The *Trithorax-mimic* Allele of *Enhancer of zeste* Renders Active Domains of Target Genes Accessible to *Polycomb*-Group-Dependent Silencing in *Drosophila melanogaster*

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ABSTRACT

Two antagonistic groups of genes, the trithorax- and the Polycomb-group, are proposed to maintain the appropriate active or inactive state of homeotic genes set up earlier by transiently expressed segmentation genes. Although some details about the mechanism of maintenance are available, it is still unclear how the initially active or inactive chromatin domains are recognized by either the trithorax-group or the Polycomb-group proteins. We describe an unusual dominant allele of a *Polycomb*-group gene, *Enhancer of zeste*, which mimics the phenotype of loss-of-function mutations in *trithorax*-group genes. This mutation, named $E(z)^{Trithorax\ mimic}$ [$E(z)^{Trm}$], contains a single-amino-acid substitution in the conserved SET domain. The strong dominant trithorax-like phenotypes elicited by this *E(z)* allele suggest that the mutated arginine-741 plays a critical role in distinguishing between active and inactive chromatin domains of the homeotic gene complexes. We have examined the modification of $E(z)^{Trm}$ phenotypes by mutant alleles of PcG and trxG genes and other mutations that alter the phosphorylation of nuclear proteins, covalent modifications of histones, or histone dosage. These data implicate some trxG genes in transcriptional repression as well as activation and provide genetic evidence for involvement of histone modifications in PcG/trxG-dependent transcriptional regulation.

SEGMENTAL identity in Drosophila is determined by two clusters of homeotic genes, the *Antennapedia*- (ANT-C; Kaufman *et al.* 1990) and the *bithorax*- (BX-C; Lewis 1978) complexes. Homeotic genes are expressed in a sequential order along the anterior-posterior axis of the fly. The complex expression pattern of the BX-C genes is due to the action of nine parasegment-specific *cis*-regulatory domains (*abx/bx*, *bxd/pbx*, *iab-2*, *iab-3*, *iab-4*, *iab-5*, *iab-6*, *iab-7*, and *iab-8*; Duncan 1987). Each domain is responsible for setting the appropriate parasegmental level of transcription of one of the three homeotic genes in BX-C. The activity patterns of these *cis*-regulatory regions are set early in development by protein products of gap and pair-rule segmentation genes (Shimell *et al.* 1994).

By midembryogenesis, when the products of the segmentation genes disappear, the regulation of the homeotic genes switches to a maintenance mode that preserves the initial pattern of activity through the remainder of development (Paro 1990, 1993). Maintenance of the inactive state requires the action of the Polycomb-group (PcG) of proteins. Loss-of-function (LOF) mutations in PcG genes cause an inappropriate expression of homeotic genes in parasegments where they are normally repressed. The resulting ectopic expression of homeotic genes leads to the transformation of parasegments to more posterior identities. By contrast, the *trithorax*-group (trxG; Shearn 1989) of genes is responsible for maintaining the active state of homeotic genes. Mutations of this group typically result in the inappropriate inactivation of homeotic genes, which leads to the transformation of parasegments toward more anterior identities. Mammalian PC-G and TRX-G protein homologs appear to have functions similar to their Drosophila counterparts (Müller *et al.* 1995; Faust *et al.* 1998; Hanson *et al.* 1999; Tomotsune *et al.* 1999; Akasaka *et al.* 2001). Establishment and maintenance of PcG-dependent silencing requires specific DNA regions, called Polycomb response elements (PREs; Simon *et al.* 1993; Gindhart and Kaufman 1995; Poux *et al.* 1996; Hagström *et al.* 1997; Mihály *et al.* 1997), which appear to be the primary targets of PcG proteins. Likewise, trxG-dependent activation requires Trithorax response elements (TREs; Tillib *et al.* 1999). PREs and TREs, although separable, may be very closely associated, suggesting the potential for molecular competition between PcG and trxG proteins.

Genetic studies suggest, and molecular evidence confirms, that PcG proteins function cooperatively and

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form multimeric complexes (Kingston *et al.* 1996; Strutt and Paro 1997; Orlando *et al.* 1998; Shao *et al.* 1999). For example, the phenotype of a mutation in one member of the PcG is usually enhanced by a mutant allele of another gene of the group, although the extent of enhancement varies with different pairwise combinations of PcG genes (Jürgens 1985; Cheng *et al.* 1994; Campbell *et al.* 1995). Antibodies directed against different PcG proteins often label common chromosomal sites (DeCamillis *et al.* 1992; Rastelli *et al.* 1993; Carrington and Jones 1996), and *in vivo* assembled PcG complexes can be isolated by immunoprecipitation (Kingston *et al.* 1996; Shao *et al.* 1999). In a number of cases, direct protein-protein interactions between different PcG proteins have also been demonstrated (Peterson *et al.* 1997; Jones *et al.* 1998; Kyba and Brock 1998; Tie *et al.* 1998). Consistent with these observations, two multimeric PcG complexes have been identified. The PRC1 complex includes the Polycomb (PC), Polyhomeotic (PH), Posterior Sex Combs (PSC), and Sex Combs on Midleg (SCM) PcG proteins (Shao *et al.* 1999). A second complex contains the Extra Sex Combs (ESC) and Enhancer of Zeste [E(Z)] PcG proteins (Ng *et al.* 2000; Tie *et al.* 2001).

The antagonistic activities of trxG and PcG proteins involve modulation of chromatin structure. One member of the trxG, *Trl*, encodes the Drosophila GAGA factor (Farkas *et al.* 1994). The Brahma (BRM) and Moira (MOR) trxG proteins are components of the BRM protein complex, which is similar to the SWI/SNF complex (Papoulas *et al.* 1998; Crosby *et al.* 1999). The ESC-E(Z) complex also includes the histone binding protein p55 and the histone deacetylase RPD3 (Tie *et al.* 2001). A possible direct molecular antagonism is implicated by the finding that the PRC1 complex can inhibit the ATP-dependent chromatin remodeling activity of the SWI/SNF complex *in vitro* (Shao *et al.* 1999).

Genetic studies have begun to blur the delineation between the Pc- and trx-groups, suggesting that some proteins, previously placed in either the PcG or trxG, may be involved in both activation and silencing (for review see Brock and van Lohuizen 2001). For example, some loss-of-function alleles of *E(z)* enhance the extent of the anteriorly directed homeotic transformations caused by a trxG gene, *abnormal small and homeotic discs-1* (*ash1*; Lajeunesse and Shearn 1996). Recently, a more extensive survey has shown that several additional PcG genes {*Psc, Scm, Additional sex combs* (*Asx*), *Enhancer of Polycomb* [*E(Pc)*], and *Suppressor of zeste 2* [*Su(z)2*]} are also phenotypic enhancers of *ash1* mutations. These genes, formally categorized as PcG members, are suggested to form a third group of maintenance genes, *Enhancer of trithorax and Polycomb* (*ETP*; Gildea *et al.* 2000). On the other hand, the product of the trxG *Trl* gene has been suggested to also be involved in PcG-dependent silencing (Hagström *et al.* 1997; Busturia *et al.* 2001; Hodgson *et al.* 2001; Mishra *et al.* 2001; Poux *et al.* 2001).

In this article, we describe an unusual dominant allele of the *E(z)* gene, the charter member of the ETP group. On the basis of the strong dominant trithorax-like phenotypes elicited by our allele, we have named it $E(z)^{Trithorax\ mimic}$ [$E(z)^{Trm}$]. The trithorax-like phenotypes of $E(z)^{Trm}$ are due to inappropriate silencing of homeotic genes and *engrailed* in regions where these genes should stay active. $E(z)^{Trm}$ phenotypes are suppressed by PcG mutations and enhanced by some trxG mutations. However, mutant alleles of several other trxG genes suppress at least some $E(z)^{Trm}$ phenotypes, suggesting that their products may be involved in silencing as well as activation and should be added to the ETP category. In addition, some mutations suppress certain $E(z)^{Trm}$ phenotypes but either enhance or have no significant effect on others.

We show that the mutant phenotype is due to a conversion of an arginine (Arg 741), conserved among different homologs of the Drosophila E(Z) SET domain, into lysine, conserved at the same position within the SET domain of TRX homologs. Significantly, a second independent mutation [$E(z)^{TrmTG}$] with identical phenotypes carries the same conversion. We interpret the $E(z)^{Trm}$ phenotype to be a result of the misidentification of active chromatin by the mutant *E(z)* gene product, suggesting that Arg 741 plays a critical role in the proper identification of active *vs.* inactive chromatin domains by E(Z) in target genes. On the basis of the molecular nature, phenotype, and genetic interactions of $E(z)^{Trm}$, we propose that the wild-type E(Z) recognizes a phosphorylated factor that marks active domains.

Our data also suggest that hyperacetylation of histones may be another important factor involved in preventing inappropriate silencing of active domains of target genes by PcG proteins.

MATERIALS AND METHODS

General procedures: Fly stocks were maintained on standard yeast-cornmeal medium containing propionic acid (0.53%) and phosphoric acid (0.053%) as mold inhibitor. Crosses were performed at 25° *en masse*. $Fab\text{-}7^{1}/Fab\text{-}7^{1}$ males were treated with 25 mm ethyl methanesulfonate (EMS) and crossed to Oregon-R homozygous females. Among ~80,000 F_1 progeny one male exhibiting strong dominant trx-like phenotype was selected and used for establishing a balanced stock of the $E(z)^{Trm}$ allele.

In experiments involving the use of Na-butyrate (Merck, St. Louis) the compound was added as an aqueous solution after the temperature of the medium dropped below 57°, and propionic acid/phosphoric acid was replaced by Tegosept M as mold inhibitor as described in Reuter *et al.* (1982).

$E(z)^{Trm}$ stocks are poorly viable and fertile. Moreover, we found that over a period of time they spontaneously acquire modifiers that suppress the dominant phenotype and increase the viability of $E(z)^{Trm}$. Therefore, to ensure that each experiment is carried out on the same genetic background, we maintained and regularly checked the phenotype of two to three parallel lines of each stock of $E(z)^{Trm}$. Only the lines that preserved the original phenotype were used in genetic experiments.

Homozygous or hemizygous $E(z)^{Trm}$ flies die as fully developed pharate adults that can be rescued by dissecting them out of their pupal case. Such rescued adults may survive for

1 or 2 days, allowing the examination of their phenotype when fully pigmented.

All mutant alleles used in this work are described in Lindsley and Zimm (1992) and Flybase (http://flybase.bio.indiana.edu).

Characterization of genetic interactions with $E(z)^{Trm}$: Genetic interactions of $E(z)^{Trm}$ were tested by examining *trans*-heterozygous adult flies under a dissecting scope. Special care was taken to avoid overcrowding and losing flies due to sticking in the media. It was especially important in cases when *trans*-heterozygotes exhibited strong enhancement of the trx-like transformations, which generally correlates with low viability.

To test for potential interactions, reciprocal crosses between stocks carrying mutant alleles of the genes to be tested and $E(z)^{Trm}$ were performed. To allow unambiguous identification of *trans*-heterozygous combinations of $E(z)^{Trm}$ with strongly suppressed Trm phenotype, we used an $E(z)^{Trm}$ line marked with the dominant marker $Fab\text{-}7^{l}$.

The effects of different mutations on the phenotype of $E(z)^{Trm}$ were assayed by evaluating the degree of homeotic transformations. To characterize the strength of the observed effect in a quantitative way, the number of flies that had two, one, or no first and third legs with apical bristles (transformation of the first or third thoracic segment toward a second thoracic segment identity) were counted. As an indication of more extreme transformations, the frequency of the presence of sternopleural bristles on the proximal lateral prothorax and metathorax was also calculated. To characterize abdominal transformations in males, a three-grade scale was set up: grade 1 corresponded to nearly wild-type (black) A5 pigmentation with at most small dispersed light spots in the anterior of the fifth tergite; grade 2 corresponded to an A5 tergite with larger lightly pigmented areas occupying at least one-third of the tergite; while grade 3 represented a higher degree of transformation toward A4 with more than one-half of the A5 tergite lacking black pigmentation. In each case, we examined >100 flies of the appropriate genotypes (except in cases indicated in the tables, when the viability of *trans*-heterozygotes was extremely low).

Analysis of the suppressor of *nos* phenotype: Suppression of the *nos* phenotype was analyzed by producing an $E(z)^{Trm}$ hb^{7M} nos^{L7} recombinant line and crossing it to either nos^{L7} or one of three different $E(z)^{son}$ nos^{L7} strains. As an internal control, the original experiments of Pelegri and Lehmann (1994) were also reproduced by crossing the $E(z)^{son}$ nos^{L7} lines with hb^{7M} nos^{L7} or nos^{L7} under the same conditions. Virgins of the desired genotype (Table 2) were collected, mated with Oregon-R males, and allowed to lay eggs for 4 hr at 25°. Embryos were allowed to develop cuticular structures and the number of abdominal segments in dechorionated embryos embedded in Hoyer's medium was scored (Wieschaus and Nüsslein-Volhard 1986).

Cuticle preparations: Adult wings and thoraces were mounted in Hoyer's solution after boiling flies of appropriate genotypes in 10% KOH for 5 min. Abdominal cuticles were mounted as described by Duncan (1982).

Immunohistochemical staining of polytene chromosomes: We first applied the method described in Carrington and Jones (1996) to detect E(Z) binding sites on polytene chromosomes, using affinity-purified rabbit E(Z) antibodies described by the same authors. Although we found no significant difference between the staining pattern of wild type and $E(Z)^{TRM}$, comparison was hampered by the inconsistent appearance of some weaker signals. To make the comparison more reliable, we modified the described method as follows. Salivary glands were quickly dissected directly in 3.7% formaldehyde-50% acetic acid and were squashed in the same solution. The protocol described for antibody staining of embryos in Sipos *et al.* (1998) was followed to stain chromosomes. Although this procedure resulted in a higher background staining, it produced good chromosome morphology and improved signal detection.

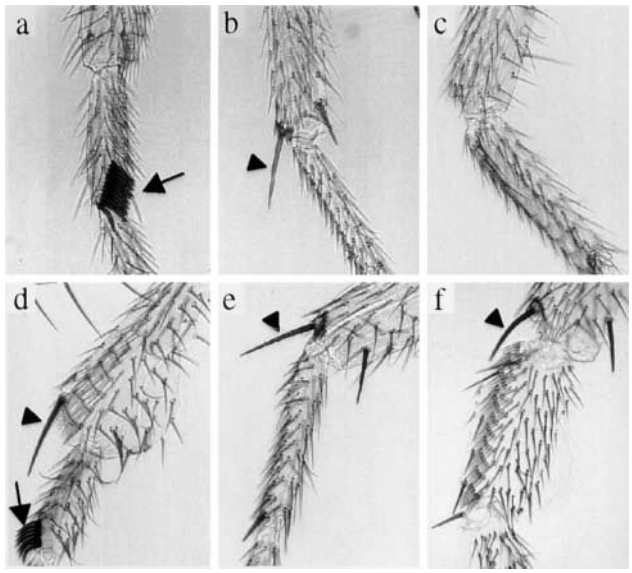

Figure 1.—Comparison of legs of wild-type and $E(z)^{Trm}$ males. (a–c) The first, second, and third legs of wild-type flies; (d–f) the first, second, and third legs of $E(z)^{Trm}$/+ heterozygous flies. Arrows point to sex combs, and arrowheads mark apical bristles. Note the reduced number of sex comb teeth on the first leg in d and the presence of ectopic apical bristles in d and f.

Wild Makroscope M24 and Hitachi KP-C550 CCD cameras were used for taking microscopic photographs, which were subsequently processed with the Adobe PhotoShop program.

Genomic DNA sequencing: For the localization of the $E(z)^{Trm}$ mutation, genomic DNA from $E(z)^{Trm}/Df(3L)Ez2$ hemizygous larvae was isolated and amplified as described for other *E(z)* point mutations in Carrington and Jones (1996). Sequencing of the PCR products carrying the $E(z)^{Trm}$ mutation revealed a single guanine-to-adenine transition at nucleotide position 2325 (Jones and Gelbart 1993) when compared to parental $Fab\text{-}7^{l}/Fab\text{-}7^{l}$ DNA.

Protein sequence comparison: Proteins containing the SET domain were selected by searching the GenBank and EMBL databanks against the E(Z) SET domain, using the FASTA, TFASTA, and BLAST algorithms.

RESULTS

***Trithorax-mimic* is an unusual gain-of-function allele of *Enhancer of zeste*:** In a screen for suppressors of *Frontabdominal-7*^{l} ($Fab\text{-}7^{l}$), a dominant gain-of-function (GOF) mutation that transforms the sixth abdominal segment (A6) into A7 (Gyurkovics *et al.* 1990), we isolated a mutation exhibiting strong dominant *trx*-like phenotypes. We termed this mutation *Trithorax-mimic* (*Trm*), as subsequent experiments proved that it was not allelic to any known members of the trxG.

Trm heterozygotes show severe anteriorly directed transformations in the segments that fall under the control of BX-C. These include the partial transformation of the third thoracic (T3) segment into T2 (Figure 1f), the fifth abdominal segment (A5) into A4, and A6 into A5 (Figure 2b). Interestingly, A7 is rarely modified. The varying degree of transformation of different segments

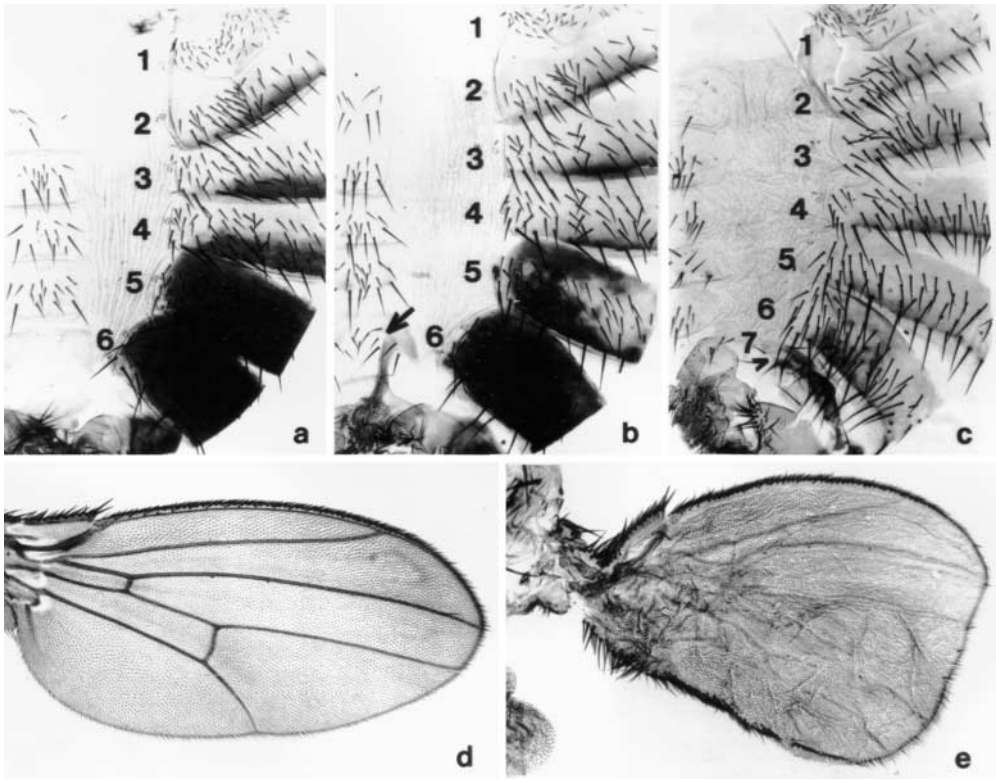

FIGURE 2.—Abdominal and wing phenotype caused by the $E(z)^{Trm}$ mutation. (a–c) Abdominal segments (numbered 1–7) of wild-type (+/+), $E(z)^{Trm}$/+, and $E(z)^{Trm}/Df(3L)Ez2$ males, respectively. (b) Patches lacking dark pigmentation on segment A5 indicate partial transformation of A5 into A4. Hairs on the sixth sternite denote partial A6 to A5 transformation (arrow). (c) Both A5 to A4 and A6 to A5 transformations are more extreme in hemizygotes than in heterozygotes. The appearance of a vestigial seventh tergite (arrowhead) is a consequence of the partial transformation of A7 into A6. (d–e) Wings of wild-type (d) and $E(z)^{Trm}/E(z)^{Trm}$ (e) flies, respectively. Note that in e the posterior compartment of the wing blade is replaced by structures characteristic of the anterior compartment (*e.g.*, appearance of thick bristles, known as the triple-row, at the posterior margin).

suggests that the mutation may affect *cis*-regulatory regions rather than the homeotic genes themselves. Expression of ANT-C is also affected, as shown by the reduced number of sex comb teeth and by the appearance of ectopic apical bristles on the first pair of legs (transformation of T1 toward T2; Figure 1d). Homozygous *Trm* flies die as fully developed pharate adults with an extremely strong trx phenotype. For example, not only the haltere (T3) but also the central part of the humerus (T1) is often transformed into wing tissue (Figure 3b). A7 is also partially transformed into A6, as indicated by the appearance of a rudimentary seventh tergite (similar to hemizygotes shown in Figure 2c). The most extreme transformation is seen in the ventral genitalia of both sexes, which are frequently replaced by leg tissue (not shown). Additionally, clones of anterior wing tissue appear on the posterior wing-blades (Figure 2e), indicating that the *engrailed* gene is inactivated in its normal domain of action (García-Bellido and Santamaría 1972; Guillen *et al.* 1995).

The *Trm* mutation was mapped by meiotic recombination to position 34.25 ± 0.5 on the third chromosome, a region not harboring any known trxG gene. The only known gene associated with a homeotic effect in this region is *Enhancer of zeste* [*E(z)*]. Based on its LOF phenotype, the *E(z)* gene is classified as a member of the *Polycomb*-group (Jones and Gelbart 1990; Phillips and Shearn 1990). However, a more recent report raised the possibility that *E(z)* may also be classified as a trxG gene (LaJeunesse and Shearn 1996). Therefore, in the absence of other obvious candidates, we crossed *Trm* to different loss-of-function mutations of *E(z)* to test them for allelism. Surprisingly, we found that *Trm/E(z) trans*-heterozygotes, like homozygous *Trm*, die as fully developed pharate adults with an enhanced trx phenotype intermediate between that of heterozygotes and homozygotes, but without a detectable Polycomb-like phenotype (Figure 2c). While noncomplementation of *Trm* by LOF *E(z)* alleles indicates allelism, the dominant trx-like phenotype suggests that *Trm* is an unusual GOF mutation of *E(z)*.

We confirmed this hypothesis by generating eight X-ray-induced phenotypic revertants of *Trm*. None of the revertants complemented the various *E(z)* alleles tested [$E(z)^1$, $Df(3L)Ez^{IR3}$, and $E(z)^{60}$; Kalisch and Rasmuson 1974; Wu *et al.* 1989; Jones and Gelbart 1990]. The phenotype and lethal phase of all revertants were comparable to those of amorphic *E(z)* alleles (Shearn 1977; Jones and Gelbart 1990). Two of the revertants carried cytologically visible breakpoints in the chromosome band 67E3–4, the cytological position of *E(z)*. Moreover, Southern blot analysis revealed molecular lesions within the *E(z)* locus in three revertants, using *E(z)*-specific cDNA probes (data not shown). These results defined *Trm* as a GOF allele of the *E(z)* gene [$E(z)^{Trm}$].

Molecular characterization of $E(z)^{Trm}$: The molecular nature of the $E(z)^{Trm}$ mutation was determined by sequencing the PCR-amplified mutant allele. A single guanine-to-adenine transition in the 741st codon was found

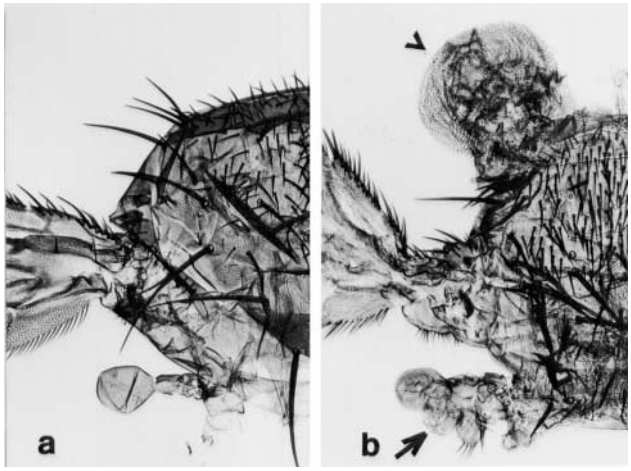

FIGURE 3.—The "trithorax" phenotype of $E(z)^{Trm}$. Wild-type (a) and $E(z)^{Trm}/E(z)^{Trm}$ (b) thoraces are shown. Transformations of T3 toward T2 and T1 toward T2 are indicated by the appearance of wing tissue in both haltera and humerus (arrow and arrowhead, respectively) in b.

to be the only difference from the parental allele, which results in the substitution of a lysine for an arginine. The affected amino acid resides near the C-terminal end of the E(Z) protein, in a region termed the SET domain. This domain is conserved in various proteins, including TRX (MAZO *et al.* 1990; BREEN and HARTE 1991; JONES and GELBART 1993; TSCHIERSCH *et al.* 1994).

We sequenced the DNA corresponding to the SET domain of a second mutation, $E(z)^{TrmTG}$ (a kind gift of Tony Greenberg), with a phenotype indistinguishable from that of $E(z)^{Trm}$. [Due to the identical phenotypes of the two mutations, $E(z)^{TrmTG}$ was not characterized in detail.] Strikingly, we found that this allele carries the same guanine-to-adenine transition as our allele does. The possibility of cross-contamination between the two mutations can be excluded, because $E(z)^{Trm}$ (and its parental chromosome) differs from $E(z)^{TrmTG}$ by having a second silent mutation (transition of G1999 to C) just 1 bp upstream of the beginning of the SET domain. This result suggests that the arginine 741-lysine substitution is critical for the phenotype of $E(z)^{Trm}$.

Comparison of the SET domains of different proteins revealed that the amino acid affected by the $E(z)^{Trm}$ mutation is a conserved arginine in the E(Z) homologs of each organism that possesses a more or less complete set of PcG proteins (Figure 4a). Conversely, TRX homologs have a conserved lysine at the same position (Figure 4b). Thus, the $E(z)^{Trm}$ mutation provides the protein with a TRX-like character at the molecular level.

Chromosomal distribution of the E(Z)^{TRM} protein: A simple explanation of the Trm phenotype could be that the mutant protein has an altered target-binding specificity. To test this possibility, we stained the polytene chromosomes of wild-type and hemizygous $E(z)^{Trm}$ with anti-E(Z) antibodies (see MATERIALS AND METHODS). We found that the binding pattern of the mutant protein is indistinguishable from the wild type both in distribution and intensity (Figure 5). This suggests that the mutant protein induces "ectopic" silencing of regions that are also the targets of the wild-type E(Z).

Interaction of $E(z)^{Trm}$ with *zeste*: The binding of the mutant protein encoded by the $zeste^{1}$ allele of the *zeste* (*z*) gene to the enhancer region of the *white* (*w*) gene renders *w* susceptible to silencing by some PcG proteins, including E(Z) (WU *et al.* 1989; JONES and GELBART 1990; PIRROTTA 1991). In fact, the *E(z)* gene was originally identified as a modifier of the $zeste^{1}$-*white* (z^{1}-*w*) interaction (KALISCH and RASMUSON 1974). While all known null and antimorphic alleles of *E(z)*, as well as all revertants of $E(z)^{Trm}$, are suppressors of the z^{1}-*w* interaction (JONES and GELBART 1990), $E(z)^{Trm}$, similarly to

locus	organism	GenBank	C-terminal end of SET domain sequences
a			
E(z)	Dm	U00180	GEELFfDY**R**Ygpt
EZH^{1}	Hs	U50315	GEELFfDY**R**Ysqa
Ezh^{1}	Mm	U60453	GEELFfDY**R**Ysqa
EZH^{2}	Hs	U61145	GEELFfDY**R**Ysqa
Clf	At	Y10580	GEELFyDY**R**Yepd
b			
trx	Dm	M31617	gEELTYDY**K**FpfE
HRX	Hm	L04284	gEELTYDY**K**FpiE
All^{1}	Mm	L17069	gEELTYDY**K**FpiE
SET^{1}	Sc	ysch8263	sEELTYDY**K**FerE
c			
Su(var)3-9	Dm	X80070	gEELsFDYiradn
MG44	Hs	L08238	gEELtFDYnmqvd
Mes-2	Ce	AF011893	sEELtFDYsysge

FIGURE 4.—The C-terminal end of the SET domains of E(Z)- and TRX-type proteins. Sequences are represented by the single-letter amino acid code. The amino acid affected by the $E(z)^{Trm}$ mutation is highlighted in boldface. Abbreviations for organisms are Ce, *Caenorhabditis elegans*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Sc, *Saccharomyces cerevisiae*; and At, *Arabidopsis thaliana.* Amino acids conserved within each protein group are in upper case. (a) Alignment of E(Z)-type SET domains. (b) TRX-type proteins aligned to the E(Z) SET domain. (c) Selected proteins aligned to the E(Z) SET domain.

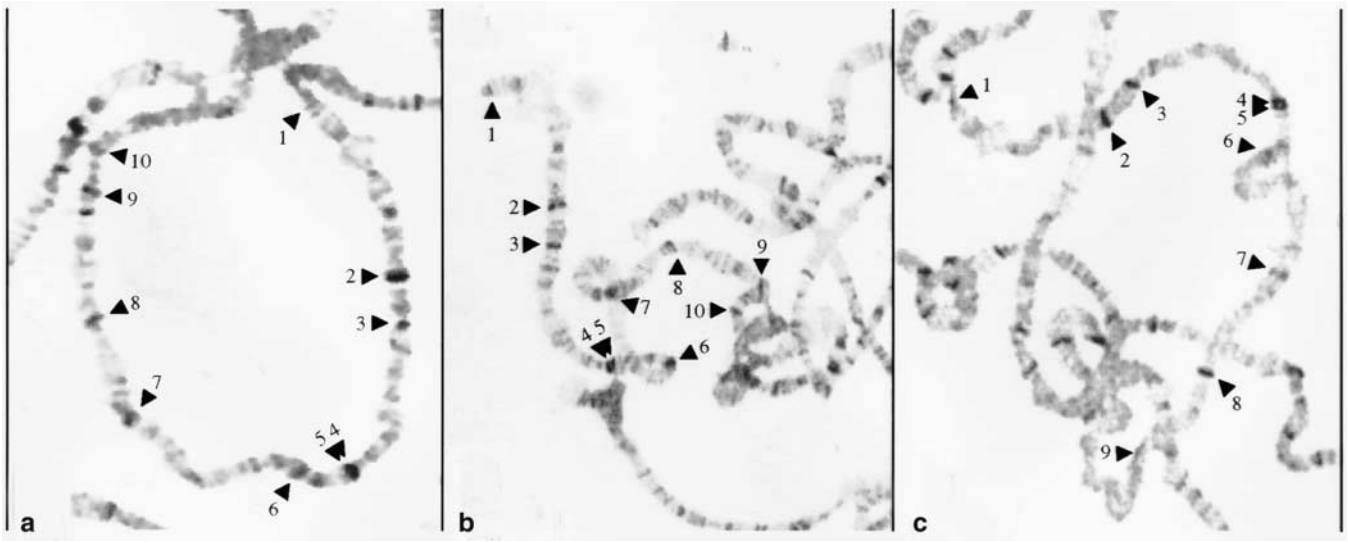

FIGURE 5.—Comparison of the binding specificity of wild-type E(Z) and E(Z)^{TRM} protein on salivary gland chromosomes. (a and c) The X chromosomes of two different *E(z)^{Trm}/Df(3L)Ez2* larvae illustrate variability in the intensity of staining. (b) The X chromosomes of wild-type larvae. Chromosomes were stained with polyclonal antibody directed against the E(Z) protein. The most prominently stained sites are marked with triangles and numbered from distal to proximal to serve as landmarks. Note the general similarity in the distribution and relative intensity of specific sites in a, b, and c.

the prototypic *E(z)^{1}* mutation, enhances the *z^{1}* phenotype. However, the *E(z)^{1}* allele requires the presence of the wild-type allele of *E(z)* for the enhancement of *z^{1}*, suggesting that the gene product of *E(z)^{1}* exerts its effect through the wild-type E(Z) polypeptide (JONES and GELBART 1990). In contrast, enhancement of the *zeste^{1}* phenotype by *E(z)^{Trm}* is less pronounced in the presence of the wild-type allele. Thus, *E(z)^{Trm}/Df(3L)Ez2* flies (rescued by dissecting out of their pupal case) have orange-brown eye color in both sexes, while *z^{1}/Y; E(z)^{Trm}*/+ males or *z^{1}*/+; *E(z)^{Trm}*/+ females differ from wild type only by having small brown spots on an otherwise wild-type background. These results suggest that the wild-type E(Z) competes with the mutant protein. This view is supported by the more severe anteriorly directed transformations exhibited by *E(z)^{Trm}/Df(3L)Ez2* hemizygotes as compared to *E(z)^{Trm}*/+ heterozygotes (compare Figure 2b and 2c). Additionally, extra copies of the wild-type *E(z)* in the form of a transgene that is able to rescue lethality of LOF *E(z)* alleles (JONES and GELBART 1993) strongly alleviate the trx-like phenotype of *E(z)^{Trm}*/+ flies (Table 1).

Interaction of *E(z)^{Trm}* with antimorphic *E(z)* alleles: Surprisingly, the antimorphic (dominant negative) alleles *E(z)^{son1}*, *E(z)^{son2}*, or *E(z)^{son3}* (PELEGRI and LEHMANN 1994) are not only viable over *E(z)^{Trm}* but strongly suppress the trx-like phenotype. This suppression is even stronger than that caused by an extra wild-type copy of *E(z)* (Table 1). For example, the phenotype of *E(z)^{son1}/E(z)^{Trm}* is nearly wild type. This efficient suppression of *E(z)^{Trm}* suggests that the mutant polypeptides encoded by these antimorphic alleles are somehow able to alter the conformation of the TRM protein, which requires a physical interaction between the protein products of *E(z)^{Trm}* and the antimorphic alleles, supporting the notion that two or more E(Z) polypeptides may form an active homomeric complex (JONES and GELBART 1993). The antimorphic allele, *E(z)^{60}*, which codes for a truncated protein (lacking the SET domain and an adjacent cysteine-rich region), also survives over *E(z)^{Trm}*, implying that the truncated E(Z)^{60} protein can still interact or compete with E(Z)^{TRM}. However, the severity of the phenotype of *E(z)^{Trm}/E(z)^{60}* is intermediate between that of *E(z)^{Trm}*/+ and *E(z)^{Trm}/Df(3L)Ez2* (Table 1), indicating that the C-terminal portion of the protein missing in E(Z)^{60} is also important for full interaction between the wild-type and E(Z)^{TRM} proteins.

The *son* alleles of *E(z)* have been isolated as strong dominant suppressors of the phenotype of maternal effect lethal *nanos* (*nos*) mutations (PELEGRI and LEHMANN 1994). In embryos derived from homozygous *nos* mothers, maternal *hunchback* (*hb*) RNA is ectopically translated in the presumptive abdomen, and the ectopic HB protein prevents the formation of the abdomen by repressing the expression of the gap-genes *knirps* (*kni*) and *giant* (*gi*). E(Z) protein is required for the continued repression of these gap-genes after the disappearance of HB (PELEGRI and LEHMANN 1994), and the presence of heterozygous, maternally derived *E(z)^{son}* alleles partially rescues the abdominal phenotype of *nos* embryos. We wondered if *E(z)^{Trm}* might modify this phenotype of *E(z)^{son}* mutations. As shown in Table 2, the effect of both *E(z)^{son2}* and *E(z)^{son3}* is suppressed by *E(z)^{Trm}*, suggesting that *E(z)^{Trm}* is an excess-of-function allele. This conclusion is supported by the finding that *E(z)^{Trm}* by itself is a weak but significant ($P = 0.1$) enhancer of *nos* (Table 2).

Interactions of *E(z)^{Trm}* with PcG and trxG mutations:

TABLE 1

Interactions of *E(z)^{Trm}* with different *E(z)* alleles

Crossed alleles (direction of crosses indicated in parentheses)	Penetrance of T1 toward T2, T3 toward T2, and A5 toward A4 transformations (%)[a]				
	T1 → T2		T3 → T2		
	ap1	stp1	ap3	stp3	A5 → A4
Oregon-R (male)	31.5	0	77.3	2.8	41.8
	n = 818	n = 212	n = 616	n = 212	n = 160
E(z)^{son1} (male)	0**	0	0.7**	0	0**
	n = 74	n = 74	n = 298	n = 298	n = 46
E(z)^{son2} (male)	2**	0	18**	0	5**
	n = 310	n = 310	n = 310	n = 310	n = 74
E(z)^{son3} (male)	0**	0	1**	0	0**
	n = 184	n = 184	n = 184	n = 184	n = 42
E(z)^{60} (male)	50**	0	75	24**	100**
	n = 152	n = 152	n = 152	n = 152	n = 22
B2 (male) [*E(z)* rescue construct]	2.9**	0	1.5**	0	1.4**
	n = 854	n = 854	n = 854	n = 854	n = 214

ap1, penetrance of weak T1 → T2 transformation indicated by the appearance of apical bristles on the first leg; stp1, penetrance of strong T1 → T2 transformation indicated by the appearance of sternopleural bristles on the proximal lateral prothorax; ap3, penetrance of weak T3 → T2 transformation indicated by the appearance of apical bristles on the third leg; stp3, penetrance of strong T3 → T2 transformation indicated by the appearance of sternopleural bristles on the metapleura. A5 toward A4 transformation was quantified by setting up a three-grade scale, grade 1 corresponding to near zero transformation, grade 2 corresponding to 50% transformed, and grade 3 corresponding to almost complete transformation of the A5 tergite toward A4 identity. By counting the number of *E(z)^{Trm} Fab-7 trans*-heterozygous males of appropriate genotype with mild, medium, and strong transformations, an average percentage of tergite transformation was calculated. *, Penetrance significantly different from Oregon-R control ($P < 0.05$); **, penetrance highly significantly different from Oregon-R control ($P < 0.001$).

[a] Penetrance is percentage of the number (n) of flies examined.

E(Z) is thought to act in concert with other PcG proteins in forming large heteromultimeric complexes that repress transcription at target loci (Franke *et al.* 1992; Rastelli *et al.* 1993; Carrington and Jones 1996). Therefore, it was of interest to test if the homeotic phenotype of *E(z)^{Trm}* is dependent on other PcG genes. For this purpose, we crossed *E(z)^{Trm}* to representative alleles of several PcG genes (Table 3). Essentially all PcG alleles tested suppressed the thoracic phenotype of *Trm* to some extent in heterozygous conditions, suggesting that most or all PcG proteins are required for the ectopic silencing. Interestingly, however, some mutations [*E(Pc)^{1}*, *Asx^{P1}*, *Psc^{1}*, and *Sce^{1}*] enhanced rather than suppressed the abdominal phenotype of *Trm*. This may be due to complicated cross-regulatory interactions among PcG (Fauvarque *et al.* 1995) and/or trxG genes (Milne *et al.* 1999). The potential outcome of these interactions may be different in the derivatives of abdominal histoblasts and imaginal discs, reflecting the difference between the mitotic cell-division patterns of the two types of imaginal precursors: while imaginal disc cells divide at regular intervals throughout the larval stages, abdominal histoblast cells go through many rapid cell cycles during early pupal stages after a long larval pause. These differences may lead to an accumulation or dilution of different PcG and trxG gene products in the two types of tissues, resulting in different homeotic phenotypes.

Considering the mild phenotype of zygotically homozygous *esc* mutations (Struhl 1981), it is surprising that

TABLE 2

Interactions of *E(z)^{Trm} hb^{7M} nos^{L7}* with different *E(z)^{son}* alleles

Crossed alleles	Penetrance of suppression of nos phenotype (%)[a]		
	nos^{L7}	*hb^{7M} nos^{L7}*	*E(z)^{Trm} hb^{7M} nos^{L7}*
nos^{L7}	0 (470)	3.69 (2140)	1.96* (1052)
E(z)^{son2} hb^{7M} nos^{L7}	0.97 (103)	9.58 (511)	3.83** (1279)
E(z)^{son3} hb^{7M} nos^{L7}	8.8 (270)	50.38 (917)	2.35** (849)

*, Penetrance significantly different from control ($P < 0.01$); **, penetrance highly significantly different from control ($P < 0.001$).

[a] Penetrance is percentage of embryos with at least three abdominal segments. The number of embryos examined is indicated in parentheses.

TABLE 3

Interactions of $E(z)^{Trm}$ with different Polycomb-group alleles

Crossed alleles (direction of crosses indicated in parentheses)	Penetrance of T1 toward T2, T3 toward T2, and A5 toward A4 transformations (%)				
	T1 → T2		T3 → T2		
	ap1	stp1	ap3	stp3	A5 → A4
Oregon-R (male)	31.5 $n = 818$	0 $n = 212$	77.3 $n = 616$	2.8 $n = 212$	41.8 $n = 160$
Oregon-R (female)	16.7 $n = 2570$	0 $n = 448$	73.6 $n = 2706$	1.7 $n = 448$	41 $n = 546$
Pc^1 (male)	0.6** $n = 342$	0 $n = 342$	39.4** $n = 292$	0 $n = 292$	14** $n = 96$
Sce^1 (male)	0.9** $n = 64$	0 $n = 64$	65.6* $n = 64$	0 $n = 64$	50 $n = 23$
E(Pc) (male)	2.1** $n = 364$	0 $n = 364$	36.5** $n = 364$	0 $n = 364$	70** $n = 86$
Psc^1 (male)	0.6** $n = 182$	0 $n = 182$	34** $n = 182$	0 $n = 182$	67** $n = 50$
Psc^{e22} (male)	41.9* $n = 124$	0 $n = 124$	12.0** $n = 124$	0 $n = 124$	14* $n = 28$
Asx^{P1} (male)	35.6 $n = 216$	0 $n = 216$	56.4** $n = 216$	0 $n = 216$	86** $n = 74$
sxc^4 (male)	15* $n = 80$	0 $n = 80$	40** $n = 80$	0 $n = 80$	40 $n = 36$
Su(z)2-5 (male)	0** $n = 184$	0 $n = 184$	31** $n = 184$	0 $n = 184$	52 $n = 56$
Scm^{D1} (male)	0** $n = 116$	0 $n = 116$	6** $n = 116$	0 $n = 116$	32 $n = 25$
esc^2 *CyO* (male)	5.5** $n = 200$	0 $n = 200$	9.5** $n = 200$	0 $n = 200$	28* $n = 50$
esc^{10} (male)	6.4** $n = 980$	0 $n = 980$	17.6** $n = 980$	0 $n = 980$	15** $n = 221$
esc^{10} (female)	1.8** $n = 272$	0 $n = 272$	5.1** $n = 272$	0 $n = 272$	2.6** $n = 75$
pho^1 (male)	0** $n = 298$	0 $n = 300$	65** $n = 300$	0 $n = 300$	11** $n = 39$
pho^1 (female)	9** $n = 458$	0 $n = 460$	19.5** $n = 460$	0 $n = 460$	0** $n = 53$
ph^{410} (female)	2** $n = 402$	0 $n = 402$	11.4** $n = 402$	0 $n = 402$	ND
Pcl^{R5} (male)	0** $n = 78$	0 $n = 78$	0** $n = 78$	0 $n = 78$	0** $n = 19$
dMi^1 (female)	5.8** $n = 102$	0 $n = 102$	20.5** $n = 102$	0 $n = 102$	22* $n = 40$
dMi^1 (male)	4.3** $n = 414$	0 $n = 414$	36.3** $n = 412$	0 $n = 414$	33 $n = 40$

See Table 1 legend. ND, not done.

even heterozygous *esc* mutations suppress the Trm phenotype (Table 3). This strong interaction probably reflects the fact that ESC is a direct binding partner of E(Z) (Jones *et al.* 1998; Tie *et al.* 1998).

As expected from the lack of Pc-like phenotype in $E(z)^{Trm}$ heterozygous or homozygous flies, combinations of $E(z)^{Trm}$ with different PcG mutations do not regularly show an enhancement of the Pc phenotype. Two notable exceptions, however, are the combinations with *Sce* and *Pc* alleles. In these cases, we detected an enhancement of the transformation of the second and third legs toward the first (extra sex combs). Interestingly, heterozygous loss-of-function *E(z)* alleles do not enhance the extra sex combs phenotype of *Sce* or *Pc*. *Sce* is a single allele of an otherwise uncharacterized gene; therefore its interaction with $E(z)^{Trm}$ is difficult to interpret. It is conceivable that the *Sce* and *Pc* genes may be direct targets of E(Z) and that TRM may downregulate these loci. However, 78C–D, the cytological position of *Pc*, is not a major binding site of E(Z) (Carrington and Jones 1996, and our unpublished results). In this case, the enhancement of *Sce* and *Pc* may be the indirect

TABLE 4

Interactions of *E(z)^{Trm}* with different trithorax-group alleles

Crossed alleles (direction of crosses indicated in parentheses)	Penetrance of T1 toward T2, T3 toward T2, and A5 toward A4 transformations (%)				
	T1 → T2		T3 → T2		
	ap1	stp1	ap3	stp3	A5 → A4
Oregon-R (male)	31.5	0	77.3	2.8	41.8
	n = 818	n = 212	n = 616	n = 212	n = 160
Oregon-R (female)	16.7	0	73.6	1.7	41
	n = 2570	n = 448	n = 2706	n = 448	n = 546
Df(3R)red-P93 (male) (*trx*)	75**	2**	91**	25**	100**
	n = 138	n = 138	n = 138	n = 138	n = 42
Tp(3;Y)ry^{506} 85C (male) (*Dptrx*)	11.4**	0	4.8**	0	11**
	n = 228	n = 228	n = 228	n = 228	n = 77
brm^2 (male)	47**	0	91*	16**	100**
	n = 100	n = 100	n = 100	n = 100	n = 28
osa^2 (male)	59**	0	65**	1.8	72**
	n = 226	n = 226	n = 222	n = 226	n = 60
vtd^2 (male)	6.2**	0	40.6**	0.4	62**
	n = 470	n = 470	n = 470	n = 470	n = 137
urd^2 (male)	10.3**	0	28.4**	0	56*
	n = 386	n = 386	n = 386	n = 386	n = 91
kto^1 (male)	29.7	0	49**	0.43	62**
	n = 318	n = 318	n = 346	n = 346	n = 87
skd^2 (male)	23.5*	0	15.7**	0.4	35.8
	n = 242	n = 246	n = 246	n = 246	n = 57
ash1^{22} (male)	72**	ND	100**	ND	100**
	n = 54		n = 54		n = 9
ash1^1 ash2^1 (male)	68.8**	1.6	89*	44**	92**
	n = 90	n = 90	n = 90	n = 90	n = 18
snr1 (male)	28	0	48**	0	81**
	n = 358	n = 358	n = 358	n = 358	n = 72
sls^1 (male)	17.8**	0	28**	0	36
	n = 314	n = 314	n = 314	n = 314	n = 77
Trl^{R85} (male)	45**	0	47**	0	91**
	n = 120	n = 120	n = 120	n = 120	n = 31
Trl^{R85} (female)	21.9*	0	30.7**	0	62*
	n = 114	n = 114	n = 114	n = 114	n = 26

See Table 1 legend. ND, not done.

consequence of downregulation of some other *Pc-G* genes. For example, the *Asx* locus (51B) is a major binding site of E(Z) (Carrington and Jones 1996), and *Asx* mutations do enhance the extra sex combs phenotype of *Pc* (Campbell *et al.* 1995).

Mutations in the trxG are expected to enhance the trx-like phenotype of *E(z)^{Trm}*. This is the case for most of the trxG alleles tested (Table 4). The mutations in the two trxG genes that code for SET domain proteins, *trx* and *ash1*, are exceptionally strong enhancers. These mutations also strongly reduce the viability of *E(z)^{Trm}*. Mutations in the genes that code for the Drosophila homologs of the SWI/SNF complex (*brm*, *osa*, *mor*, and *snr1*) are also strong enhancers of *E(z)^{Trm}*, although *snr1* enhances only the abdominal Trm phenotype. *kto^1*, *urd^2*, *vdt^1*, and *Trl^{R85}* mutations are similar to *snr1* in this respect, again emphasizing the potential difference between imaginal disc and abdominal histoblast derivatives in response to homeotic effects (see above). Finally, *skd^2* and *sls^1*, mutations in genes that are also considered to be members of the trxG, suppress rather than enhance the Trm phenotype. One possible explanation for this unexpected finding is that the proteins encoded by this latter group may be essential for the normal expression of some PcG genes. Thus, heterozygosity for mutations in these genes may lead to a subtle reduction in the levels of the respective PcG proteins, which, in turn, would result in a weaker *E(z)^{Trm}* GOF phenotype. Although the basis of this interaction is not clear, it may provide an assay for classifying the heterogeneous trxG genes. Since suppression of the trx-like phenotype may be considered as functionally equivalent to the enhancement of the Pc phenotype, *skd* and *sls* may be classified as members of the ETP group of genes.

The phenotype of *E(z)^{Trm}* is sensitive to changes of the global level of histone acetylation: PcG-dependent

TABLE 5

Interactions of $E(z)^{Trm}$ with different *Su(var)2-1* alleles and histone cluster deletions

Crossed alleles (direction of crosses indicated in parentheses)	Penetrance of T1 toward T2, T3 toward T2, and A5 toward A4 transformations (%)				
	T1 → T2		T3 → T2		
	ap1	stp1	ap3	stp3	A5 → A4
Oregon-R (male)	31.5	0	77.3	2.8	41.8
	$n = 818$	$n = 212$	$n = 616$	$n = 212$	$n = 160$
Oregon-R (female)	16.7	0	73.6	1.7	41
	$n = 2570$	$n = 448$	$n = 2706$	$n = 448$	$n = 546$
Su(var)2-1^{1} (female)	13.7**	0	62.5**	0	35.2
	$n = 240$	$n = 240$	$n = 240$	$n = 240$	$n = 61$
Su(var)2-1^{5} (male)	11**.3	0	38.5**	0	27
	$n = 118$	$n = 118$	$n = 270$	$n = 270$	$n = 35$
Df(2L)TW161 (male)	26.5	0	34.8**	0	29*
(38A6;40A4–B1)	$n = 132$	$n = 132$	$n = 132$	$n = 132$	$n = 42$
Df(2L)TW65 (male)	22.4*	0	26**	0	4.5**
(37F5–38A1;39E2–F1)	$n = 250$	$n = 250$	$n = 250$	$n = 250$	$n = 28$
Df(2L)DS6 (male)	13.8**	0	10**	0	34
(38F5;39E7–F1)	$n = 254$	$n = 260$	$n = 260$	$n = 260$	$n = 65$

See Table 1 legend.

silencing has often been compared to silencing by heterochromatin (Paro 1990; Boivin and Dura 1998). However, while heterochromatin-induced silencing [or *p*osition *e*ffect *v*ariegation (PEV)] is known to respond to alterations in chromatin structure, a similar link between PcG silencing and basic chromatin structure has not yet been possible to establish. For example, PEV can be suppressed by reducing histone gene dosage and by mutations that lead to the hyperacetylation of histones H3 and H4 (Reuter *et al.* 1982; Dorn *et al.* 1986; Lu and Eissenberg 1998). In contrast, PcG-mediated silencing does not appear to respond to these effects (Pirrotta 1997). We reasoned that the ectopic silencing induced by $E(z)^{Trm}$ might differ from normal PcG silencing in being sensitive to small perturbations in chromatin structure. To explore this possibility, we tested whether a reduction in histone gene dosage has any effect on the Trm phenotype. As shown in Table 5, deletions that remove all or part of the histone gene cluster suppress the Trm phenotype. Moreover, as is observed for PEV, *Trm* is also suppressed by mutant alleles of *Su(var)2-1*, which cause the hyperacetylation of histones H3 and H4 (Dorn *et al.* 1986; Table 5). In the rare homozygous escapers of the hypomorphic allele *Su(var)2-1*^{3}, the Trm phenotype is completely suppressed, as *Su(var)2-1*^{3}/*Su(var)2-1*^{3}; $E(z)^{Trm}$/+ flies look wild type (not shown).

To test if this interaction depends upon the presence of the wild-type E(Z), we dissected out some *Su(var)2-1*^{3}/+; $E(z)^{Trm}/E(z)^{Trm}$ pharate adults from their pupal cases. Examination of these flies indicated that *Su(var)2-1*^{3} may suppress the Trm phenotype even in the absence of wild-type protein (not shown). To test this possibility more rigorously, we checked the presence of apical bristles on the third pair of legs in the viable $E(z)^{Trm}/E(z)^{60}$ combination. As shown in Table 6, *Su(var)2-1* alleles clearly suppress the Trm phenotype in this allelic combination.

These results strongly suggest the involvement of histones/nucleosomes, and their covalent modification, in PcG-mediated silencing. However, since $E(z)^{Trm}$ induces partial inactivation of normally active chromatin domains, these results would be compatible with the view that the role of acetylated histones is restricted to active chromatin domains, as part of the mechanism maintaining active chromatin conformation in the homeotic gene complexes. In accordance with this possibility, we found that neither histone deletions nor *Su(var)2-1* mutations enhance the phenotype of dominant PcG mutations (data not shown). The *Su(var)2-1* gene is not characterized molecularly, and the mechanism by which this gene modifies the level of histone acetylation is unknown. Therefore, it could be argued that the genetic interactions between $E(z)^{Trm}$ and *Su(var)2-1* mutations may be mediated by a direct interaction between the mutant proteins. To provide further support to the idea that the phenotype $E(z)^{Trm}$ responds to the elevated level of histone acetylation, we tested the effect of Na-butyrate. To produce a sufficiently high level of butyrate during the developmental stage, which appears to be critical for the establishment PcG silencing (Cavalli and Paro 1999), we treated $E(z)^{Trm}$/+ embryos by feeding their mothers for 6–7 days with a media containing 0.05 M or 0.01 M Na-butyrate and subsequently reared them on standard media. We observed a suppression of the $E(z)^{Trm}$ phenotype (Table 7). These findings support the view that increasing the level of histone acetylation at some early stage of development establishes a

TABLE 6

The effect of *Su(var)2-1*^{3} on the homeotic phenotype of *E(z)*^{Trm}/*E(z)*^{60} *trans*-heterozygotes

	Penetrance of T1 toward T2, T3 toward T2, and A5 toward A4 transformations (%)				
	T1 → T2		T3 → T2		
Genotypes	ap1	stp1	ap3	stp3	A5 → A4
E(z)^{Trm}/*E(z)*^{60}	50	0	75	24	100
	n = 152	n = 152	n = 152	n = 152	n = 22
Su(var)2-1^3/+;	10**	0	9**	0	14**
E(z)^{Trm}/*E(z)*^{60}	n = 214	n = 214	n = 214	n = 214	n = 55
SM6/+;	53	0	60**	0	78**
E(z)^{Trm}/*E(z)*^{60}	n = 94	n = 94	n = 94	n = 94	n = 16

Genetic interaction between the butyrate-sensitive PEV suppressor *Su(var)2-1* and *E(z)*^{Trm} was tested in the absence of the wild-type *E(z)* gene product by crossing *Su(var)2-1*^{3}*/SM6; E(z)*^{Trm}*/TM3* females to *E(z)*^{60}*/TM6* males, and the homeotic phenotype of the viable *E(z)*^{Trm}*/ E(z)*^{60} offspring was examined in the presence and absence of the mutant *Su(var)2-1*^{3} allele. See also Table 1 legend.

change in the chromatin structure of PcG target genes that is propagated through the rest of development, and this imprinted state strongly interferes with the effect of *E(z)*^{Trm}.

The dominant phenotype of *E(z)*^{Trm} responds to the dosage of the *protein phosphatase 1* gene: On the basis of the observations that arginine, unlike lysine, has high binding affinity to anionic ligands (Fromm *et al.* 1995) and that arginine residues are involved in establishing phosphoryl-bonds in protein-protein interactions (Tian and Martin 1996), we speculated that the inability of one or more putative E(Z) interacting phosphorylated factor(s) to interact with TRM may be the cause of the Trm phenotype. However, if TRM is able to form a heterooligomeric complex with the wild-type E(Z) polypeptide, as suggested by the interaction of *E(z)*^{Trm} with strong antimorphic alleles (see above), such "hybrid" complexes might still be able to interact with the supposed phosphorylated partner(s) in *E(z)*^{Trm} heterozygotes. In this case, the efficiency of the residual interaction should be reflected in the severity of the phenotype.

TABLE 7

Effect of butyrate on the *E(z)*^{Trm} phenotype

	Penetrance of T1 toward T2, T3 toward T2, and A5 toward A4 transformations (%)		
	T1 → T2	T3 → T4	
Genotypes	ap1	ap3	A5 → A4
E(z)^{Trm}/+ (nipagin)	19	92	42
	n = 1184	n = 1134	n = 243
E(z)^{Trm}/+ (after 6 days 0.01 M Na-butyrate feeding)	11**	53**	12**
	n = 454	n = 454	n = 86
E(z)^{Trm}/+ (after 6 days 0.05 M Na-butyrate feeding)	11**	51**	8**
	n = 384	n = 388	n = 99

Crosses between *E(z)*^{Trm}*/TM3* females and Oregon-R males were transferred from control (nipagin-containing) media to the same media supplemented with Na-butyrate at 0.01 M or 0.05 M concentration. After the flies were fed butyrate-containing media for 6–7 days, they were transferred into bottles containing control media and allowed to lay eggs for 3 days, and offspring hatching from these transfers were analyzed. See also Table 1 legend.

We reasoned that the degree of phosphorylation of the supposed protein factor(s) might be modified by mutations in the major protein phosphatase, PP1 87B, encoded by the *Su(var)3-6* gene (Axton *et al.* 1986). The *Su(var)3-6* gene is responsible for ~80% of the total protein phosphatase 1 activity in the fly. Loss-of-function mutations of *Su(var)3-6* dominantly suppress position effect variegation (Reuter *et al.* 1987; Baksa *et al.* 1993), indicating that PP1 may be involved in dephosphorylation of chromosomal proteins. *Su(var)3-6* mutations alone do not have detectable homeotic phenotypes nor do they modify the phenotype of *trx*, *Pcl*, or *Pc*^{2} (data not shown). In contrast, we found that amorphic alleles of *Su(var)3-6* significantly suppress the homeotic transformations caused by heterozygous *E(z)*^{Trm}, while extra wild-type copies of the gene enhance it (Table 8). On the other hand, the phenotype of homozygous *E(z)*^{Trm}, or its combination with *E(z)*^{60} that codes for an E(Z) protein lacking the SET domain, could not be modified by mutations of *Su(var)3-6* (data not shown), suggesting that Trm completely lost its ability to interact with the putative phosphorylated partner(s). Although it is still possible that the effect of *Su(var)3-6* mutations on *E(z)*^{Trm} is indirect, these results indicate that phosphorylation/dephosphorylation of some protein substrates is part of the mechanism that distinguishes active and inactive domains in the homeotic gene complexes and that the E(Z) protein participates in this process.

In an attempt to identify factors that may be involved in the phosphorylation of the putative protein partner

TABLE 8

Interactions of *E(z)^{Trm}* with different *Pp1* and *aurora* alleles

Crossed alleles (direction of crosses indicated in parentheses)	Penetrance of T1 toward T2, T3 toward T2, and A5 toward A4 transformations (%)				
	T1 → T2		T3 → T2		
	ap1	stp1	ap3	stp3	A5 → A4
Oregon-R (male)	31.5	0	77.3	2.8	41.8
	n = 818	n = 212	n = 616	n = 212	n = 160
Oregon-R (female)	16.7	0	73.6	1.7	41
	n = 2570	n = 448	n = 2706	n = 448	n = 546
Pp1-87B^{87Bg-3} (male)	24.8*	0	25.6**	0	6**
	n = 250	n = 250	n = 250	n = 250	n = 54
Pp1-87B^{87Bg-3} (female)	16.8	0	19.2**	0	11**
	n = 480	n = 480	n = 702	n = 702	n = 122
Pp1-87B^{87Bg-6} (male)	8.1**	0	34.2**	0	39
	n = 374	n = 374	n = 294	n = 294	n = 51
Df(3R)E079 (male)	14.7**	0	39.6**	0	30**
	n = 470	n = 470	n = 480	n = 480	n = 210
P15 (female) (*Pp1* rescue construct)	66.7**	5.9	83.9*	4.8*	100**
	n = 168	n = 168	n = 168	n = 168	n = 26
7/2/1 (female) (*Pp1* rescue construct)	31.1	0	85.2*	20**	94**
	n = 228	n = 228	n = 228	n = 228	n = 54
aur^{87Ac-4} (female)	78.5**	0	80.5	19.3**	80**
	n = 224	n = 224	n = 224	n = 218	n = 98
aur^{87Ac-3} (female)	32.3	0	82.2*	2.1*	74**
	n = 760	n = 760	n = 760	n = 760	n = 133

See Table 1 legend.

of TRM, we crossed mutant alleles of *fu*, *polo*, and *fs(1)h* (genes known to encode protein kinases with a nuclear localization) to *E(z)^{Trm}* and checked if they modify the Trm phenotype. While most of these mutations have little or no effect on *E(z)^{Trm}*, all strong loss-of-function mutations in the *aurora* gene enhanced the Trm phenotype (Table 8), suggesting that at least one of the substrates of the aurora kinase plays a significant role in E(Z)-dependent silencing.

DISCUSSION

Loss of *E(z)* activity disrupts binding of other PcG proteins to polytene chromosomes (Rastelli *et al.* 1993), suggesting a key role for wild-type E(Z) protein in organizing repressive heteromultimeric complexes of PcG proteins. In this article we describe a point mutation in the SET domain of the PcG gene, *E(z)*, resulting in a phenotype like that of loss-of-function trxG alleles, indicating the functional importance of the SET domain of E(Z) in distinguishing between the inactive and the active chromatin state of PcG target genes.

***E(z)^{Trm}* is an unusual gain-of-function mutation of a PcG gene that results in the ectopic inactivation of target genes:** It has been suggested that *E(z)* may be classified as a member of both the *Pc-G* and the *trx-G* (LaJeunesse and Shearn 1996; Gildea *et al.* 2000). One possibility is that E(Z) has two distinct functions, one in gene activation and another in gene silencing. In this view, *E(z)^{Trm}* could be considered as a dominant antimorphic mutation in the activating function with an essentially wild-type silencing function. On the basis of formal criteria, some of our observations appear to support this hypothesis. For example, the assumed antimorphic character of *E(z)^{Trm}* would be compatible with the findings that *E(z)^{Trm}* can be completely reverted by LOF mutations *in cis*, and its phenotype is enhanced by LOF *E(z)* alleles *in trans* and suppressed by extra copies of the *E(z)* gene. However, other observations are not compatible with this assumption. Thus, while simple LOF and well-characterized antimorphic alleles suppress the phenotype of *zeste^{1}* and *nanos*, both of these phenotypes are enhanced by *E(z)^{Trm}*, suggesting that *E(z)^{Trm}* is an excess-of-function allele with respect to silencing. The fact that a single-amino-acid change is responsible for both features makes it unlikely that the *E(z)^{Trm}* [and *E(z)^{TrmTG}*] mutation affects two distinct and antagonistic functions. Rather, it suggests that the trithorax-like phenotype is the direct consequence of the hyperactivity of the mutant protein in silencing. This is supported by the observation that *E(z)^{Trm}* is not only reverted by LOF mutations *in cis* but it is also suppressed by antimorphic alleles, clearly deficient in silencing, *in trans* (see also below). Therefore, it is conceivable that a subfunction of the E(Z) protein is to prevent ectopic or excessive inactivation of target genes by E(Z) itself. We hypothesize that

the mutation in $E(z)^{Trm}$ impairs this subfunction and consequently the mutant protein (partially) inactivates target genes in domains where they should stay active.

One possible explanation of the phenotype associated with $E(z)^{Trm}$ could be that the mutant protein binds to ectopic sites. However, our data do not support this explanation. First, the distribution of TRM protein on polytenic chromosomes suggests a binding specificity for TRM indistinguishable from wild type. Second, increasing the dose of wild-type *E(z)* gene proportionally suppresses the $E(z)^{Trm}$ phenotype, indicating that wild-type E(Z) competes with TRM for common targets.

These observations raise the possibility that E(Z) may be present in both active and inactive domains of target genes and that it functions differently in the two domains. Indeed, preliminary genetic data suggest that E(Z) is required not only for maintaining a silent state of inactive domains but also for setting the appropriate "strength" of enhancers in active domains of BX-C. Strong reduction of E(Z) activity together with a reduction in the number of PREs within a *cis*-regulatory domain results in a hyperactivation of the affected domain (L. Sipos, I. Bajusz, J. Gausz and H. Gyurkovics, unpublished results). In contrast to the wild-type protein, $E(Z)^{TRM}$ may be unable to differentiate between active and inactive chromatin domains of the target genes and, therefore, induces inappropriate silencing in active domains. This explanation implies that active or inactive domains are marked by a specific molecular label, which is recognized by the wild-type E(Z) protein but not by $E(Z)^{TRM}$.

Detailed comparison of $E(z)^{Trm}$ to another GOF mutation, $E(z)^{1}$, supports this hypothesis. Although both *Trm* and $E(z)^{1}$ are dominant enhancers of the z^{1}-w^{+} interaction, $E(z)^{1}$, in sharp contrast to *Trm*, suppresses z^{1} when an insufficient amount of wild-type E(Z) protein is produced by the homolog (Jones and Gelbart 1990). This suggests that the mutant protein encoded by $E(z)^{1}$ exerts its effect on the z^{1}-w^{+} interaction through the wild-type protein, possibly by forming a heteromeric complex with an altered conformation, which allows the heteromeric complex to generate a more efficient silencing of *w*. The *white* gene is not a normal target of *E(z)*; only the binding of the mutant Z^{1} protein renders *w* susceptible to silencing mediated by some PcG proteins, including E(Z). Unlike $E(z)^{Trm}$, $E(z)^{1}$ does not cause an inappropriate inactivation of the homeotic genes or *engrailed*, suggesting that the $E(Z)^{1}$-$E(Z)^{+}$ heteromeric complex recognizes some specific label present in the active state of its normal target genes (but not in *white*). In contrast to the $E(Z)^{1}$-$E(Z)^{+}$ complex, the mutant TRM protein inappropriately inactivates target genes in regions where they are normally active, suggesting that it is unable to recognize this label.

The E(Z) SET domain contributes to PcG-dependent silencing: It is noteworthy that the antimorphic $E(z)^{son1}$ and $E(z)^{son3}$ alleles, which strongly suppress $E(z)^{Trm}$, and the GOF $E(z)^{1}$ allele all contain point mutations within the SET domain (E. A. Carrington and R. S. Jones, unpublished data). Although there is no direct biochemical evidence supporting the multimerization of E(Z), these data would nevertheless indicate that two or more SET domains of E(Z) form an interactive surface (a "composite" SET domain). Taken together, our data suggest that this composite SET domain carries out two related subfunctions of E(Z): It senses signals tethered to the active (or inactive) conformation of target genes and, in response to these signals, modulates PcG silencing.

How does the E(Z) SET domain contribute to the modulation of PcG silencing of target genes? One possibility is suggested by the similarity between the mutant SET domain of TRM (and TRMTG) and the SET domain of wild-type TRX. The recent study of Rozenblatt-Rosen *et al.* (1998) demonstrated that the SET domain of TRX can directly interact with two other trx-group proteins, ASH1 and SNR1, which are also thought to antagonize PcG silencing (Dingwall *et al.* 1995). TRM may counteract the activating effect of TRX by competing for one or both of these activators. This competition model would be consistent with our finding that the phenotype of $E(z)^{Trm}$ is strongly alleviated by a duplication that provides an extra wild-type copy of *trx* (Table 4). Since duplications of *trx* magnify the phenotypes of all loss-of-function alleles of *E(z)* (our unpublished results), TRX and wild-type E(Z) may also compete for common factors in the inactive domains of target genes. It is conceivable that one of the functions of E(Z) is to promote PcG-mediated silencing by competing with TRX.

***E(z)*^{Trm} and the ETP group:** The partial ectopic inactivation of target genes by $E(Z)^{TRM}$ provides a useful system for testing the effect of factors that are required for, or antagonize, PcG-dependent silencing. For example, all PcG mutations tested, including alleles of the ETP group, modify the $E(z)^{Trm}$ phenotype. Interestingly, however, using the frequency of transformation of the third leg into the second as an indicator (Gildea *et al.* 2000), we found that none of the ETP alleles enhances the trx-like phenotype of $E(z)^{Trm}$ in T3. In fact, most of the ETP alleles suppress the T3 > T2 transformations as other "classical" PcG alleles do. On the other hand, some of the ETP mutations do enhance the trx-like phenotype of $E(z)^{Trm}$ in the abdomen. In some cases, even different alleles of the same gene may give opposite results (*e.g.*, Psc^{1} and Psc^{e22}). Moreover, while many of the trxG mutations enhance the $E(z)^{Trm}$ phenotype as expected, others clearly suppress it in all or in some tissues, which might qualify the genes represented by the latter alleles as members of the ETP group. Two of these genes (*sls* and *skd*), originally identified as suppressors of *Pc*, were not previously linked to gene silencing. These results show that the assignment of members of the trxG or the PcG to the ETP group greatly depends on the test system

used and suggest that in some cases the unexpected or paradoxical phenotypes resulting from the combination of certain trxG and PcG mutations *in trans* may simply be the consequence of tissue- and allele-specific alterations of a global balance between activators and repressors of homeotic genes. Possible differences in target specificity and complex regulatory interactions among members of the trxG and PcG may be main factors in setting the actual activator/silencer ratio that is reflected in the final level of expression of target genes.

Histone acetylation may be a factor marking active domains of PcG target genes: We found that the phenotype of $E(z)^{Trm}$ is highly sensitive to the dosage of histone genes, indicating that some components of PcG complexes are able to interact with nucleosomes and that this interaction is necessary to establish efficient silencing. On the other hand, as suggested by the effect of *Su(var)2-1* mutations and early exposure to Na-butyrate, high levels of histone acetylation appear to be incompatible with the establishment of ectopic PcG-dependent silencing. Involvement of acetylated histones in antagonizing PcG-dependent silencing is supported by the findings of Cavalli and Paro (1999). These authors found that when a transgene containing a PRE is forcibly transcribed early in development, the PRE is unable to silence the reporter gene, concomitant with the appearance of a high level of acetylated histone H4 (but not H3) at the site of the insertion of the transgene.

A direct link between E(Z) and histone deacetylation is suggested by the finding that the Drosophila E(Z) binds directly to ESC (Jones *et al.* 1998; Tie *et al.* 1998), and the E(Z)-ESC complex is associated with the histone deacetylase RPD3 (Tie *et al.* 2001). Moreover, RPD3 is required for silencing mediated by a PRE *in vivo*. Suppression of $E(z)^{Trm}$ by *Su(var)2-1* mutations and early exposure to Na-butyrate is consistent with conserved inclusion of histone deacetylase activity in Drosophila ESC-E(Z) complexes. However, since the interaction of ESC is mediated through an N-terminally located region of E(Z), it is unclear how a mutation in the C-terminal SET domain can modify the functioning of HDAC2-ESC-E(Z) complexes. Detailed studies of mutations like $E(z)^{Trm}$ may shed some light on this question.

A possible role of phosphoproteins in marking active domains of PcG target genes: We argued that active domains of PcG target genes should be labeled for recognition by E(Z). The putative molecular label in the target domains is unlikely to be the acetylated histones, because *Su(var)2-1* mutations suppress not only heterozygous but also homozygous $E(z)^{Trm}$, indicating that PcG complexes containing only the mutant $E(Z)^{TRM}$ protein are still able to recognize the difference in the degree of histone acetylation and that $E(z)^{Trm}$ is not mutant in this respect. In contrast, homozygous $E(z)^{Trm}$ is not affected by *Su(var)3-6* mutations, indicating that the mutant protein is unable to respond to a decreased level of PP1. However, the presence of a wild-type allele renders $E(z)^{Trm}$ suppressible by *Su(var)3-6* mutations, suggesting that wild-type E(Z) is able to respond to the level of phosphorylation of some proteins in the active domains. Protein phosphorylation has already been suggested to play a role in PcG silencing by the finding that ESC protein appears to become phosphorylated upon inclusion into the complex formed with E(Z). However, this phosphorylation event is likely to be required for the normal ESC function and not for avoiding ectopic silencing, since replacement of the putatively phosphorylated amino acids in ESC results in an esc^{-} (weakened silencing) phenotype (Ng *et al.* 2000). Our results, however, suggest that protein phosphorylation may also play a role in marking active domains of PcG target genes. It is tempting to speculate that binding of a putative moderator phosphoprotein by the SET domain might reduce interactions of the wild-type E(Z) with associated PcG proteins or reduce the ability of E(Z) to compete with TRX, thus preventing inappropriate formation of the silencing complex in domains of target genes that are designated to be active. The replacement of arginine-741 by lysine in $E(z)^{Trm}$ [and in $E(z)^{TrmTG}$] would prevent binding of the putative moderator, resulting in ectopic inactivation that mimics the consequence of the decreased abundance of TRX. In this view, lysine in the same position of the TRX SET domain would be preserved by selection to avoid its interaction with the moderator.

We found that mutations in the aurora kinase, a protein known to be involved in the phosphorylation of H3 (Hsu *et al.* 2000), enhance the Trm phenotype. Inhibition of PP1 (and PP2A) with okadaic acid increases the level of histone H3 phosphorylated at the amino acid residue Ser 10 in cultured cells, suggesting that PP1 may play a role in the dephosphorylation of phospho-H3 (Mahadevan *et al.* 1991). Moreover, biochemical evidence suggests that PP1 and aurora kinases are associated with the chromatin in Xenopus and PP1 regulates the activity of these kinases (Murnion *et al.* 2001). These data are compatible with the assumption that the putative moderator of E(Z) is the phosphorylated form of histone H3.

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