any of the five proteins under discussion has a direct effect on heterochromatin since, in contrast to the *Su(var)205* product (James and Elgin, 1986), none of them is found associated with heterochromatic regions. They might, however, affect PEV by facilitating the spread of heterochromatinization to regions that are normally euchromatic. More likely, their involvement in PEV is indirect, exerted by regulating the expression of other genes such as the *Su(var)* genes themselves.

Collective action of Pc genes

The synergistic action of the estimated 30–40 members of the *Pc* group in controlling homeotic gene expression raises the question of how so many proteins might be involved in the same regulatory mechanism. Some of the genes may simply regulate or coordinate the expression of other members of the group forming a regulatory network or hierarchy that might account for the common phenotypes. Nevertheless, the genetic evidence strongly suggests that many of the *Pc* group genes act together and supports models that envision the assembly of *Pc* group products to form extensive multiprotein complexes at a number of distinct chromosomal sites. Franke *et al.* (1992) have shown in fact that *Pc* and *ph* proteins are entirely co-localized on polytene chromosomes and that they can be isolated from nuclei in the form of a complex. In such an assembly process, some

gene products might act at early steps, some at later steps; some might interact directly with DNA while others might need to form complexes before binding to DNA and still others might only interact with previously bound proteins. Our results favor this assembly model since lack of *E(z)* function leads not to the loss of *Psc* and *Su(z)2* expression but to the inability of their products to bind to most chromosomal sites. A few experiments using anti-*ph* antibody indicate that loss of *E(z)* function has a similar effect on the binding of *Pc* and *ph* proteins. This approach may reveal a hierarchy of binding or a pathway for the assembly of the functional complex.

While *Pc* protein has no recognizable DNA-binding structural motifs, the *ph* product contains a zinc finger and both *Su(z)2* and *Psc* have a modified finger-like motif. However, bacterially expressed *Pc* protein does not bind to DNA *in vitro* (R.Paro, unpublished results cited by Zink *et al.*, 1991) and although the *Su(z)2* and *Psc* products have DNA binding activity (L.Rastelli, C.S.Chan and V.Pirrotta, unpublished results) their affinity is not sequence-specific as would be expected from their distribution in polytene chromosomes. The fact that *in vivo* their interaction with chromosomes is dependent on *E(z)* function suggests that the binding specificity is supplied by other cofactors. Nevertheless, when overproduced by induction of *hsp70*-driven constructs, both *Su(z)2* and *Psc* products are found associated with most, possibly all, polytenic bands, implying that at high concentrations they bind independently and non-specifically also *in vivo*. The binding of *Psc* and *Su(z)2* proteins to most sites is affected by the loss of *E(z)* function, while the *zeste* bands remain apparently unaffected. Since the *E(z)*-independent binding sites are not simply the strongest, it is likely that binding at these sites involves interactions with other cofactors or with a different chromatin conformation. The importance of the chromatin structure surrounding the target sites is shown by the observation that transposons containing potential *Pc* binding sites bind *Pc* protein when integrated in some genomic sites but not in others in correlation with the expression or repression of the reporter gene in salivary glands (Zink *et al.*, 1991). Another indication of the importance of the state of the chromatin is given by the remarkable effect of *E(z)* function on the appearance of the polytene chromosomes. The general decondensation, loss of contrast and the disappearance of many characteristic constrictions imply that *E(z)* product or the chromatin complexes that depend on it play a major role in maintaining the integrity of the chromosomes and help explain the low mitotic index, chromosome breakage and irregular condensation observed in mitotic cells carrying *E(z)* mutations (Gatti and Baker, 1989).

Interaction of zeste and its modifiers

The *zeste* cytological locus (3A3,4) is one of the sites at which *Su(z)2* and *zeste* proteins but not *Psc* are found. It is possible therefore that at least some of the *modifiers of zeste* exert their effect on the *zeste−white* interaction by regulating the expression of the *zeste* gene itself. Loss-of-function *E(z)* mutations generally act as suppressors of *zeste*, implying that the *E(z)* protein is necessary for the *zeste−white* effect (Jones and Gelbart, 1990). However, the fact that loss of *E(z)* function does not affect the binding of *zeste* to chromatin suggests that the *modifiers of zeste* do not act directly on the *zeste* gene but rather that *E(z)* affects a chromatin structure at the *white* gene, very probably involving *Su(z)2* and *Psc*, that is necessary for the z^1 product to have its repressive effect. The effect of *E(z)* on the *zeste−white* interaction might be indirect: loss of function may prevent the binding of other proteins such as *Psc* and *Su(z)2* and therefore relieve repression; gain of function might result in assembly of greater complexes and enhance repression of *white* by *zeste*. One possibility is that the *Su(z)2*, *Psc* and *E(z)* products do not normally bind at the *white* locus but are induced to do so by the formation of large aggregates of the z^1 mutant protein bound there. This would then result in the condensation of the chromatin into a less active state. Unfortunately, we cannot detect *zeste*, *Su(z)* or *Psc* at the *white* locus in salivary gland polytene chromosomes although the *white* gene is known to be a direct target at least for the zeste product. We suppose that this is due to the fact that the *white* gene is not active in this tissue and its chromatin is in a form inaccessible to these products.

Materials and methods

Fly strains and mutants

The wild type strain used in this work was Canton S. The host strain for all germ line transformation experiments was $Df(1)w^{67c23(2)}$, which carries a deletion removing the proximal part of the *white* gene including the promoter and first exon (Pirrotta *et al.*, 1983). Mutants carrying deletions of the *Psc* or *Su(z)2* genes were $Psc^{Arp\text{-}1}$ and $Su(z)2^{Arp\text{-}1}$ (Brunk *et al.*, 1991b). The *Polycomb* allele used was Pc^3 and the temperature sensitive $E(z)^{S2}$ allele was induced by EMS treatment by Jones and Gelbart (1990).

Plasmid and transposon constructs

Expression clones to produce full length *Su(z)2* or *Psc* proteins were constructed by inserting the entire coding region of the two cDNAs (from clones obtained from P.Adler) into the pET11A vector of Studier and Moffat (1986). Transposon constructs for germ line transformation were made by inserting the same cDNA sequences into the CaSpeR-hs vector (Thummel and Pirrotta, 1991) where they are expressed under the control of the *hsp70* promoter. The pUKK-*zeste* expression construct was as previously described (Bickel and Pirrotta, 1990).

Antibody purification

Antibodies were raised in rabbits against β-galactosidase fusion proteins containing amino acids 477−558 of *Su(z)2* protein or amino acids 819−926 of *Psc* protein. The sera were first passed through a CM Affigel Blue column (Bio-Rad) and then affinity-purified by applying them through an affinity column containing total insoluble *Escherichia coli* protein equilibrated with PBS buffer. The protein in the flow through was then passed through a second affinity column containing bound β-galactosidase-*Su(z)2* or *Psc* fusion protein. The material bound to the column was eluted with buffer containing 200 mM glycine, pH 2.7, and 137 mM NaCl. The peak fractions were immediately neutralized, dialysed against PBS and concentrated.

Western blot analysis

Bacterial extracts were prepared from 400 ml cultures of *E.coli* BL21(DE3) expressing the *Su(z)2* or *Psc* proteins. The cells were pelleted, resuspended in 10 ml extraction buffer (100 mM KCl, 25 mM HEPES, pH 7.6, 12.5 mM $MgCl_2$, 1 mM DTT, 0.1% NP-40, 20% glycerol and 1 mM PMSF) and lysed by sonicating in ice three times for 20 s. The insoluble inclusion bodies were isolated by centrifugation at 4°C for 30 min. The pellet was resuspended in urea sample buffer. Total *Drosophila* protein extracts were prepared by homogenizing flies directly in SDS sample buffer (3% SDS, 100 mM Tris pH 6.8, 10% glycerol, 1 mM PMSF and 1 μg/ml leupeptin). The homogenates were sonicated to solubilize the chromatin, boiled for 5 min and the cell debris removed by centrifugation in 18 000 r.p.m. for 10 min. For heat shock induction, flies carrying CaSpeR hs-Su(z) or CaSpeR hs-Psc transposons were incubated for 2 h at 37°C and then immediately homogenized. To prepare embryonic extracts deficient for *Psc* or *Su(z)2* proteins, we used flies heterozygous for the $Su(z)2^{Arp\text{-}1}$ or the $Psc^{Arp\text{-}1}$ deletions, balanced with a *CyO* chromosome carrying a reporter gene expressing the *lacZ* gene under the control of the *wg* regulatory region. After ageing for ~20 h, the embryos were dechorionated, treated with heptane for 10 min and stained with X-gal for 10 min. All but the

homozygous mutant embryos developed a blue color. Embryos that remained white were collected under the microscope and used to prepare mutant extracts by the same procedure as used for the flies. Extract samples were separated on a 10% discontinuous SDS–polyacrylamide gel, electroblotted onto Immobilon P membranes (Bio-Rad) and detected after incubation with primary antibodies with secondary antibodies conjugated to peroxidase (Nordic).

Immunostaining of salivary glands and chromosomes

Salivary gland polytene chromosomes were stained following a modified version of the protocol of Pirrotta *et al.* (1988). The glands were fixed for 5–20 s for *zeste*, 60 s for *Su(z)2* and 90 s for *Psc* in a drop of 3.7% formaldehyde in PBS, then transferred to a drop of 45% acetic acid, 3.7% formaldehyde in PBS. Reproducible staining for *Psc* protein requires the longer fixing time. After spreading and squashing the chromosomes, the slides were washed in 100% ethanol for 1–24 h. The chromosomes were rehydrated in PBT (PBS containing 0.1% Tween 20) for 1 h, blocked in the same buffer with 5% bovine serum albumin for 30 min and then incubated with the primary antibodies overnight at 4°C. A Vectastain kit (Vector Laboratories) with goat anti-rabbit secondary antibody was used and the chromosomes were stained with HRP according to the manufacturer's instructions. The color was intensified by development in the presence of 0.008% $NiCl_2$ and $CoCl_2$. To help in the cytological analysis and to distinguish cytological bands from staining bands, we often prestained the chromosomes with 0.1 mg/ml DAPI for 30 s. Even faint HRP staining will completely quench the DAPI fluorescence of a chromomere, increasing the sensitivity of detection of the stain (Karr and Kornberg, 1989). The slides were finally rinsed in PBT and counterstained with Giemsa diluted 1:100 in 10 mM sodium phosphate, pH 7.0. The chromosomes of $E(z)^{S2}$ larvae were studied in larvae raised at 29°C or in larvae raised at permissive temperatures, transferred to 37°C for 1 h and maintained for various times above 30°C before dissecting and fixing the glands. As controls we used wild type larvae treated in parallel and mutant larvae raised at 23°C.

For whole mount staining, salivary glands were fixed with 3.7% formaldehyde in PBS for 5 min, rinsed several times in PBS containing 0.1% bovine serum albumin and incubated for 15 min in the same buffer containing 0.3% H_2O_2 to eliminate endogenous peroxidase activity. After several washes, the glands were blocked with PBT containing 5% serum albumin or 2% goat serum for 2 × 30 min and incubated with primary antibody overnight at 4°C. Staining with HRP-conjugated secondary antibody followed as before and the stained glands, washed several times with PBT, were mounted in a solution of 50% glycerol, 50% ethanol.

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