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The *Drosophila Enhancer of zeste* gene encodes a chromosomal protein: examination of wild-type and mutant protein distribution

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SUMMARY

The *Drosophila Enhancer of zeste* [*E(z)*] gene is a member of the Polycomb-group and, as such, is involved in maintaining the transcriptional repression of the homeotic genes of the Antennapedia (ANT-C) and bithorax (BX-C) complexes. It has been proposed that Polycomb-group (Pc-G)-mediated silencing requires the formation of heteromeric protein complexes which modify the chromatin structure of target genes. We describe the in vivo distribution of the E(Z) protein and show it to be ubiquitously present in embryonic and larval nuclei. In salivary gland polytenized nuclei, the identifiable E(Z) chromosome binding sites are a subset of those described for other Polycomb-group proteins, suggesting that E(Z) may also participate in Polycomb-group complexes. E(Z) binds to chromosomes in a DNA sequence-dependent manner, as illustrated by the creation of a new E(Z)-binding site at the location of a P element reporter construct that previously has been shown to contain a Polycomb response element (PRE). We also present the sequences of one null and three temperature-sensitive *E(z)* alleles, describe the effects these mutations have on the in vivo distribution of E(Z) protein and discuss their implications concerning putative functional domains. Finally, we describe the effect a *trithorax* mutation has on E(Z) chromosome binding.

Key words: *Enhancer of zeste*, *Polycomb*, *trithorax*, transcription, temperature-sensitive mutations, *Drosophila*

INTRODUCTION

In the development of multicellular organisms, the process of determination requires the maintenance of specific patterns of gene expression through many cycles of cell division. A well-documented example involves the control of segmental identity by the genes of the *Drosophila* Antennapedia (ANT-C) and bithorax (BX-C) complexes (Lewis, 1978; Kaufman et al., 1980). The transcriptional activity of the ANT-C and BX-C segment identity genes must be not only initiated correctly, but accurately maintained in order to maintain the determined states of cells under their control. The initial patterns in which the segment identity genes are expressed are established early in embryogenesis by transcription factors that are encoded by the segmentation genes (White and Lehmann, 1986; Harding and Levine, 1988; Irish et al., 1989; Reinitz and Levine, 1990). However, shortly after these patterns are set up, the products of the segmentation genes dissipate. For example, *hunchback* (hb) represses the transcription of *Ultrabithorax* (*Ubx*) and thereby establishes the anterior border of *Ubx* expression (White and Lehmann, 1986). However, by mid-germ band elongation the anterior domain of hb expression is no longer detectable (Tautz, 1988). At that point, maintenance of *Ubx* repression becomes the responsibility of the products of the Polycomb-Group (Pc-G) (Struhl and Akam, 1985; Jones and Gelbart, 1990). The Pc-G plays a similar regulatory role in maintaining the repression of the other ANT-C and BX-C genes (Wedeen et al., 1986; Glicksman and Brower, 1988; Simon et al., 1992). The products of a second group of genes, known as the trithorax-group (trx-G), are required to maintain ANT-C and BX-C activity in those cells in which the respective segment identity genes are initially turned on (reviewed by Kennison, 1993).

There are 13 characterized Pc-G loci: *Polycomb* (*Pc*) (Lewis, 1978; Duncan, 1982), *extra sex comb* (*esc*) (Struhl, 1981), *Polycomblike* (*Pcl*), *pleiohomeotic* (*pho*, synonym *l(4)29*) (Duncan, 1982), *Additional sex combs* (*Asx*), *Posterior sex combs* (*Psc*), *Sex combs on midleg* (*Scm*) (Jurgens, 1985), *polyhomeotic* (*ph*) (Dura et al., 1985), *multi sex combs* (*mxc*) (Santamaria and Randsholt, 1995), *Sex comb extra (Sce)* (Breen and Duncan, 1986), *super sex combs* (*sxc*) (Ingham, 1984), *cramped* (*crm* aka *sparse arista*) (Rayle and Green, 1968) and *Enhancer of zeste* [*E(z)*] (Shearn et al., 1978a; Phillips and Shearn, 1990; Jones and Gelbart, 1990). Mutant alleles in additional loci such as *Suppresser 2 of zeste* [*Su(z)2*] (Wu et al., 1989; Campbell et al., 1995) and *Enhancer of Pc* [*E(Pc)*] (Sato et al., 1983), enhance the homeotic phenotypes produced by Pc-G mutations and their products likely participate in similar aspects of transcriptional regulation. Based on enhancement of Pc-G homeotic phenotypes by randomly selected deficiencies, it has been proposed that up to 40 loci may be involved (Jurgens, 1985). Of the Pc-G and related proteins that have been sufficiently studied, all have been shown to encode chromatin proteins that bind to chromosomes in a DNA-sequence-dependent manner

(Zink and Paro, 1989; Zink et al., 1991; DeCamillis et al., 1992; Martin and Adler, 1993; Rastelli et al., 1993; Lonie et al., 1994). It has been proposed that Pc-G proteins form heteromeric protein complexes (Locke et al., 1988). This is supported by several observations. First, double Pc-G mutant combinations often display synergistic interactions (Jurgens, 1985; Cheng et al., 1994; Campbell et al., 1995). Second, PC, PH, PCL and PSC are either completely or extensively co-localized on polytene chromosomes (Franke et al., 1992; Rastelli et al., 1993; Martin and Adler, 1993; Lonie et al., 1994). Third, PC and PH co-immunoprecipitate from nuclear extracts as components of a large protein complex (Franke et al., 1992). Fourth, lack of $E(Z)^+$ activity disrupts PSC, SU(Z)2, PH and PC binding at most chromosomal sites (Rastelli et al., 1993; Platero et al., 1995). Fifth, *Xenopus* homologs of PC and PSC have been shown to directly interact in vitro (Reijnen et al., 1995). It thus appears that many of the Pc-G proteins function as components of heteromeric complexes and that some likely are in direct contact with one another. Other members of the complexes may be critical for its formation, and are therefore required for chromosome binding by other Pc-G proteins, yet do not come into direct physical contact. It is also possible that other proteins are bound to closely linked chromosomal sites and affect local chromatin conformation in such a way as to influence the ability of other proteins to bind.

In addition to its role in maintaining the repression of ANT-C and BX-C genes, *E(z)* is involved in other examples of negative gene regulation including the repression of *white* by *zeste*^{1} (Kalisch and Rasmuson, 1974; Wu et al., 1989; Jones and Gelbart, 1990) and repression of *knirps* by maternal hb (Pelegri and Lehmann, 1994). *E(z)* is also required for cell proliferation and may play a role in maintaining gross chromosomal integrity (Gatti and Baker, 1989; Phillips and Shearn, 1990; Rastelli et al., 1993). The E(Z) protein contains a region of sequence similarity, which has been named the SET domain, that is shared with trithorax (TRX) (Jones and Gelbart, 1993) and the product of *Su(var)3-9* (Tschiersch et al., 1994), which is involved in position effect variegation. Based on the presence of the SET domain in two proteins, E(Z) and TRX, that have antagonistic effects on transcription, it has been proposed that it may permit these proteins to interact with a common, or similar, target and that other domains of the proteins are responsible for their respective effects on transcription (Jones and Gelbart, 1993).

In order to better understand the role of *E(z)* in Pc-G activity and its role in maintaining overall chromosome integrity, we have examined the in vivo distribution of the E(Z) protein. We find that it is ubiquitously distributed in embryonic and larval nuclei and that it binds many of the same polytene chromosome loci as other Pc-G proteins, including a reporter construct whose expression in embryos is in part controlled by E(Z). We also present the sequences of one null and three temperature-sensitive *E(z)* alleles and describe their effects on the E(Z) protein. It has been reported that lack of $E(Z)^+$ activity disrupts chromosome binding by TRX (Kuzin et al., 1994). In a reciprocal experiment, we find that *trx*^{1}, a weak temperature-sensitive allele, reduces polytene chromosome binding by E(Z) at restrictive temperature.

MATERIALS AND METHODS

Drosophila stocks and culture

Descriptions of marker mutant alleles and balancer chromosomes may be found in Lindsley and Zimm (1992). Descriptions of the *E(z)*^{61} and *E(z)*^{63} alleles [aka *E(z)*^{S2} and *E(z)*^{S4}, respectively] may be found in Jones and Gelbart (1990). Descriptions of the *E(z)*^{28} and *E(z)*^{32} alleles (aka *pco*^{ox736} and *pco*^{pco25hs}, respectively) may be found in Phillips and Shearn (1990). The *trx*^{1} homozygous mutant stock was a gift from Peter Harte. Descriptions of this allele may be found in Ingham and Whittle (1980) and Breen and Harte (1991). The transgenic line 85-39 was a gift from Jeff Simon. Descriptions of this line may be found in Simon et al. (1990).

Sequencing mutant *E(z)* alleles

DNA was extracted from adult flies that were either hemizygous or homozygous for temperature-sensitive alleles and that had been reared at permissive temperature (≤21°C). Larvae that were hemizygous for the null *E(z)*^{63} allele were obtained by crossing *E(z)*^{63}*/TM6B,Tb* males to *Df(3L)lxd*^{15}*/TM6B,Tb* females and collecting Tb^+ larvae. DNA from adults or larvae was extracted and the entire *E(z)* transcribed region was amplified using Taq DNA Polymerase and Taq Extender (Stratagene) according to the manufacturer's suggestions. Nested PCR primers were used to amplify the entire gene and internal PCR primers were used to amplify overlapping regions within the secondary PCR products. The tertiary PCR products were gel-isolated from low melting point agarose using Gelase (Epicentre) and directly sequenced following the procedure described by Drebot and Lee (1994).

Generation of antibodies

The *Ava*I-*Bam*HI fragment from *E(z)* cDNA e32 (Jones and Gelbart, 1993), which encodes E(Z) amino acids Pro_8-Asp_{155}, was subcloned into both pQE32 (Qiagen) and pGEX-2T (Smith and Johnson, 1988) bacterial expression vectors and the fusion proteins affinity purified using Ni-NTA agarose and glutathione agarose, respectively. Rabbit polyclonal antibodies were generated against the His_6-E(Z) fusion protein (all injections and bleeds were conducted at the Pocono Rabbit Farm & Laboratories). Antibodies were affinity purified by first mixing the antiserum overnight (4°C) with total bacterial extract that included a GST-C-terminal E(Z) fusion protein [E(Z) amino acids Gly_{637} to Val_{760}], which had been attached to CnBr-activated Sepharose, and then mixing the unbound antiserum with affinity-purified GST-E(Z) (Pro_8-Asp_{155}) fusion protein (attached to CNBr-activated Sepharose) and then pouring the slurry into a column. After thorough washing, bound antibodies were eluted from the column with 0.2 M glycine, 0.2 M NaCl (pH 2.5), neutralized with Tris-HCl (pH 8.0), dialized against PBS and stored at −80°C. Antibodies were diluted 1:100 for staining whole-mount embryos and larval tissues, 1:40 for staining polytene chromosomes and 1:800 for western blots.

Western analysis

Nuclear and whole embryo extracts were prepared from 0-12 hour embryos according to the protocol described by Franke et al. (1992) and Dingwall et al. (1995), respectively. Larval extracts were prepared from *Oregon-R*, *E(z)*^{63}*/Df(3L)Ez2*, *E(z)*^{28}*/E(z)*^{28}, *E(z)*^{32}*/E(z)*^{32} and *E(z)*^{61}*/E(z)*^{61} late third instar larvae. *Oregon-R* and *E(z)*^{63} hemizygous larvae were reared at 25°C and 18°C, respectively. Homozygous temperature-sensitive allele stocks were maintained at 21°C, then shifted to 29°C approximately 24 hours prior to selection of larvae. For each preparation, four larvae were homogenized in 2% SDS, 60 mM Tris-HCl, pH 6.8, 25% glycerol, 5% β-mercaptoethanol, 1 mM PMSF, 10 μg/ml leupeptin and 1 μg/ml aprotinin in TBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl), followed by brief sonication. After boiling, samples were centrifuged, then run on 8% SDS-PAGE gels. Immunoblots were then incubated with anti-E(Z) primary antibody followed by horseradish peroxidase-conjugated goat anti-rabbit

secondary antibody diluted 1:10,000 (Jackson Labs). Signal was detected using ECL reagents (Amersham). Molecular weights were estimated by comparison to a broad range molecular weight marker (Bio-Rad). In order to confirm that equal amounts of total protein were present in each lane, the blots were stained with Ponceau S (Sigma).

Immunolocalization of the E(Z) protein on polytene chromosomes

Polytene chromosome spreads were prepared from climbing third instar larvae prior to spiracle eversion. *Oregon-R* chromosomes were stained to determine the wild-type distribution of the E(Z) protein. Homozygous *E(z)* temperature-sensitive mutants were reared at 21°C until climbing third instar larvae were present, then the cultures were placed in a 29°C incubator for approximately 24 hours prior to dissection. Homozygous trx^1 larvae were derived from homozygous trx^1 parents that had been maintained at 29°C for at least 4 days prior to egg collection. For control chromosome spreads, larvae that were homozygous for temperature-sensitive *E(z)* alleles or trx^1 were reared continuously at 21°C. The staining of salivary gland chromosomes essentially followed the procedure of Zink and Paro (1989) with the following changes. The first fixative solution was 3.7% formaldehyde in PBT (PBS: 150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.3; PBT: PBS with 1% Triton-X-100). Slides were incubated with affinity-purified rabbit anti-E(Z) primary antibody overnight at 4˚C, and then washed for a total of 30 minutes in PBS supplemented with 300 mM NaCl, 0.2% np-40 and 0.2% Tween-20. Slides were then incubated with biotinylated goat anti-rabbit secondary antibody diluted 1:5,000 (Jackson Labs) for 2 hours at room temperature, washed as before and then incubated with streptavidin-horseradish peroxidase (SA-HP, Jackson Labs) diluted 1:1,000 in PBT for 1 hour at room temperature. For photography, slides were mounted in glycerol after counter staining with Giemsa.

Immunostaining of wild-type and mutant embryos

Oregon-R embryos were collected at 25°C and used to study the normal distribution of the E(Z) protein. *Oregon-R* embryos were also collected at 18°C and 29°C and used as controls in experiments involving the homozygous *E(z)* mutant embryos. Homozygous $E(z)^{28}$ and $E(z)^{61}$ stocks were maintained at 21°C. Homozygous $E(z)^{28}$ and $E(z)^{61}$ embryos were collected from the respective homozygous stocks at 18˚C or 29˚C. Embryos were collected at 0-18 hour intervals at 18˚C and 0-8 hour intervals at 29˚C. Immunodetection of the E(Z) protein in embryos followed the procedure previously described in Jones and Gelbart (1990). Goat anti-rabbit biotinylated secondary antibodies were diluted 1:5,000. Embryos were incubated with a 1:1,000 dilution of SA-HP for one hour at room temperature, washed, then suspended in 0.5 mg/ml diaminobenzidine (DAB) in 0.1 M Tris-HCl (pH 7.0), 0.2% $NiCl_2$, 0.003% H_2O_2. After approximately 15 minutes, the reaction was stopped by washing with PBS. Embryos were then dehydrated and mounted in methyl salicylate (Sigma).

Whole-mount immunostaining of larval salivary glands, brain and imaginal discs

Immunohistochemical staining of imaginal discs, CNS and salivary glands was performed according to the protocol of Brower (1987), except that following incubation with biotinylated goat anti-rabbit secondary antibodies protein localization was visualized by horseradish peroxidase staining (MacDonald and Struhl, 1986) with the addition of $NiCl_2$ and $CoCl_2$ to 0.008% each. Tissues were mounted and stored in 70% glycerol, 30mM Tris-HCl (pH 9.0).

RESULTS

Sequence analysis of E(z) mutant alleles

We have sequenced one null and three temperature-sensitive *E(z)* alleles. Each was found to contain a single base pair substitution (Fig. 1). The null $E(z)^{63}$ allele contains a G to A transition that eliminates the translation initiation codon (M1I). The next in-frame ATG is codon 103. We are unable to detect a hypothetical N-terminally truncated $E(Z)^{63}$ product in larval extracts using our anti-E(Z) antibodies (Fig. 2B). However, since the E(Z) antigen against which our antibodies were generated (Pro_8 to Asp_{155}) only partially overlaps with this hypothetical $E(z)^{63}$ product, it is possible that they would not detect such a protein. Since $E(z)^{63}$ genetically behaves as a true null allele (Jones and Gelbart, 1990) and expression of other truncated E(Z) proteins [e.g. $E(Z)^{60}$] produce gain-of-function phenotypes, it is more likely that $E(z)^{63}$ is a protein null.

Each of the three temperature-sensitive *E(z)* alleles contains a single G to A transition that results in a Cys to Tyr substitution. The Cys to Tyr mutations in $E(z)^{32}$ (C545Y) and $E(z)^{61}$ (C603Y) lie within the Cys-rich region of the E(Z) protein, which spans amino acids 538-603 (Jones and Gelbart, 1993). $E(z)^{28}$ contains a Cys to Tyr mutation in the middle portion of E(Z) (C363Y). It is important to note that each of the Cys residues affected in the three *E(z)* temperature-sensitive alleles are conserved in two human proteins [ENX-1 (Hobert et al., 1996b) and E(Z)H1/ENX-2 (Rommens et al., 1995; Hobert et al., 1996b; Abel et al., 1996] and one mouse protein [Enx-1 (Hobert et al., 1996a)] that appear to be homologs of the *Drosophila* E(Z) protein.

Immunodetection of the E(Z) protein

Rabbit anti-E(Z) antibodies were generated against a His_6-E(Z) fusion protein [E(Z) amino acids Pro_8-Asp_{155}] and affinity-purified with a GST-E(Z) fusion protein that contains the same E(Z) residues (see Methods). These antibodies detect a M_r 89×10^3 protein on immunoblots of total and nuclear wild-type embryo extracts (Fig. 2A). This is very close to the predicted molecular weight of the 760 amino acid protein encoded by the *E(z)* cDNA e32 (Jones and Gelbart,

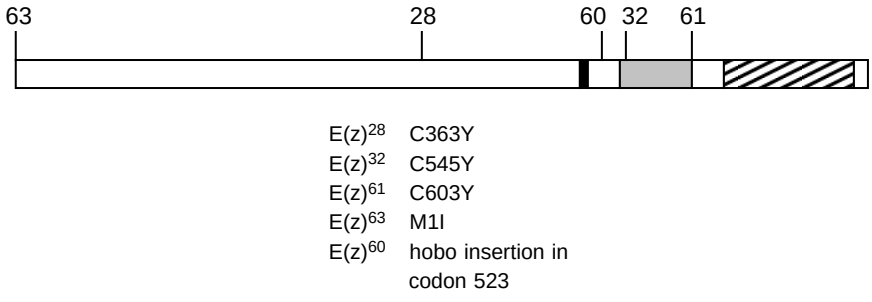

Fig. 1. A schematic depiction of the E(Z) protein and the positions of mutations in *E(z)* alleles. E(Z) domains are represented as filled-in boxes: putative nuclear localization signal (amino acids 504 to 510, solid black), Cys-rich region (amino acids 538 to 603, stippled), SET domain (amino acids 633 to 747, diagonal lines). The position of the mutation in each allele is indicated by the allele's name above the rectangular diagram. Using the coordinates of *E(z)* cDNA e32 as described in Jones and Gelbart (1993), these alleles contain the following base pair substitutions: $E(z)^{28}$, G_{1191} to A; $E(z)^{32}$, G_{1737} to A; $E(z)^{61}$, G_{1911} to A; $E(z)^{63}$, G_{106} to A. $E(z)^{60}$ contains a *hobo* element inserted into codon 523 and was previously described (Jones and Gelbart, 1993). (Wild-type cDNA accession number is Genbank U00180.)

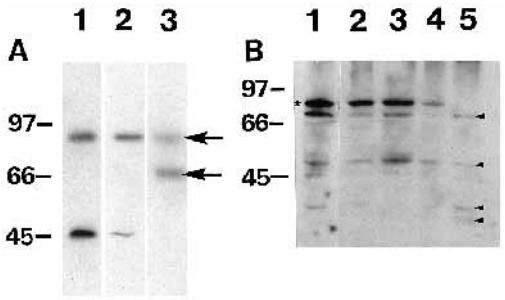

Fig. 2. Characterization of the E(Z) protein by western analysis. Polyclonal rabbit antibodies were raised against a 6His-E(Z) fusion protein containing E(Z) residues 8-155. (A) Detection of E(Z) protein in nuclear and total embryo extracts. Lanes 1 and 2, nuclear and total embryo extracts, respectively, derived from *yellow white* embryos showing full-length E(Z) protein (M_r 89×10^3, top arrow). The lower molecular weight band in lanes 1 and 2 represents a degradation product. Lane 3, total embryo extract derived from *E(z)^{60}/TM3* embryos showing both full-length E(Z)$^+$ (top arrow) and truncated E(Z)^{60} (M_r 67×10^3, bottom arrow) proteins. (B) Detection of E(Z) protein in total larval extracts. Lane 1, extracts derived from *Oregon-R* larvae reared at 29˚C [* indicates full-length E(Z)]. Lanes 2-4, extracts from homozygous *E(z)^{28}*, *E(z)^{32}* and *E(z)^{61}* larvae, respectively, reared at 29˚C. Note that the overall levels of E(Z) are decreased in *E(z)^{61}* larvae compared to *E(z)^{28}* and *E(z)^{32}*. Lane 5, extracts from *E(z)^{63}/Df(3L)Ez2* larvae show the absence of full-length E(Z). Several lower molecular weight bands are also detected in each lane (see arrowheads next to lane 5). Given their presence in *E(z)^{63}/Df(3L)Ez2* larval extracts and their absence in embryo extracts, it is likely that these are cross-reacting larval proteins and not E(Z) degradation products.

1993). This result also indicates that *E(z)* encodes a nuclear protein. As a control for specificity, extracts from embryos derived from *E(z)^{60}/TM3* parents were also analyzed. The antimorphic *E(z)^{60}* allele contains a hobo element inserted into codon 523, and has therefore been predicted to encode a C-terminally truncated protein that lacks amino acids 524 to 760 (Jones and Gelbart, 1993). In agreement with this prediction, our anti-E(Z) antibodies detect both full length (M_r 89×10^3) and truncated (M_r 67×10^3) proteins in *E(z)^{60}/TM3* extracts (Fig. 2A).

The same antibodies also detect a M_r 89×10^3 protein in *Oregon-R* late third instar larval extracts (Fig. 2B, lane 1, see *), and both M_r 89×10^3 and truncated M_r 67×10^3 proteins in extracts from *E(z)^{60}/TM3* larvae (data not shown). We also examined extracts from larvae that were hemizygous for the null *E(z)^{63}* allele and find that the M_r 89×10^3 protein is not present (Fig. 2B, lane 5). Several cross-reactive bands are detected in larval immunoblots (Fig. 2B, arrowheads), but not embryo extracts (Fig. 2A). These bands are not detected with preimmune serum (data not shown). The intensity of these bands are much weaker in immunoblots of isolated larval salivary glands, imaginal discs or central nervous systems (data not shown). These are not E(Z) degradation products since they are unchanged in *E(z)^{63}* larvae. It is our estimation that these cross-reactive species may be diffuse larval cytoplasmic proteins or components of the hemolymph, since no staining is detectable in whole-mount tissues from larvae that are hemizygous for the null *E(z)^{63}* allele (described below).

Immunolocalization of wild-type and mutant E(Z) protein

Anti-E(Z) antibodies were used to examine the distribution and subcellular localization of the E(Z) protein in wild-type and mutant embryos (Fig. 3). During cleavage stages, E(Z) is uniformly distributed and nuclear localization is seen in some embryos. By syncytial blastoderm nuclear staining is clearly evident and by cellular blastoderm virtually all staining is nuclear, including pole cells. This ubiquitous nuclear localization continues in later embryos with no obvious differences between tissues. This is consistent with developmental northern blots, which show very high levels of both maternal and zygotic *E(z)* transcript in early embryos (Jones and Gelbart, 1993). Homozygous *E(z)^{28}* and *E(z)^{61}* embryos derived from homozygous parents at 29˚C exhibit staining

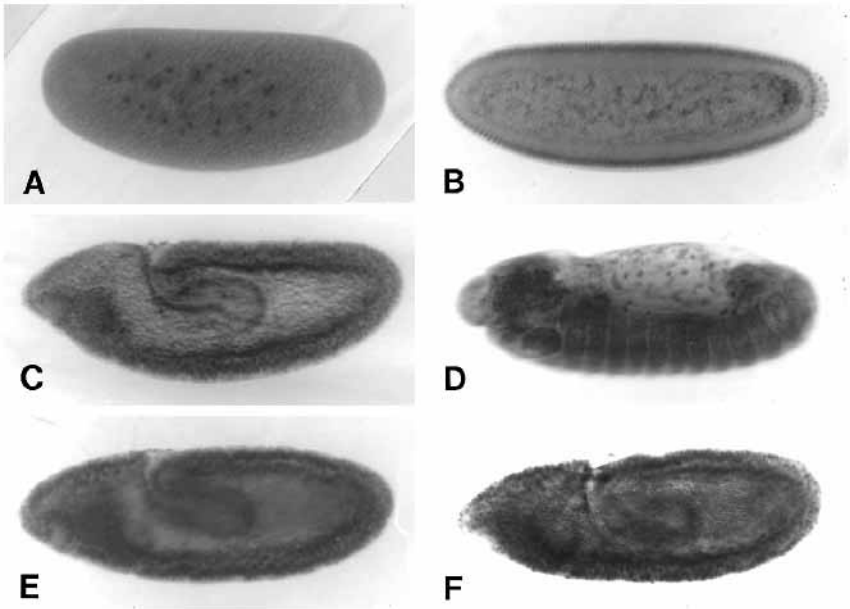

Fig. 3. Expression of the E(Z) protein in wild-type and mutant embryos. (A-D) *Oregon-R* embryos collected at 25°C. (E,F) Homozygous temperature-sensitive *E(z)^{61}* and *E(z)^{28}* embryos, respectively, both of which were derived from homozygous parents at 29°C. (A) Cleavage stage embryo showing ubiquitous distribution and early nuclear localization. (B) Embryo at the cellular blastoderm stage showing ubiquitous nuclear staining, including staining of pole cell nuclei. (C,E,F) Germ-band-extended embryos showing high levels of nuclear staining remaining ubiquitous and strong. (D) Germ-band-retracted embryo in which the E(Z) protein is still detected at high levels. (E,F) The pattern of E(Z) staining in these mutant embryos is indistinguishable from that in wild-type embryos.

patterns that are essentially indistinguishable from wild type (Fig. 3).

In order to determine the distribution of the E(Z) protein in larval tissues, whole-mount staining using the same anti-E(Z) antibodies was performed. In all cases, climbing third instar larvae were selected that had not yet undergone spiracle eversion. E(Z) is essentially ubiquitously distributed in larvae, but at considerably lower levels than seen in embryos. Again, this is consistent with developmental northern blots, which show very low levels of *E(z)* mRNA in larvae (Jones and Gelbart, 1993). E(Z) is present in essentially all imaginal discs, central nervous system (CNS) and salivary gland cells (Fig. 4). The small size of diploid imaginal disc and CNS cells makes it difficult to ascertain its subcellular distribution in these tissues, but nuclear localization is clearly seen in salivary gland polytenized nuclei (Fig. 4). At higher magnification (Fig. 4E), it is apparent that the staining pattern is not uniform, but rather resembles the globular coiling patterns of polytene chromosomes (Mathog et al., 1984). This suggests that the vast majority of E(Z) is associated with chromatin and that it may be somewhat generally distributed along the chromosomes. Considerable variability in overall levels of staining between nuclei is often, but not always, seen within the same salivary gland. However, even in nuclei where overall staining is low, this 'chromosomal' pattern is still evident (Fig. 4F). This appears to be different from the intranuclear distribution of the PC protein, which has been described as showing a 'bright-banded pattern' in immunofluorescently stained salivary gland nuclei (Messmer et al., 1992).

As a negative control for the specificity of these antibodies for E(Z) in larval tissues, whole-mount staining of hemizygous $E(z)^{63}$ larvae was performed. No staining of salivary glands, imaginal discs or CNS was observed (Fig. 4). In addition, we also see a decrease in immunohistochemical staining of tissue derived from *E(z)* temperature-sensitive larvae reared at restrictive temperature. Therefore, although these anti-E(Z) antibodies detect additional larval proteins on western blots, we conclude that they are specific for E(Z) in immunohistochemical staining of these tissues.

We also analyzed the effects of the temperature-sensitive $E(z)^{61}$, $E(z)^{28}$ and $E(z)^{32}$ alleles on E(Z) protein levels and distribution. Embryos were collected from homozygous parents at permissive temperature (21°C) and allowed to develop at 21°C until climbing third instar larvae were detected. Cultures were then shifted up to restrictive temperature (29°C) and climbing third instar larvae were dissected approximately 24 hours later. Controls included *Oregon-R* larvae reared continuously at 29°C, as well as homozygous temperature-sensitive mutant larvae continuously reared at 21°C. Both types of controls closely resemble immunohistochemically stained tissues from wild-type larvae reared at lower temperatures. Staining of imaginal discs and CNS is not noticeably altered in these mutants at 29°C (Fig. 5). However, the overall levels of temperature-sensitive E(Z) protein is generally reduced in the salivary gland and the intranuclear distribution of the remaining protein is more diffuse. Almost all nuclear staining in the salivary gland is abolished in $E(z)^{61}$ homozygotes at 29°C (Fig. 5F). In those nuclei in which $E(Z)^{61}$ protein is still detectable, staining is weak and the protein does

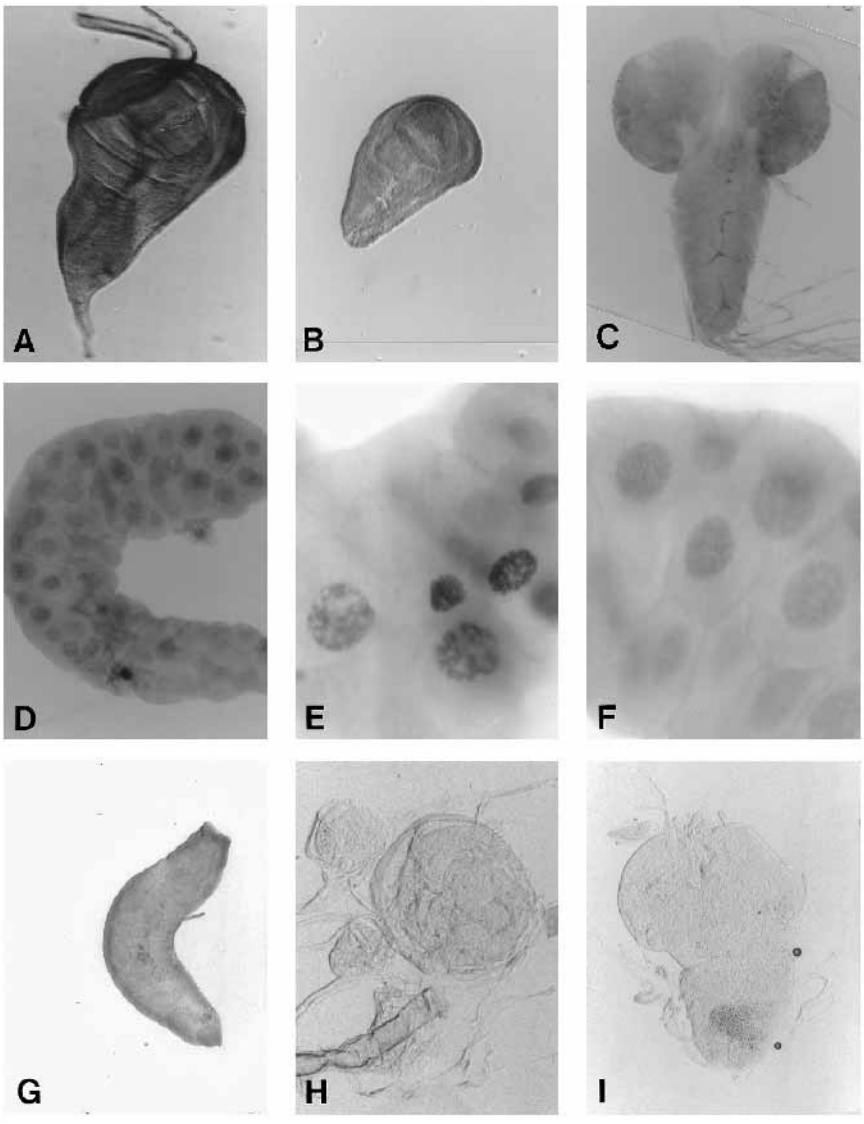

Fig. 4. Distribution of E(Z) in third instar larval tissues. (A-F) Tissues from *Oregon-R* larvae. (G-I) Tissues from larvae that were hemizygous for the null $E(z)^{63}$ allele, used as controls to determine specificity of the E(Z) antibodies. (A,B) Wing and haltere imaginal discs, respectively, showing the ubiquitous nuclear distribution of E(Z) protein; (C) the CNS showing relatively weak staining; (D) representative salivary gland showing nuclear staining; (E,F) close-up images (magnified 400×) of salivary glands showing the intranuclear distribution of E(Z) in addition to the variability of staining seen in these tissues; (G) $E(z)^{63}/Df(3L)Ez2$ salivary gland showing absence of staining; (H) $E(z)^{63}/Df(3L)Ez2$ wing, haltere and metathoracic leg imaginal discs and (I) CNS showing absence of staining.

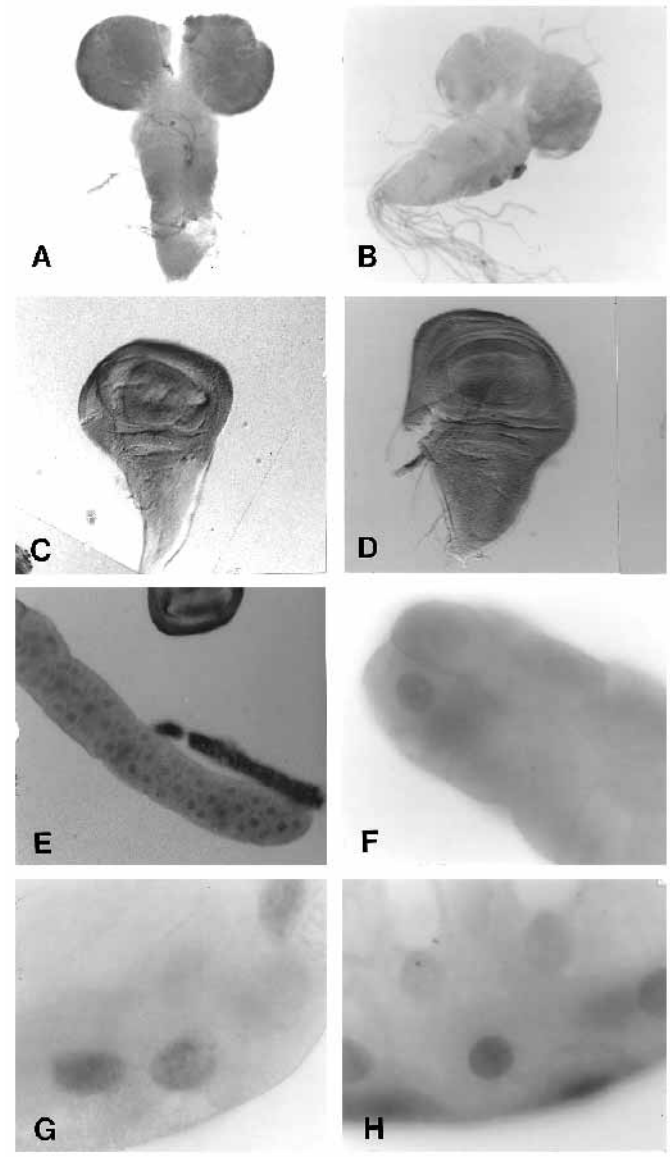

Fig. 5. Distribution of E(Z) in third instar larval tissues from *E(z)* temperature-sensitive mutants. (A,C,E) Tissues from $E(z)^{61}$ larvae reared continuously at 21°C. (B,D,F) Tissues from $E(z)^{61}$ larvae that had been shifted from 21°C to 29°C one day prior to dissection. (A,B) Normal distribution of E(Z) in the CNS from $E(z)^{61}$ larvae at both 21°C and 29°C, respectively. The staining in B is weaker than in A, however, it is similar to the wild-type staining seen in *Oregon-R* (see Fig. 4C). (C,D) Essentially all nuclei are stained in the wing disc from $E(z)^{61}$ larvae reared at both 21°C and 29°C, respectively. (E) Normal distribution of E(Z) in a salivary gland from an $E(z)^{61}$ larva reared at 21°C. (F) A close-up image of the salivary gland from an $E(z)^{61}$ larva following one day at 29°C. Note the near absence of staining. Also note that the intranuclear distribution of E(Z) appears homogenous in these mutants. (G,H) Close-up images of salivary glands from homozygous $E(z)^{28}$ and $E(z)^{32}$ larvae, respectively, that had been shifted from 21°C to 29°C one day prior to dissection. Note that while overall levels of staining appear to be reduced in $E(z)^{28}$, some protein still appears to be associated with the chromosomes. In $E(z)^{32}$, some protein is still nuclear, however, it is homogeneously distributed.

Table 1. Polytene chromosome binding sites for the E(Z) protein

Chromosome 1	Chromosome 2	Chromosome 3
1A	21A	61A
2D	21C*	61C
4C	22A	61F
5A	22B	68A
8A	24A	68C
8B	25E/F	69C
8F/9A	29E	70DE
12D	33F/34A	82D/E
	35AB	84A/B
	49EF	86C
	51A	87B
	56C	87F-88A
	60E	89B
	60F	89C
		89E
		90E
		93E
		96B/C
		99AB
		100A
		100B*
		100F

With two exceptions (*), all sites overlap with known Pc-G binding sites. SU(Z)2 also binds to the 21C region (Rastelli et al., 1993), but the 100B site appears to be novel.

not appear to be associated with chromosomes. $E(z)^{28}$ homozygotes show relatively weak or low levels of staining in salivary gland nuclei at 29°C. Within nuclei still staining for E(Z), the protein appears to retain at least some of its chromosomal pattern (Fig. 5G). Salivary gland nuclear staining varies from strong to weak in $E(z)^{32}$ homozygotes at 29˚C. The $E(Z)^{32}$ protein most often appears to be uniformly distributed within the nuclei (i.e., not specifically associated with chromosomes) (Fig. 5H).

Immunostaining of salivary gland polytene chromosomes with anti-E(Z) antibodies has allowed us to identify 44 binding sites for the E(Z) protein (Fig. 6A; Table 1). Additional weak sites are sometimes detectable, but are usually obscured by general chromosomal background signal when the staining reaction is allowed to continue. With the possible exception of one site at 21C5-6 (although SU(Z)2 is also reported to bind within the 21C region; Rastelli et al., 1993), and another at 100B, all other sites appear to overlap with known binding sites of other Pc-G proteins. To show specificity of the E(Z) antibodies, we stained chromosomes from hemizygous $E(z)^{63}$ larvae. Only a few faint bands are detected in some cases and are presumably residual maternally encoded protein (Fig. 6B).

A new E(Z)-binding site is seen in the P element transformant line 85-39 at the P element insertion site (62A) (Fig. 7). This reporter P element contains 14.5 kb from the *bxd cis*-regulatory region of *Ubx* (Simon et al., 1990), which has been shown to require the Pc-G, including *E(z)*, in order to maintain its anterior border of expression in embryos (Simon et al., 1993; R.J., unpublished observations). It thus contains a Polycomb response element (PRE) (Simon et al., 1993). This particular construct, and others that contain smaller fragments from the same *bxd* region, have been shown to direct chromosome binding by PH (DeCamillis et al., 1992), PSC, SU(Z)2 (Chan et al., 1994) and PC (Chiang et al., 1995). Therefore,

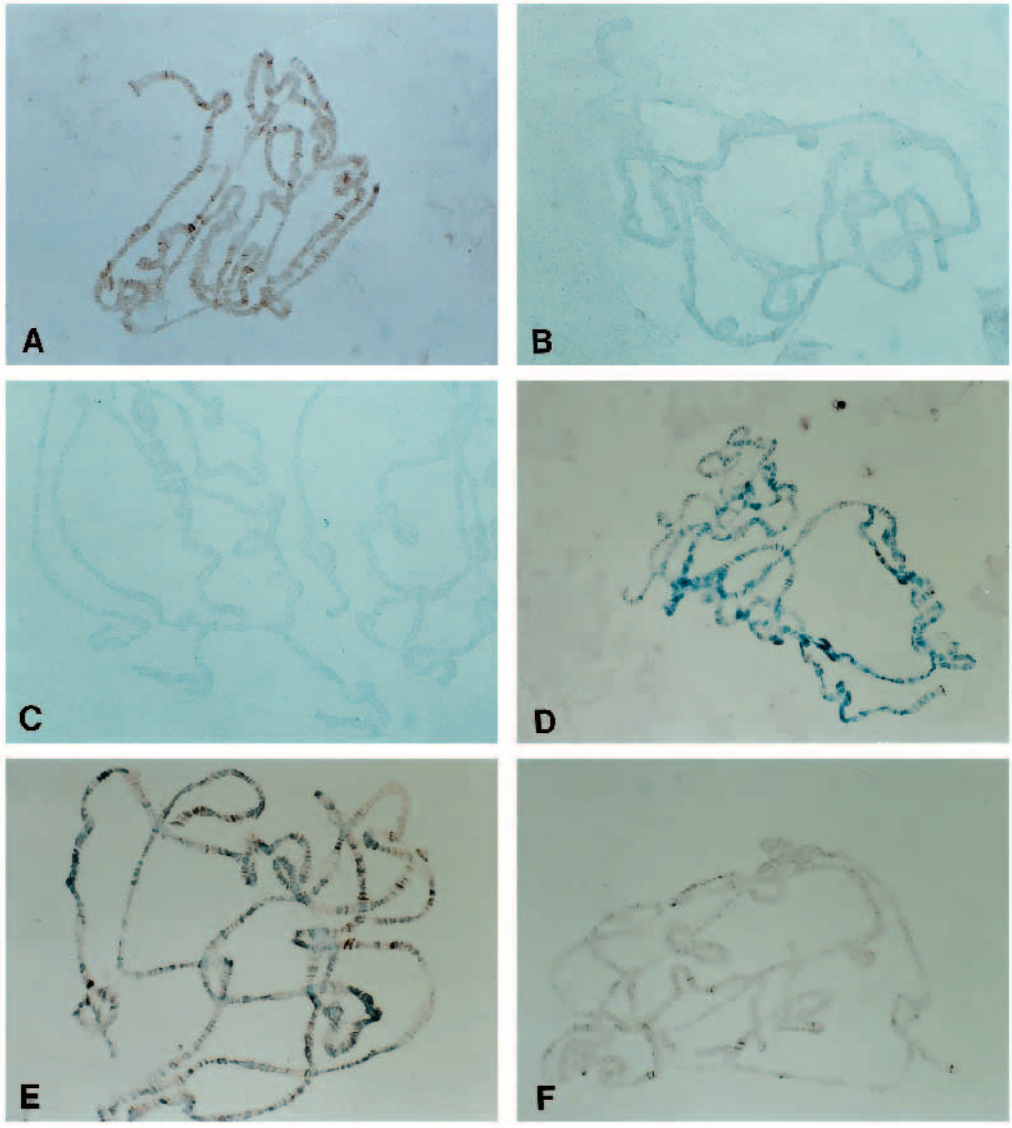

Fig. 6. Immunolocalization of E(Z) protein on salivary gland polytene chromosomes. (A) *Oregon-R* chromosomes showing multiple bands. The background staining described in the text is also evident here. (B) $E(z)^{63}/Df(3L)Ez2$ chromosomes; a few very faint bands are sometimes visible, and are presumably due to the perdurance of maternal $E(Z)^{+}$. (C) Chromosomes from homozygous $E(z)^{61}$ larvae shifted to 29°C show almost complete absence of staining. (D) Chromosomes from homozygous $E(z)^{32}$ larvae shifted to 29°C have some detectable bands, however, overall staining is strongly reduced. This is similar to the results obtained with homozygous $E(z)^{28}$ larvae at 29°C (not shown). (E,F) Chromosomes from homozygous trx^{1} mutants reared at 21°C and 29°C, respectively. Note the overall reduced levels of staining in trx^{1} mutants that were reared continuously at 29°C.

E(Z), like these other Pc-G proteins, binds to chromosomes in a DNA sequence-dependent manner.

Whole-mount staining of salivary glands from homozygous temperature-sensitive mutants suggested that the intranuclear distribution of these mutant proteins is altered at restrictive temperatures. In order to directly examine the effects of these mutations on chromatin binding, polytene chromosomes from homozygous $E(z)^{28}$, $E(z)^{32}$, and $E(z)^{61}$ larvae, which were reared as described above for whole-mount immunohistochemical staining, were probed with the anti-E(Z) antibodies. To control for differences in fixation and squashing, chromosomes from the same mutants reared continuously at 21°C were squashed on the same slides as their siblings that had been shifted to 29°C. All three temperature-sensitive alleles show wild-type chromosome binding at permissive temperatures (data not shown). However, chromosomes from larvae that were shifted to 29°C

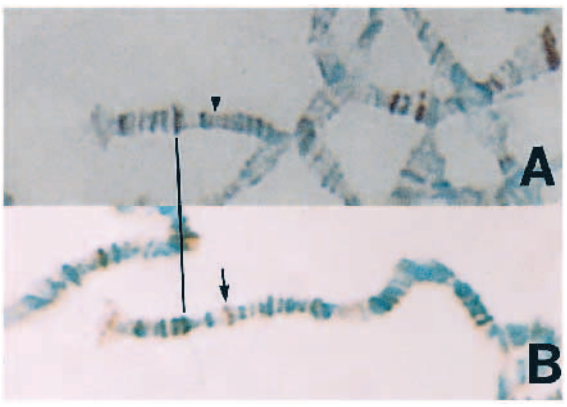

Fig. 7. The E(Z) protein binds to the 5′ *Ubx* regulatory region. (A) Wild-type 3L chromosome showing the absence of an E(Z)-binding site within 62A (arrowhead). (B) A new E(Z)-binding site is created in the P-element transformant line 85-39 (Simon et al., 1990) at the P element insertion site in 62A (arrow). This P element contains a 14.5 kb segment from the *bxd/pbx Ubx* regulatory region. The endogenous E(Z)-binding site at position 61F is indicated by the straight line. This site is partially obscured by Giemsa in the 85-39 line.

24 hours prior to dissection showed strongly reduced chromosome binding by the mutant proteins. Virtually all chromosome binding is eliminated in $E(z)^{61}$ chromosomes; however, a few faint bands are sometimes visible (Fig. 6C). One copy of $E(z)^{+}$ in *trans* to $E(z)^{61}$ at 29°C is sufficient to produce the normal E(Z)-binding pattern (data not shown). Both $E(z)^{28}$ and $E(z)^{32}$ (Fig. 6D) strongly reduce chromosome binding, but the effect is not quite as severe as that produced by $E(z)^{61}$. Nevertheless, only a few residual binding sites are usually visible in each case. For all three alleles, the effect on chromosome binding is more dramatic when the larvae have been shifted to 29˚C more than one day prior to dissection (E.C., unpublished observation).

Distribution of the E(Z) protein in trx^{1} larvae

The trithorax protein has been reported to bind polytene chromosomes at 16 (Kuzin et al., 1994) or 63 sites (Chinwalla et al., 1995). Although the total number of binding sites is in dispute, both Kuzin et al. (1994) and Chinwalla et al. (1995) report that many of the sites overlap with binding sites of Pc-G proteins. In homozygous trx^{1} temperature-sensitive mutants reared continuously under restrictive conditions (29°C), only a few TRX-binding sites remain (Chinwalla et al., 1995). trx^{1} contains a 9 kb insertion in the 5′ untranslated portion of the *trx* gene (Breen and Harte, 1991), which may result in altered expression of *trx* at restrictive temperature. Due to the fact that E(Z) shares significant homology with TRX in the C-terminal region, and because TRX binding decreases in *E(z)* mutants (Kuzin et al., 1994), we wanted to test the effect of reduced *trx* activity on chromosome binding by E(Z). We found that E(Z) binding is noticeably weaker in trx^{1} homozygotes derived from homozygous parents brooded continuously at 29°C compared to control trx^{1} homozygotes reared at permissive temperature (21°C) (see Fig. 6E,F). Overall, the same E(Z)-binding sites are detectable in this mutant background at permissive as well as restrictive temperature.

DISCUSSION

Distribution of E(Z) protein in wild-type embryos and larvae

Consistent with the function of *E(z)* in regulating ANT-C and BX-C genes throughout the anterior-posterior axis, the E(Z) protein is ubiquitously present in embryonic nuclei. We presume that much of this protein is maternally encoded, either directly loaded during oogenesis, or the translation product of abundant maternal *E(z)* mRNA (Jones and Gelbart, 1993). However, the ability of paternal copies of $E(z)^{+}$ to partially rescue the maternal effect of $E(z)^{61}$ (Jones and Gelbart, 1990) suggests that some of the embryonic protein is produced from zygotic mRNA. Survival of *E(z)* null individuals to late larval or early pupal stages (Shearn et al., 1978b) is probably due to perdurance of the abundant maternally encoded E(Z) protein. Indeed, a few faint E(Z)-binding sites are usually detectable on polytene chromosomes from larvae that are hemizygous for the $E(z)^{63}$ allele, which we have shown to be a protein null. Only actively dividing cells (e.g. imaginal disc and CNS cells) require continued zygotic expression of *E(z)* for proliferation and proper development. E(Z) is also ubiquitously expressed in these tissues, but is considerably less abundant than in embryos and the staining is more variable from individual to individual.

Chromosomal distribution of the E(Z) protein

Loci have been assigned to the Pc-G based on their involvement in the negative regulation of the ANT-C and BX-C segment identity genes. However, mutant alleles of most Pc-G loci reveal overlapping, and in some cases distinct, pleiotropic functions. For example, *Psc*, *Scm* and *E(z)* behave as modifiers of the $zeste^{1}$-*white* interaction (Wu et al., 1989; Jones and Gelbart, 1990), null alleles of *E(z)* block cell proliferation (Gatti and Baker, 1989; Phillips and Shearn, 1990), *mxc* is a tumor suppressor and is required for germ-line proliferation (Santamaria and Randsholt, 1995; Docquier et al., 1996), mutant alleles of *ph* and *pho* produce nervous system defects (Smouse et al., 1988; Girton and Jeon, 1994), and several Pc-G loci have been demonstrated to be involved in the regulation of segmentation genes (Breen and Duncan, 1986; Moazed and O'Farrell, 1992; Sinclair et al., 1992; McKeon et al., 1994; Pelegri and Lehmann, 1994). Therefore, it is unlikely that Pc-G proteins act as a monolithic group in the regulation of all target genes. Nevertheless, growing evidence supports a model in which Pc-G proteins repress the transcription of target genes through the formation of heteromeric complexes (Locke et al., 1988; Jurgens, 1985; Cheng et al., 1994; Campbell et al., 1995; Franke et al., 1992; Rastelli et al., 1993; Lonie et al., 1994; Reijnen et al., 1995). In support of this model, we find that the vast majority of E(Z)-binding sites are a subset of those described for other Pc-G proteins. Attempts to detect additional weak E(Z)-binding sites by prolonging the immunohistochemical staining reactions generally result in increased overall chromosomal staining. We are not sure if this represents a general chromosomal distribution of E(Z), with increased concentrations at those loci that we detect as bands, or if low abundance of E(Z) and/or the quality of our antibodies may permit us to identify only a subset of discrete E(Z)-binding sites. In the latter case, the background staining may simply represent a technical barrier of this detection method. Some support for the former alternative is provided by the general chromosomal pattern of staining that we observe in the nuclei of whole-mount salivary glands, which seems to be unlike the bright banded pattern described for the PC protein (Messmer et al., 1992). In addition, unlike other Pc-G proteins, absence of $E(Z)^{+}$ activity blocks cell proliferation and disrupts gross chromosome organization (Shearn et al., 1978b; Gatti and Baker, 1989; Phillips and Shearn, 1990; Rastelli et al., 1993). Therefore, low levels of generally distributed E(Z) protein may be involved in maintaining chromosome/chromatin integrity, but higher concentrations might be required to assist in the transcriptional repression of Pc-G target genes. Alternatively, the effects of *E(z)* mutations on cell proliferation and/or chromosome morphology may be due to misregulation of a target gene(s) or other indirect effects on cellular physiology. Regardless of which of these models is correct, it is highly likely that E(Z) is present at additional sites that we cannot identify as discrete bands. This conclusion is based on our inability to detect E(Z) binding at many loci at which PH, PSC, SU(Z)2 and PC binding is disrupted by heat inactivation of the $E(Z)^{61}$ product (Rastelli et al., 1993; Platero et al., 1996). In fact, six of the loci at which PSC and SU(Z)2 binding is retained in these *E(z)* mutants (4C, 21A, 49EF, 69CD, 84AB;

Rastelli et al., 1993) are among the most readily identifiable E(Z)-binding sites. Therefore, retention of PSC and SU(Z)2 at these sites is probably due to residual E(Z) protein at those loci where it is normally most abundant. Alternatively, other Pc-G proteins may not be equally dependent on E(Z) for binding at all loci. We have shown that association of the $E(Z)^{61}$ protein with chromosomes is disrupted at restrictive temperature. In addition, E(Z) binds in a sequence-dependent manner to a P element that contains a PRE from the *bxd* region of the *Ubx* gene. This PRE has been shown to direct chromosome binding by other Pc-G proteins (DeCamillis et al., 1992; Chan et al., 1994; Chiang et al., 1995). Taken together, these observations support a model in which the E(Z) protein is physically involved in the assembly and/or stabilization of Pc-G complexes at most, if not all, loci.

Recently, Platero et al. (1996) have provided additional evidence, which suggests that E(Z) plays a specific role in the protein-protein interactions that are required for Pc-G complex formation, even if generally distributed E(Z) may be involved in maintaining gross chromosome integrity. A chimeric protein, in which the HP1 chromodomain was replaced with the corresponding PC chromodomain, binds to both the heterochromatic chromocenter (as does endogenous HP1) and the euchromatic binding sites of PC (Platero et al., 1995). This chimeric protein is also able to recruit endogenous PC and PSC to the chromocenter. Heat inactivation of $E(Z)^{61}$ has no effect on heterochromatin binding by this chimera (or that of endogenous HP1), which is consistent with the absence of E(Z) from the chromocenter. However, it does disrupt HP1-PC chimera binding to euchromatic loci (Platero et al., 1996). In addition, inactivation of $E(Z)^{61}$ disrupts recruitment of PC and PSC to the chromocenter by the chimera. Since E(Z) is not normally detectable in the chromocenter, nor is necessary for HP1 chromosome binding, this suggests that E(Z) acts as an integral structural component of Pc-G complexes that is needed to mediate and/or stabilize Pc-G complex formation and that the effects of temperature-sensitive *E(z)* alleles on chromosome binding by other Pc-G proteins is not due to general effects on chromatin structure. Unfortunately, lack of conditional alleles of other Pc-G loci precludes reciprocal experiments that would permit further dissection of the individual roles played by each Pc-G protein in complex formation/stabilization. It is possible that inactivation of many (or even any) Pc-G proteins would destabilize the complex. It is also possible that removal of others, which may be essential for silencing but not the structural integrity of the complex, would have little or no effect on chromosome binding by other members of the group.

Analysis of mutant *E(z)* alleles reveals putative functional domains

We have shown that three *E(z)* temperature-sensitive alleles each contains a single point mutation that leads to a Cys to Tyr substitution. $E(z)^{32}$ and $E(z)^{61}$ contain amino acid substitutions at Cys_{545} and Cys_{603} respectively, which lie within a very Cys-rich region of the E(Z) protein (amino acids 538-603; Jones and Gelbart, 1993). $E(z)^{28}$ contains a substitution at Cys_{363}. $E(z)^{32}$ and $E(z)^{61}$ exhibit similar temperature-sensitive phenotypes (Phillips and Shearn, 1990; Jones and Gelbart, 1990). For example, at restrictive temperature females that are homozygous for either of these alleles produce maternal effect embryonic lethality and their embryos display strong posteriorly directed homeotic transformations. Chromosome binding by both the $E(Z)^{32}$ and $E(Z)^{61}$ proteins is disrupted at restrictive temperature. These observations support a model in which the Cys-rich region is a functional domain that is involved in chromosome binding. Preliminary results suggest that this domain does not bind to DNA in vitro (R. J. and Clark Jones, unpublished observations). Therefore, it may function as a protein-protein interaction domain. Presumably, these Cys residues are involved in stabilizing the tertiary structure of this domain, enabling it to interact with other chromatin proteins that mediate E(Z) chromosome binding. Of these three temperature-sensitive alleles, $E(z)^{28}$ exhibits the most severe phenotypes at restrictive temperature. For example, when homozygous $E(z)^{28}$ females are initially shifted up to 29°C, their embryos display the same strong homeotic phenotypes as do $E(z)^{32}$ or $E(z)^{61}$ embryos (R. J., unpublished observations). However, after prolonged exposure to restrictive temperature, fecundity of $E(z)^{28}$ females drops dramatically due to blockage in early oogenesis (Phillips and Shearn, 1990). These observations suggest that the Cys_{363} to Tyr substitution in $E(z)^{28}$ more completely inactivates the protein. It is therefore somewhat surprising that staining of intact polytene nuclei shows that $E(Z)^{28}$ chromosome binding is not as severely disrupted as is binding by $E(Z)^{32}$ or $E(Z)^{61}$. It may be that the Cys_{363} substitution more completely disrupts a domain that is required for E(Z) activity, but does not play as great a role in chromosome binding. Alternatively, $E(Z)^{28}$ may have a less reversible effect on chromosome binding. That is, once the $E(Z)^{28}$ protein assembles into Pc-G complexes at permissive temperature, its association may be at least partially stabilized by other domains (e.g., the Cys-rich region). Two human genes [*ENX-1* (Hobert et al., 1996b) and *E(z)H1*/*ENX-2* (Rommens et al., 1995; Hobert et al., 1996b; Abel et al., 1996] and one mouse gene [*Enx-1* (Hobert et al., 1996a)] that appear to be *E(z)* homologs have been cloned and sequenced. Each of the Cys residues that are mutated in these temperature-sensitive alleles is conserved in these mammalian homologs. In addition, both the entire Cys-rich region and several smaller regions immediately N-terminal to the $E(Z)^{28}$ mutation are highly conserved in these proteins, lending support to the model that these mutations disrupt distinct functional domains.

Effects of trx^1 on chromosome binding by the E(Z) protein

Kuzin et al. (1994) showed that polytene chromosome binding by TRX is strongly reduced in homozygous $E(z)^{61}$ and $E(z)^{32}$ larvae at restrictive temperature. Intriguingly, many of the TRX sites that are disrupted do not overlap with E(Z) or other PcG-binding sites. Again, this suggests that E(Z) may be present at more chromosomal sites than we can detect due to its low abundance or inaccessibility to antibodies. It is not clear whether the dependence of TRX on E(Z) for chromosome binding is due to their direct, or indirect, interaction within protein complexes, or whether this reflects a more general effect of E(Z) on chromatin structure. In a somewhat reciprocal experiment, we find that overall polytene chromosome binding by E(Z) is reduced, but not eliminated, in larvae from a homozygous trx^1 stock at restrictive temperature. Immunoblots of trx^1 salivary gland extracts show that the overall level of E(Z) protein itself is not reduced in these

mutants (data not shown). *trx*^{1} is an unusual temperature-sensitive allele. Unlike typical temperature-sensitive alleles, which contain point mutations that destabilize a protein's secondary or tertiary structure, *trx*^{1} contains a transposable element inserted into its 5′ untranslated region (Breen and Harte, 1991). Presumably, this somehow causes a reduced level of *trx* expression at elevated temperatures. However, in order to see an effect on chromosome binding by the TRX^{1} protein, it is necessary to collect embryos from *trx*^{1} adults at 29°C and then continuously maintain the embryos and resulting larvae at restrictive temperature (Chinwalla et al., 1995). Therefore, a reduced level of E(Z) chromosome staining in larvae reared under these conditions may not necessarily suggest a physical interaction between these two proteins. Instead, it is very possible that reduced *trx* activity alters the expression of one or more other proteins that are involved in E(Z) chromosome binding. For example, TRX has been shown to bind to the cytogenetic location of the *Psc* and *Su(z)2* genes, implying a potential regulatory interaction (Chinwalla et al., 1995). In support of this model, all E(Z)-binding sites are roughly equally affected, with no detectable differences between those that overlap with known TRX-binding sites and those that do not. If such regulatory interactions exist, they must be taken into consideration when interpreting genetic interactions between Pc-G and trx-G mutations.

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