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# Self-association of the *Drosophila zeste* protein is responsible for transvection effects

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**The *zeste* gene product is required for transvection effects that imply the ability of regulatory elements on one chromosome to affect the expression of the homologous gene in a somatically paired chromosome. The $z^1$ mutation causes a pairing dependent inhibition of the expression of the *white* gene. Both of these phenomena can be explained by the tendency of *zeste* protein, expressed in bacteria or in flies, to self-associate, forming complexes of several hundred monomers. These large aggregates bind to DNA and are found in nuclear matrix preparations, probably because they co-sediment with the matrix. The principal determinants of this self-association are located in the C-terminal half of the protein but some limited aggregation is obtained also with the N-terminal half, which contains the DNA binding domain. The $z^1$ and $z^{op6}$ mutant proteins aggregate to the same degree as the wild type but the $z^{11G3}$ product, a pseudorevertant of $z^1$, has a reduced tendency to aggregate. This mutation, which *in vivo* is antagonistic to $z^1$ and does not support transvection effects, can be made to revert its phenotype when the mutant protein is overproduced under the control of the heat shock promoter. These results indicate that both the *zeste – white* interaction and transvection effects require the formation of high order aggregates. When the $z^1$ protein is overproduced *in vivo*, it reduces the expression of an unpaired copy of *white*, indicating that the normal requirement for chromosome pairing is simply a device to increase the size of the aggregate bound to the *white* regulatory region.**

*Key words:* chromosome pairing/nuclear matrix/protein aggregation/transcription factors

## Introduction

The *zeste* gene is known genetically from two types of interaction generically referred to as transvection phenomena. One of these is the interallelic complementation that occurs between certain regulatory and structural mutations at the *Ultrabithorax (Ubx), decapentaplegic (dpp)* and *white* genes, provided that the two copies of the genes involved are brought together by homologous pairing. The other, which we shall call the *zeste – white* interaction or the *zeste* effect, results from the inhibitory action of a particular mutant of *zeste*, $z^1$ (Gans, 1953), on the transcription of a wild type *white* gene (Bingham and Zachar, 1985). The effect is strongly dependent on and enhanced by the presence of two or more copies of the *white* gene in close physical proximity to one another, due to chromosome pairing or tandem duplication (Jack and Judd, 1979). Both of these phenomena require a functional *zeste* gene and imply that the *zeste* product can interact with two chromosomes at the same time, allowing regulatory elements of a gene on one chromosome to affect the promoter of a second copy of the gene on a homologously paired chromosome.

*In vitro, zeste* protein binds to DNA (Benson and Pirrotta, 1987, 1988; Mansukhani *et al.*, 1988a) and can be shown to stimulate transcription from a nearby promoter (Biggin *et al.*, 1988). Co-transfection experiments in tissue culture cells indicate that *zeste* also stimulates transcription *in vivo* (P.Miller and V.Pirrotta, in preparation). The *zeste* product therefore has at least two kinds of functions: one is transvection, a long range effect so far detectable only *in vivo*. The other is a short range promoter stimulation effect that can be demonstrated both *in vivo* and *in vitro*. We do not know whether these two functions of *zeste* are related or whether they are separable. We have argued that the long range transvection effects can be understood as a generalization of the action of a distant regulatory region upon a promoter. In both cases a mechanism must exist to bring together a distant regulatory element and a promoter region. The *zeste* product may provide one such mechanism. For example, some of the regulatory elements of the *Ubx* gene are as much as 50 kb distant from the promoter they control. Benson and Pirrotta (1988) have shown that the *zeste* protein binds to DNA at several sites in the *Ubx* gene, including one immediately preceding the transcription start site and others distributed in the vicinity of various genetically defined control regions. They proposed therefore that *zeste* might be able to juxtapose distant control sequences and promoter by binding in the vicinity of both and bringing them together in a single large complex. Transvection would then be explained as the same kind of long range looping but occurring between two paired DNA molecules rather than within the same DNA molecule. Some evidence in support of such a model was the fact that the *zeste* protein could bind simultaneously to two DNA molecules *in vitro* (Benson and Pirrotta, 1988). Further support comes from the physical state of the *zeste* protein in solution. In this paper we show that the *zeste* protein undergoes extensive self-association *in vitro* and probably also *in vivo*. This is the form of the protein which binds efficiently to DNA *in vitro*. We examine the effects of a variety of mutations on the aggregation properties of *zeste* and correlate them with the ability to sustain transvection-like effects *in vivo*.

## Results

### *Zeste protein from bacteria forms aggregates*

Bacteria expressing the *zeste* cDNA under the control of the *tac* (Brosius, 1984) or T7 p10 promoter (Tabor and Richardson, 1985) do not accumulate large amounts of *zeste*

protein. However, the *zeste* product is readily detectable in Western blots using antisera directed against various parts of the *zeste* polypeptide, provided that the bacteria are extracted with strong solubilizing agents. If the bacteria are lysed under standard mild conditions, in high or low salt conditions, virtually no *zeste* protein is recovered in the supernatant. The most effective extraction occurs with 8 M urea, 1% SDS. To isolate the *zeste* protein in soluble and functional form we resorted to lysing the bacteria in the presence of 4 M urea, centrifuging at high speed to remove the DNA and dialyzing to remove the urea.

A rapid test shown in Figure 1 shows that much of the *zeste* protein in such preparations is present as a very fast sedimenting aggregate, able to pellet after centrifugation for 10 min in a microfuge. Depending on the preparation, from 30 to 90% of the total *zeste* protein present pellets under these conditions, indicating an S value > 200 or an apparent molecular weight > 15 million. The degree of aggregation is not affected by ionic strength (10 mM−2 M NaCl) but depends on the concentration of *zeste* protein present while the protein is renaturing during the dialysis to remove urea. If a concentrated extract in 4 M urea is diluted with extract from bacteria not expressing *zeste*, after dialysis, the proportion of *zeste* found in the pellet decreases and that found in the supernatant increases in step with the degree of dilution (Figure 2). However, if the dilutions are made under non-denaturing conditions, the state of aggregation does not appear to be affected, at least over an interval of 15−30 min, suggesting that dissociation is a slow process.

In contrast to most proteins that form inclusion bodies when overexpressed in bacteria, this fast sedimenting *zeste* form is active and, in fact, binds to DNA better than the slow sedimenting form (Figure 1). This property enabled us to dispense with the antibody and protein A−Sepharose beads used in our standard DNA immunoprecipitation assay (Benson and Pirrotta, 1987): the *zeste* preparation, incubated with labeled DNA fragments and then centrifuged in a microfuge will cause the specific sedimentation of the DNA fragments containing *zeste* binding sites with an efficiency equal to or higher than that of the immunoprecipitation method. *Escherichia coli* extracts not containing *zeste*, prepared in parallel, cause no labeled DNA to sediment. We conclude that the fast sedimenting form of *zeste* binds to DNA better than the form which does not pellet under these conditions.

The aggregation is not caused by interaction with bacterial proteins, as shown by a dilution experiment (Figure 2), nor by interaction with DNA molecules contaminating the extract. No difference was detected in the sedimentation assay if the extract contained the total sonicated bacterial genome, or if the unsonicated chromosomal DNA was removed by centrifugation or if the extracts were treated with DNase I. Furthermore, the fast sedimenting property persists in *zeste* protein purified by affinity chromatography to near homogeneity.

### *Zeste protein from flies also aggregates*

*Zeste* produced in bacteria might lack important modifications which might otherwise affect its properties and conceivably prevent hyperaggregation. For example, Jackson and Tjian (1988) have found that *zeste* isolated from embryos is O-glycosylated, although they find that this has no detectable effect on DNA binding or on the ability to enhance

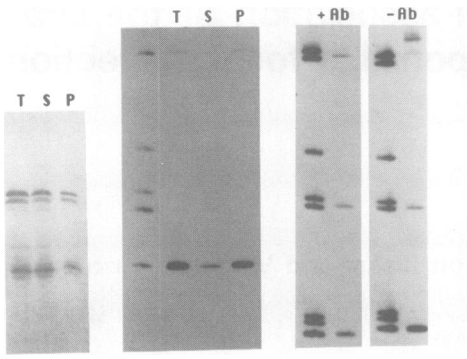


**Fig. 1.** *Zeste* protein aggregate binds DNA. (**Left**). Western blot of 30 μg of total bacterial extract (**T**), supernatant of 30 μg bacterial extract spun 15 000 *g* 10 min (**S**) and corresponding 15 000 *g* pellet (**P**). (**Middle**). Immunoprecipitation (see Materials and methods) of *Ubx* DNA using equivalent amounts of total (**T**), supernatant (**S**), or pellet (**P**) fractions shown in Western blot. (**Right**). DNA binding using *white* DNA and 30 μg total bacterial extract with the addition of anti-*zeste* antibody and protein A−Sepharose (**+Ab**) or protein and DNA fragments incubated and pelleted without the addition of antibody or protein A−Sepharose (**−Ab**).

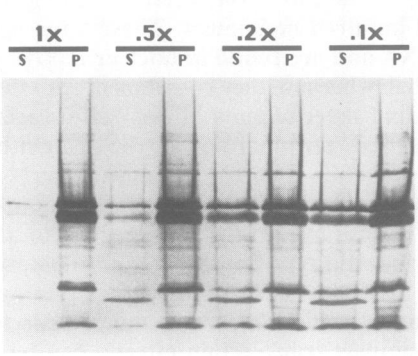


**Fig. 2.** Effect of *zeste* concentration on aggregation. Western blot of the 15 000 *g* supernatant (**S**) and pellet (**P**) fractions when $z^{1G3}$/pUKK bacterial extract in 4 M urea is dialyzed after no dilution (**1×**), 2-fold dilution (**.5×**), 5-fold dilution, (**.2×**), or 10-fold dilution (**.1×**) with pUKK bacterial extract containing no *zeste*.

transcription. Many transcription factors are also known to become phosphorylated (Gay *et al.*, 1988; Sorger and Pelham, 1988; Krause and Gehring, 1989). We therefore extracted *zeste* from flies containing a *zeste* gene under the control of the *hsp70* promoter (*hs-zeste*). We have previously shown that, after heat shock, such flies contain > 10 times the normal amount of *zeste* protein (Pirrotta *et al.*, 1988). We isolated nuclei of heat shocked flies and prepared extracts by the urea method. Figure 3 shows that *zeste* produced in *Drosophila* also associates to form aggregates that sediment by low speed centrifugation. These aggregates, as before, contain most of the specific DNA binding activity. The aggregation phenomenon is not simply caused by overproduction of the protein. In similar extracts made from Canton S flies, although much less *zeste* protein is present, a sizable proportion of the polypeptide can be pelleted by low speed centrifugation.

Could the aggregation of *zeste* be due to improper refolding of the polypeptide when renaturing after the urea extraction? As alternatives to the urea extraction, we explored two other methods. One was lysis of the nuclei with 0.36 M ammonium sulfate, a procedure similar to that used

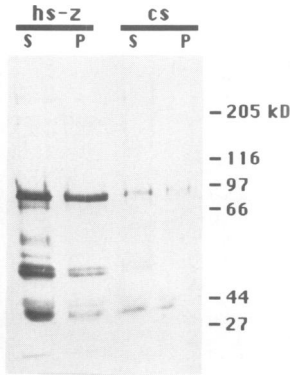


**Fig. 3.** Sedimentation properties of *zeste* extracted from *hs−zeste* (**hs-z**) and Canton S (**cs**) flies. 100 μg of Canton S and 50 μg of *hs−zeste* nuclear extracts were adjusted to 50 μl with buffer A/G (see Materials and methods), centrifuged at 15 000 *g*, 10 min and the supernatant (**S**) and pellet (**P**) compared by Western blotting. Note that even in the presence of several protease inhibitors, several degradation products are visible, some of which appear to be associated with the aggregate.

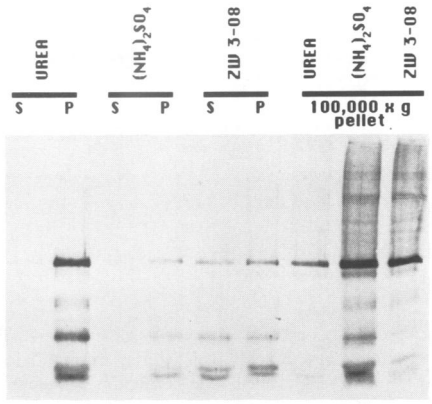


**Fig. 4.** Aggregation as a function of extraction conditions. The Western blot shows the extraction of *zeste* from *hs−zeste* flies by three different methods (described in Materials and methods), 4 M urea (**urea**), 0.36 M ammonium sulfate [**$(NH_4)_2SO_4$**], or 1% Zwittergent 3-08 (**Zw 3-08**). After dialysis each extract was spun at 15 000 *g*, 10 min and the supernatant (**S**) and pellet (**P**) compared. Note that regardless of the extraction procedure, *zeste* retains the ability to associate. In addition, the 100 000 *g* pellet from each extraction procedure after DNase I digestion and solubilization in gel sample buffer shows the amount of *zeste* not extracted by each procedure.

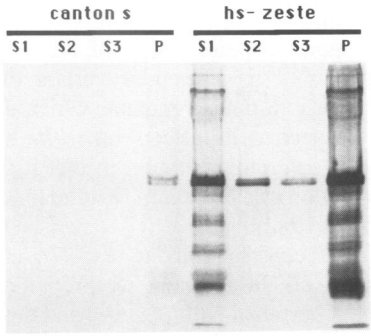


**Fig. 5.** Western blot of *zeste* protein in nuclear matrix preparation. **S1**, **S2** and **S3** represent the supernatants from three consecutive salt washes which leave behind the final insoluble matrix fraction (**P**).

to prepare other transcription factors (Soeller *et al.*, 1988; Biggin and Tjian, 1988). The other was lysis of the nuclei with a zwitterionic detergent. Such detergents have been found to be effective in extracting sensitive proteins without causing denaturation and loss of activity (Bailyes *et al.*, 1982; Matuo *et al.*, 1985). Figure 4 shows that, regardless of the method of preparation, *zeste* retains the ability to form aggregates large enough to sediment during low speed centrifugation. We conclude that denaturation and incorrect folding are not likely to be the cause of this association. However, the three procedures differ greatly in their ability to extract *zeste* from nuclei as shown by the quantity of *zeste* remaining in the 100 000 *g* pellet. The urea method is clearly most efficient. Zwittergent 3-08 is also effective but leaves behind a substantial amount of *zeste*. The ammonium sulfate extraction, though it may be effective in preparing other transcription factors, is unable to solubilize the greater part of *zeste* protein. These results do not tell us if the losses in extraction are due to aggregation of *zeste* or to residual association with genomic DNA or other nuclear structures.

### *Zeste and the nuclear matrix*

The preceding results show that *zeste* synthesized in bacteria or extracted from flies forms large aggregates *in vitro* and strongly suggest that it may exist as a high order oligomer also *in vivo*. Only extraction procedures that reduce or disrupt this extensive association would allow the separation of *zeste* from cellular debris and chromatin by high speed centrifugation. This persistent association under different extraction conditions is reminiscent of the solubility properties of the components of the nuclear matrix or chromosome scaffold.

The following results demonstrate that a commonly used procedure to prepare nuclear scaffold yields a fraction highly enriched in *zeste*. We used the isolation procedure of Cockerill and Garrard (1986) to prepare a nuclear scaffold pellet which was then resuspended, sonicated and analyzed by gel electrophoresis. Figure 5 shows scaffold preparations from both Canton S and *hs−zeste* flies. In both cases the major portion of the *zeste* protein remains in the pellet after extraction with 2 M NaCl although in the *hs−zeste* extract, a substantial amount of *zeste* can be removed with the salt wash. This may represent newly synthesized *zeste* that has not yet segregated in the appropriate compartment. It is also possible that after heat shock the nucleus becomes saturated with *zeste*, a portion of which can be easily removed. Instead, in the Canton S extracts virtually all the *zeste* in the nucleus co-sediments with the scaffold. This indicates that, in the normal fly, *zeste* is present in the nucleus in a very rapidly sedimenting form not due to its association with bulk chromatin. These experiments do not distinguish whether *zeste* fractionates with the scaffold because it is associated with it or simply because it forms very large aggregates as it does *in vitro*.

### *Zeste mutants*

Three mutant *zeste* alleles, $z^1$, $z^{op6}$ and $z^{11G3}$, have previously been cloned and sequenced (Pirrotta *et al.*, 1987). Each of these mutations can be attributed to a single amino acid change in a small region near the C-terminal of the protein (Figure 6). The $z^1$ mutation results in a *zeste* product that has different effects at the three loci that exhibit *zeste* dependent phenotypes. At the *white* locus, $z^1$ causes

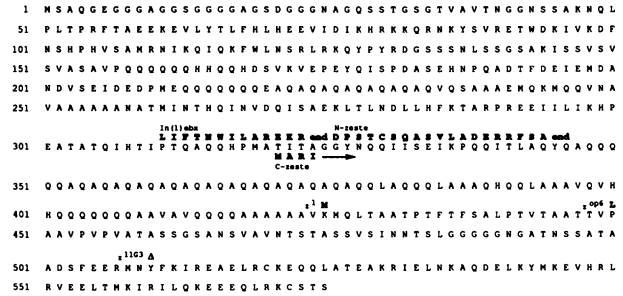

**Fig. 6.** Amino acid sequence of wild type *zeste* (numbered lines). For several mutants, those amino acids which differ from $z^+$ are shown in bold type. Note that a single nucleotide correction of our published sequence (Pirrotta *et al.*, 1987) now brings the amino acid sequence in agreement with that of Masukhani *et al.* (1988b).

a specific underexpression of the gene in the eye when two copies of the *white* gene are physically apposed by chromosome pairing or by tandem duplication. Expression of *white* in other tissues is not affected. The $z^1$ product behaves like $z^+$ in promoting transvection effects at the *Ubx* locus but fails to permit transvection at the *dpp* locus. The $z^{op6}$ allele was derived from $z^1$ (Lifschytz and Green, 1984) and is therefore a double mutation. Its behavior at *Ubx* and *dpp* mimics $z^1$ but at *white* it is able to repress expression even when a single, unpaired copy of the target gene is present. Finally, $z^{11G3}$ is a pseudorevertant of $z^1$ (Gans, 1953), therefore also a double mutant, which can complement $z^1$ with respect to its effects on *white* but behaves otherwise like a *zeste* null mutation in that it is unable to promote transvection at *Ubx* or *dpp* (Kaufman *et al.*, 1973; Gelbart and Wu, 1982).

We engineered constructions to express each mutant polypeptide in *E.coli*. In addition, we prepared constructs that express the N-terminal half (*N-zeste*) and the C-terminal half (*C-zeste*). *N-zeste* mimics the polypeptide encoded by the *In(1)e(bx)* mutation, an inversion with breakpoint in the middle of the *zeste* coding region that results in a truncated *zeste* mRNA (Mariani *et al.*, 1985) and, presumably, in a truncated protein. *In vivo*, this mutation does not complement $z^1$ and cannot promote transvection effects. The sequences of this and of the other mutations used are illustrated in Figure 6.

When tested for DNA binding ability by the immunoprecipitation assay, the $z^1$, $z^{op6}$ and $z^{11G3}$ proteins bind to the *Ubx* promoter as well as wild type *zeste* (Figure 7A). When examined by a footprinting assay, their interaction with DNA is also indistinguishable from that of wild type (M.Benson and V.Pirrotta, unpublished experiments). In the immunoprecipitation assay, *N-zeste* also binds efficiently while *C-zeste* does not interact significantly with DNA, in agreement with the results of Mansukhani *et al.* (1988a) which localize the DNA binding domain near the amino terminal region of *zeste*. Footprinting experiments show that *N-zeste* also protects the same sequences as wild type *zeste* although its binding is highly sensitive to $Mg^{2+}$ ions (M.Benson, S.Bickel and V.Pirrotta, unpublished experiments). If the $z^1$, $z^{op6}$, $z^{11G3}$ and *e(bx)* phenotypes do not result from altered protein−DNA interactions then perhaps these mutations affect protein−protein interactions, either between *zeste* monomers or between *zeste* and other proteins.

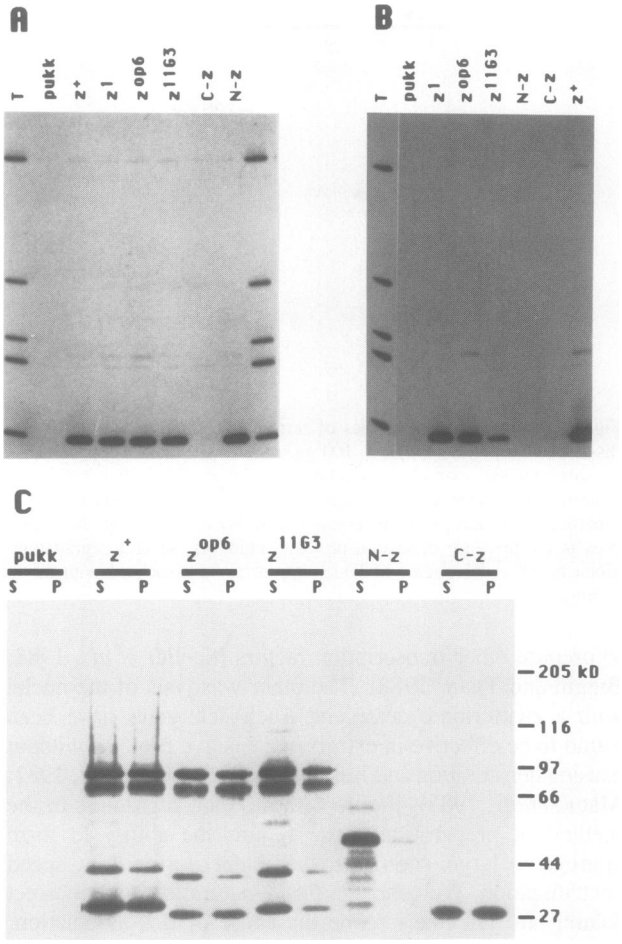


**Fig. 7.** Aggregation and DNA binding properties of *zeste* mutants. (**A**). Immunoprecipitation of *Ubx* fragments with extracts prepared from bacteria containing the pUKK vector with no insert or expressing different *zeste* polypeptides. (**B**). Binding of *Ubx* DNA with each *zeste* polypeptide without the addition of antibody or protein A−Sepharose. (Note that pUKK alone does not pellet DNA fragments.) (**C**). Western blot showing the 15 000 *g* supernatant (**S**) and pellet (**P**) of each *zeste* protein. (Note that, like full length *zeste* (mol. wt = 62 kd), *N-zeste* (mol. wt = 35 kD) migrates with an apparent molecular weight much higher than predicted while *C-zeste* (mol. wt = 27 kd) migrates normally. Also, the degradation products of $z^{op6}$ consistently migrate faster than those of the other full length *zeste* proteins. We are unable to explain either observation.)

### *Self-aggregation of mutant proteins*

The ability of the different mutant proteins to aggregate is illustrated in Figure 7C. After a 10 min centrifugation at 15 000 *g*, the proportion of *C-zeste* protein found in the pellet is approximately the same as for wild type *zeste* protein. In contrast, under the same conditions, the vast majority of *N-zeste* remains in the supernatant, indicating that sequences in the C-terminal region are required for the massive aggregation that results in pelletable protein. The lack of extensive association means that, while it binds efficiently to DNA in the immunoprecipitation or footprinting assay, *N-zeste* cannot cause a DNA fragment to pellet in the absence of antibody or protein A−Sepharose (Figure 7B).

Gel filtration experiments indicate that *N-zeste* is still able to aggregate to form a lower order oligomer. Figure 8 shows the elution profile of *N-zeste* protein from a Sepharose 6 column. The majority of *N-zeste* elutes with an apparent

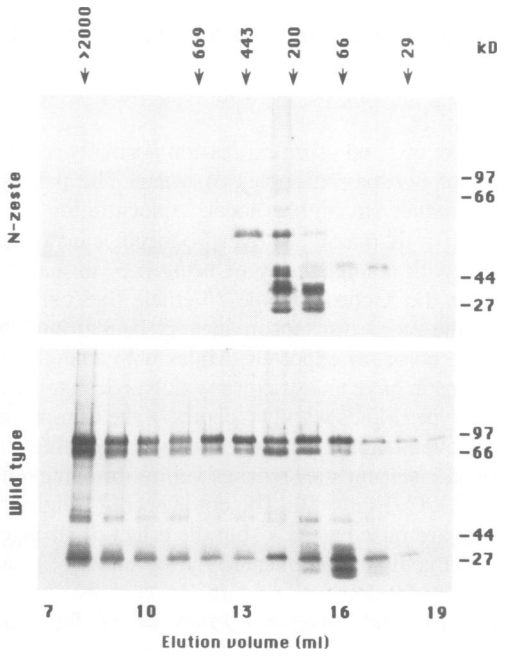


**Fig. 8.** Western blot of the gel filtration profile of *N-zeste* (**top**) and wild type *zeste* (**bottom**). The arrows across the top show the elution profile of Sigma gel filtration standards. Sigma SDS−PAGE molecular weight standards are shown on the right. (Note that both *N-zeste* and $z^+$ degradation products also appear to be associated with the oligomer. Although most of wild type *zeste* elutes at or above its expected molecular weight, a small amount appears to be trailing off the column, probably because of non-specific adsorption to the resin.)

molecular weight of 440 000 daltons, ~12 times the predicted molecular weight. For comparison, the figure also shows the profile of wild type *zeste*. In this experiment only the 15 000 *g* supernatant was loaded on the Sepharose to avoid clogging the column. Even in this supernatant, however, the wild type protein is present in forms ranging in apparent molecular weight from > 5 million down to the monomer. While *N-zeste* exhibits a sharp cut off in the extent of association, the wild type protein may only be limited by the concentration at which it is present while renaturing. We conclude that sequences in *N-zeste* can also contribute to self-association but to a much lower degree than those in the C-terminal half.

The $z^1$ and $z^{op6}$ mutant proteins appear to behave much like wild type *zeste* with respect to aggregation, at least by our crude sedimentation assay. Figure 7C shows however, that the $z^{11G3}$ protein has a distinctly lower ability to aggregate than wild type, though substantially higher than that of *N-zeste*. The proportion of the total *zeste* protein found in the 15 000 *g* pellet varied from preparation to preparation but in all cases the distribution of $z^{11G3}$ protein was shifted towards the supernatant relative to that of wild type *zeste*. While the assay does not permit a quantitative measurement, we conclude that in $z^{11G3}$, the deletion of the Tyr510 residue decreases the degree of association.

### *Phenotypes of mutant protein overproduction*

We have argued previously that the transvection and transvection-like phenotypes strongly suggest that *zeste* is capable of interacting simultaneously with two chromosomes, possibly by binding at the same time to two DNA molecules. We have demonstrated that *zeste* can bind *in vitro* to two DNA molecules at the same time (Benson and Pirrotta, 1988). The formation of high order aggregates provides a ready explanation for such behavior and predicts that mutants deficient in aggregation should also be defective in transvection but not necessarily in their interaction with DNA or in their ability to activate transcription. That this is the case is suggested by the *N-zeste* product and its *in vivo* equivalent, the product of the *In(1)e(bx)* mutation. This truncated protein fails to aggregate extensively *in vitro*, does not support transvection effects *in vivo* and does not complement $z^1$ effects at *white*. However, the flies are normal in eye color and other phenotypes and the truncated protein not only binds to DNA *in vitro* but can be shown to stimulate transcription from target promoters in tissue culture cells (P.Miller and V.Pirrotta, in preparation).

Another partially defective mutant is $z^{11G3}$. This mutation does not support transvection but it can, in large part, complement $z^1$ in its effect at the *white* gene. Female flies heterozygous for $z^1$ and $z^{11G3}$ have mottled red-brown eyes instead of yellow eyes like $z^1/z^1$ flies. *In vitro*, the $z^{11G3}$ product binds to DNA but is less able than $z^+$ or $z^1$ to form extensive aggregates. If the degree of aggregation is dependent on concentration and if the ability to form a large aggregate is important for *zeste−white* interaction or transvection, then overexpression should be able to alter or revert the phenotypes of $z^{11G3}$ and perhaps other *zeste* mutants. We assembled transposon constructs in which various *zeste* mutant genes were placed under the control of the *hsp70* promoter. A *hsp70−zeste*$^+$ construct and its behavior has already been described (Pirrotta *et al.*, 1988). Flies carrying this transposon and raised at room temperature produce 2−3 times more *zeste* protein than wild type, due to the basal level of activity of the uninduced *hsp70* promoter. After heat shock, they produce at least 10 times higher levels of *zeste*.

### *Phenotypic zeste effects depend on concentration*

Flies carrying one copy of the *hs-*$z^{11G3}$ transposon in a $z^a$ (*zeste* defective) background have a nearly wild type eye color, similar to the normal $z^{11G3}$ mutant, but if the gene dosage is doubled by making them homozygous for the transposon, the eye color becomes orange-brown to pale yellow (in females), depending on the endogenous $z^a$ allele used (Figure 9). In one transformed line this color is attained even with a single copy of the chromosome bearing the transposon. Upon examination of its genomic DNA, this line was found to contain at least two and possibly three copies of the transposon inserted on the third chromosome. Heat shock treatment in the late third instar stage makes even females with a single copy of the transposon develop a yellow eye color indistinguishable from that of $z^1$ females. Depending on the line, the eye color of the males that received the heat treatment also becomes lighter, reaching a muddy orange-brown.

We interpret these results to mean that the *zeste−white* effect requires the formation of large $z^1$ aggregates. The $z^{11G3}$ product is functionally equivalent to the $z^1$ product in its effect at *white*, but less efficient in forming aggregates of the size required for transvection-like effects. In a $z^1/z^{11G3}$ heterozygote with the endogenous $z^{11G3}$ allele, the $z^{11G3}$ protein limits the aggregation of the $z^1$ product and results in a degree of complementation of the $z^1$ phenotype.

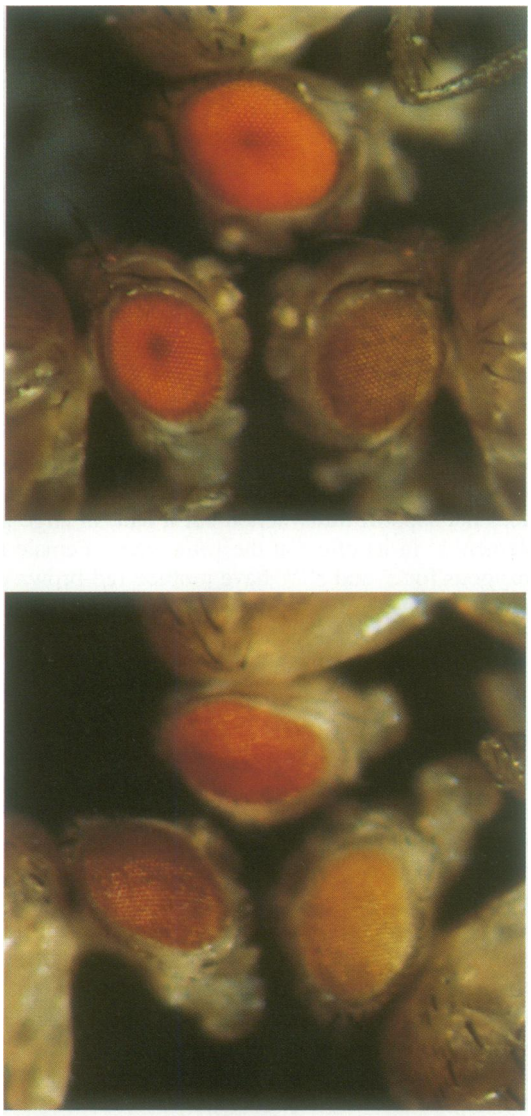

**Fig. 9.** Mutant *zeste* overproduction in flies. (**Top panel**). Flies carrying two copies of the *hs*−$z^{11G3}$ transposon in a In(*1*)*e*(*bx*) background. Male on the left, female on the right; female homozygous for the endogenous $z^{11G3}$ mutation is upside down. (**Bottom panel**). Flies carrying two copies of the *hs*−$z^{1}$ transposon in a *In(1)e(bx)* background. Male on the left, female on the right; male *In(1)e(bx)* upside down. All flies were raised at 23°C and photographed 3−4 days after eclosion.

However, if the $z^{11G3}$ product is overexpressed with the *hs*-$z^{11G3}$ transposon, it reaches concentrations that allow it to aggregate to a degree comparable with that of $z^{1}$, resulting in the *zeste* eye color. This concentration dependent reversion of the $z^{11G3}$ phenotype argues that $z^{11G3}$ protein does not compete with $z^{1}$ but rather that it interacts with it, reducing its ability to achieve its physiologically active state.

The endogenous $z^{11G3}$ allele cannot support transvection effects at *Ubx*. However, flies carrying one copy of the *hsp70*−$z^{11G3}$ transposon and the *Cbx Ubx*/+ + configuration, show weak but distinct signs of wing to haltere transformation. This is indicative of transvection effects at the *Ubx* locus between the *Cbx* allele on one chromosome and the wild type allele on the homologous chromosome (Gelbart and Wu, 1982). These effects are not detectable when the flies are raised at 24°C but are easily demonstrable when the flies had been heat shocked for 30 min at the late third instar larval stage. A heat shock at this stage has no effect on the wing phenotype of wild type flies or of $z^{a}$; *Cbx Ubx*/+ + flies.

The effect of $z^{1}$ on *white* expression normally requires the presence of two paired copies of *white*. The pairing may simply produce a higher local concentration of *zeste* protein. If $z^{1}$ is massively overproduced, can we achieve the effect with a single copy of *white* (i.e. in males, since *white* is on the X chromosome)? Female flies carrying one copy of the *hs*-$z^{1}$ transposon in a $z^{a}$ background have a yellow eye color, as expected. Males with a single copy of the transposon have an eye color which is detectably lighter than wild type. Males with two copies of the transposon have eyes of even lighter color, which, depending on the transposon insertion site, reaches yellow-brown even when raised at 23°C (Figure 9). The color becomes lighter when the larvae are raised at 28°C but the effect of stronger heat shock treatments is complicated by the lethality caused by overproduction of the $z^{1}$ protein. In contrast, heat shock treatment of wild type or $z^{1}$ flies or of flies carrying *hs*−*lacz* constructs does not affect the eye color and has no lethal effect. The fact that, given a sufficient level, $z^{1}$ is able to elicit the *zeste* effect even with a single copy of *white* leads us to suspect that the pairing dependence of the classical *zeste*−*white* interaction is simply a device to accumulate a sufficient concentration of *zeste* in the vicinity of the *white* promoter. We suppose that, through a mechanism still to be elucidated, the inhibitory effect of $z^{1}$ requires very large aggregates of the mutant protein to bind at the *white* regulatory region. This can be achieved by coalescing the *zeste* protein bound to two copies of the *white* gene brought together by chromosome pairing. Our result shows that it can also be achieved by overproducing $z^{1}$, presumably through a concentration dependent hyper-aggregation of the protein *in vivo*. Flies carrying a *hs*−$z^{op6}$ transposon in a $z^{a}$ genetic background have yellow eyes in both males and females, indicating that they produce the *zeste* effect, as expected. Heat shock treatment of these flies in the larval stage does not result in further eye color changes.

## Discussion

### *Self-aggregation of transcription factors*

The *zeste* protein aggregates to a remarkable degree *in vitro*. Although it is very difficult to prove it, our results strongly suggest that it does so also *in vivo*. It might be argued that, in the nucleus, *zeste* is prevented from excessive aggregation by being DNA bound and not free to diffuse (Pirrotta *et al.*, 1988). It is also possible that *in vivo*, some specific modification of the protein (glycosylation, phosphorylation) might limit the degree of self-association. However, the bulk of the *zeste* protein isolated from flies behaves in a way entirely analogous to that of the protein isolated from bacteria from the moment the cells are lysed. *Zeste* protein extracted from bacteria or from flies with urea, detergent or ammonium sulfate behaves very similarly with respect to aggregation, DNA binding and stimulation of transcription in an *in vitro* assay (Biggin *et al.*, 1988). *In vitro*, the degree of aggregation reached in the final preparation depends on the concentration of the protein present during the dialysis to remove the urea. In the fly, the absolute concentration

of the protein present in the nucleus is considerably higher than that present in nuclear extracts. The fact that in high salt nuclear lysates the *zeste* protein has very fast sedimentation properties argues that it is either correspondingly aggregated or bound to other fast sedimenting structures. While we cannot exclude the latter, the properties of the protein extracted from flies strongly suggest that *zeste* itself forms high aggregates in *Drosophila* nuclei as it does in bacterial cells and *in vitro*.

The strongest argument in favor of the self-association of *zeste in vivo* is provided by the behavior of the $z^{11G3}$ mutant. At low or normal concentrations, this mutant protein has no detectable effect on the expression of *white*, is deficient in transvection at *Ubx* and is antagonistic to $z^1$. When expressed at higher levels, it reverses all these effects: it has a $z^1$-like effect on the expression of *white*, it supports transvection at *Ubx* and it no longer antagonizes $z^1$. We interpret this to mean that endogenous $z^{11G3}$ is itself unable to aggregate sufficiently and interferes with $z^1$ aggregation. Our results with $hs-z^{11G3}$ strongly argue that this highly aggregated state is responsible for the transvection and transvection-like phenomena that *zeste* is known to mediate. The aggregated form of *zeste* binds to DNA with much greater avidity than the protein which remains in lower states of association. This is not surprising if we imagine a conglomerate of several hundred *zeste* molecules, each in principle able to recognize and bind to DNA. Such a multimeric form accounts for the ability of *zeste* to bind to two DNA molecules at the same time (Benson and Pirrotta, 1988) and, in principle, to mediate contacts between two chromosomes. Other proteins known or believed to act as transcriptional regulators, also aggregate extensively when expressed in *E.coli* (Stanojevic *et al.*, 1989; Treisman and Desplan, 1989) and still bind to DNA targets. It might be argued from our results with *zeste* that also these regulatory proteins should be able to mediate transvection-type phenomena. This is entirely possible. That no such effects have been reported so far might be explained in several ways. Specific experiments designed to detect such transvection effects have not been done. A particular distribution of binding sites might be necessary to achieve transvection. *Zeste* protein might simply have a higher degree of association which would favor interchromosomal effects. In fact, several cases of transvection-like effects have been reported, some very recently, (Stern and Heidenthal, 1944; Ashburner, 1970; Korge, 1981; Kornher and Brutlag, 1986; Henikoff and Dreesen, 1989; Geyer *et al.*, 1990). Some of these have been shown not to involve *zeste* and suggest that transvection phenomena may be more widespread than was previously believed. We suspect that transvection effects can be mediated by a variety of transcription factors, provided that a suitable disposition of binding sites and regulatory elements exists at the target gene.

Our experiments showing a cohabitation of *zeste* with what has been called the nuclear matrix or chromosome scaffold do not allow us to conclude that *zeste* is physically associated with such structures in the nucleus. If such an association exists, it cannot be demonstrated by this approach. However, our results illustrate the dangers of interpreting matrix or scaffold binding experiments (Gasser and Laemmli, 1986; Cockerill and Garrard, 1986) as evidence for the specific binding of genomic sequences to the structural matrix visualized by electron microscopy. *Zeste* or any protein that behaves like *zeste* would co-sediment with and therefore contaminate scaffold preparations whether or not the protein is actually associated with such nuclear structures. If extensive aggregation properties are frequent characteristics of transcription regulating factors, most gene regulatory regions would appear to be scaffold-attached in these experiments.

### *Zeste* mutants *in vivo*

The behavior of the $z^1$ mutation *in vivo* strongly implies that the $z^1$ product interacts differently from $z^+$ with some component of the transcriptional or regulatory machinery. It is antagonistic to $z^+$ (Lifschytz and Green, 1984) and can be suppressed or enhanced by second site mutations at other loci (Green, 1967; Kalisch and Rasmuson, 1974; Wu, 1984; Phillips and Shearn, 1990). Although $z^1$ itself has an inhibitory effect on transcription at *white* (Bingham and Zachar, 1985), it is still able to produce transvection-like effects both at *white* and at *Ubx*, consistent with its unimpaired ability to aggregate. Our results suggest that the *zeste–white* effect requires two features of the *zeste* protein. One of these is the ability of the protein to form large aggregates. When this ability is decreased, as in the $z^{11G3}$ mutant, both transvection and the *zeste–white* effect are lost and $z^{11G3}$ acts as an antagonist of $z^1$. The other feature is the Lys425 to Met425 change caused by the $z^1$ mutation. When large enough aggregates of the mutant protein are formed, by the pairing of two homologous *zeste* binding sites or by overproduction of the protein, they have an inhibitory effect on transcription. An additional mutation, found in $z^{op6}$, allows this negative effect on transcription even on a single unpaired copy of the *white* gene. This could be interpreted in our scheme as due to enhanced aggregation of the $z^{op6}$ mutant protein or to an enhanced ability of the $z^{op6}$ protein to cause transcriptional interference even in relatively small aggregates.

Our assay for the extent of aggregation is a very crude and not very quantitative one, able to measure only the amount of protein present in aggregates with a sedimentation rate equal or greater than that necessary to cause pelleting in the microfuge. Moreover, the range of concentrations that can be examined this way is limited. With these limitations we were not able to detect any appreciable difference in the tendency of the $z^+$, $z^1$ or $z^{op6}$ proteins to self-associate either in bacterial extracts or in fly nuclear extracts. We suppose therefore that although all three proteins form aggregates, only the aggregates of the mutant proteins have inhibitory effects. Furthermore, since the $z^{op6}$ mutation does not appear to affect the tendency to aggregate, we conclude that even the smaller aggregates of this mutant protein cause inhibitory effects on *white* transcription. This conclusion is supported by the behavior of the mutant proteins in tissue culture co-transfection experiments. These show that, while the wild type *zeste* protein can activate transcription of a reporter gene, the $z^1$ protein activates only at low concentrations but not at high, while $z^{op6}$ is defective in activation altogether (P.Miller and V.Pirrotta, manuscript in preparation).

The results reported in this paper, combined with the transcriptional effects that *zeste* can elicit *in vitro* and in tissue culture, lead us to propose that *zeste* can have two distinct functions. One is the effect exemplified by transvection, where *zeste* plays a largely neutral role, usually limited to

bringing together two DNA regions either on the same or on different DNA molecules. In this case, the transcriptional effects are most likely due to other proteins that are brought to bear on the promoter. A different function is one in which *zeste* itself acts upon the promoter to stimulate the transcriptional machinery. We know that *zeste* can act as a transcriptional activator *in vitro* and in tissue culture cells. In the fly however we have only indirect evidence of this role, which cannot in any case be an essential one since *zeste* null flies are viable (Goldberg *et al.*, 1989; V.Pirrotta and E.McGuffin, unpublished). The action of the $z^+$ product appears to be partially but not absolutely required for expression of the *white* gene in the eye, since *zeste* null flies have a dull brown eye color instead of bright red. In the $z^1$ effect on *white* we have an antimorphic transcriptional effect combined with a requirement for interaction between chromosomes to achieve the size of aggregate required for the antimorphic effect. We do not yet understand the basis for the antimorphic effect but its promoter and tissue specificity suggests that it depends on the particular configuration of regulatory elements acting on a particular promoter. Antimorphic transcriptional effects, combined with transvection effects might also explain why $z^1$ is even more defective than *zeste* hypomorphic mutants in permitting interallelic complementation at the *dpp* locus (Gelbart and Wu, 1982) while it is fully active in supporting such effects at the *Ubx* locus.

## Materials and methods

### Bacterial expression clones

We have expressed a *Bal*31 digested *zeste* cDNA (Benson and Pirrotta, 1987) in *E.coli* using several expression systems. For convenience we now use an expression vector, pUKK, which contains pKK223-3 (Brosius, 1984) sequences from *Pvu*I (3620) to *Nae*I (286) fused to pUC9 from *Pvu*II (628) to *Pvu*I (2066). pUKK uses the expression cassette of pKK223-3 in addition to the replication origin of pUC9 resulting in higher copy number. Induction with IPTG produces levels of *zeste* protein not readily visible by Coomassie staining of total bacterial extracts.

In order to express $z^1$, $z^{op6}$ and $z^{11G3}$ mutant polypeptides, hybrid cDNAs were constructed by fusing the wild type cDNA with each mutant genomic clone at the *Nae*I site (2059). This was possible because both introns precede the *Nae*I site and in all three cases the mutations have been localized downstream of the *Nae*I site (Pirrotta *et al.*, 1987). *N-zeste* was expressed by truncating the cDNA at the *Nae*I site and joining to the *Sma*I site in pUKK, thereby utilizing a translation termination codon in pUKK. This protein is similar to that produced in *In(1)e(bx)* flies (see Figure 6). *C-zeste* was expressed by cloning the 3′ half of the cDNA in pT7-7 (gift from S.Tabor). The *Nae*I site was fused to the filled in *Eco*RI site placing the vector AUG in frame with the *zeste* coding sequence. The *Xba*I–*Hin*dIII fragment which included the pT7-7 AUG was then subcloned into pUKK.

### Bacterial protein extraction

We based our procedure on the method by Desplan *et al.* (1985) with modifications. Cultures were grown in 100 μg/ml ampicillin L Broth at 37°C until $A_{600}$ = 0.8, induced by addition of IPTG to 1 mM, and grown for 2 h. Cells were spun and resuspended by addition of 0.05 volumes (original culture volume) of cold extraction buffer (25% sucrose, 0.2 mM EDTA, 40 mM Tris–HCl pH 7.6, 1 mM DTT) and digested on ice for 1 h with 1 mg/ml lysozyme. An equal volume of 8 M urea was added and the lysate rocked gently at 4°C for 1 h. Samples were spun 1 h at 30 000 r.p.m. in a Beckman Ti75 rotor at 4°C (≈ 60 000 *g*). The supernatant was dialyzed 12 h at 4°C first against 1 l of 2 M urea dissolved in cold buffer A: 10 mM Tris–HCl pH 7.6, 25 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF and 0.1 mM benzamidine (DTT, PMSF and benzamidine are added just before use) and then twice for 12 h against buffer A (no urea). Extracts were adjusted to 50% glycerol and stored at −20°C. Total protein concentration was determined using BioRad Protein Assay with IgG as a standard.

To examine *zeste* aggregation as a function of its concentration, $z^{11G3}$ bacterial extract was prepared and dialyzed as above without dilution or after 2, 5 or 10-fold dilution with pUKK extract containing no *zeste*. Similar results were obtained with $z^+$ extract, although the absolute amount of aggregation was higher.

### DNA binding reactions

Based on Desplan *et al.* (1985), a 25 μl reaction contained 15 ng labeled DNA, 1.5 μg calf thymus DNA and 2.5 μl of 10× binding buffer (without glycerol) and up to 5 μl of total bacterial extract (in 50% glycerol). If < 5 μl of protein extract was used, the difference was adjusted with buffer A/G (buffer A adjusted to 50% glycerol). Binding activity decreases when final glycerol concentration exceeds 10%. For experiments comparing the binding activity of different mutants, equal μg of each total bacterial extract were used. Protein extract was incubated with DNA at 0°C for 30 min. 1 μl of antiserum was added and incubated 30 min at 0°C followed by addition of 25 μl Pharmacia protein A–Sepharose (0.1 g swollen in 500 μl 1× binding buffer) and a further 30 min incubation. The reaction was then diluted with 300 μl 1× binding buffer (1×BB), microfuged 5 min and the pellet washed three more times with 350 μl 1×BB, extracted with saturated phenol, then chloroform:iso-amyl alcohol. The DNA was ethanol precipitated and analyzed on acrylamide gels. 1× binding buffer: 150 mM NaCl, 20 mM Tris–HCl pH 7.6, 0.2 mM EDTA, 1 mM DTT, 10% glycerol. Analogous binding reactions were also performed as described above but without the addition of antibody or protein A–Sepharose (Figures 1 and 7).

The *Ubx* DNA used for binding was a pUC8 plasmid containing nucleotides −268 to +96 of the *Ubx* promoter, cut with *Hin*fI and *Hin*dIII and end labeled. The *white* clone contained nucleotides 3231–5660 (O'Hare *et al.*, 1984) cloned into pUC8 and cut with *Hin*fI before labeling.

### Isolation of Drosophila adult nuclei

Flies containing the *hs–zeste* transposon (Pirrotta *et al.*, 1988) were induced at 37°C for 1 h and allowed to recover at 25°C 1 h. Canton S or *hs–zeste* adult flies were homogenized (10 ml/g flies) at 4°C in cold Buffer I: 350 mM sucrose, 15 mM HEPES, 10 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 1 mM NaBisulfite, 1 mM PMSF, plus protease inhibitors (2 μg/ml aprotinin, 0.5 μg/ml leupeptin and 0.7 μg/ml pepstatin). Small samples (1 ml) were homogenized using 10 strokes with the B pestle of a Dounce homogenizer and filtered through glass wool. Large samples (10 ml) were homogenized using a motorized teflon pestle followed by five strokes with the B pestle of a Dounce homogenizer and filtered through prewetted Nitex. One volume homogenate was layered onto a cushion consisting of one volume 1.6 M sucrose/Buffer I and one volume of 0.8 M sucrose/Buffer I and centrifuged in a Beckman JS-13 rotor, 6000 r.p.m., 20 min at 4°C. The supernatant was removed by aspiration, the sides of the tube wiped clean with a kimwipe, and the nuclei washed once in Buffer I and centrifuged again.

### Extraction of nuclei

For extraction with urea, nuclei were resuspended (1 ml/g flies) in cold 4 M urea, 40 mM Tris–HCl pH 7.6, 25 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.1% Zwittergent 3-08 (Calbiochem) and inhibitors (see above) and rocked gently 1 h at 4°C. Samples were then spun in a Beckman 42.2Ti rotor at 30 000 r.p.m. (≈ 106 000 *g*) 1 h at 4°C. Supernatant was removed and dialyzed as the bacterial extract above.

For the ammonium sulfate extraction based on a procedure by Soeller *et al.* (1988), nuclei were resuspended (0.9 ml/g flies) in cold 14 mM HEPES pH 7.6, 114 mM KCl, 4.8 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 1 mM NaBisulfite, 1 mM PMSF and inhibitors (see above). Nuclei were lysed by the addition of 4 M $(NH_4)_2SO_4$ pH 7.9 to a final concentration of 0.36 M and gentle rocking at 4°C for 1 h. Samples were spun as above and the supernatant dialyzed at 4°C with three changes against buffer A (no urea).

Extraction with zwitterionic detergent was based on a procedure by Matuo *et al.* (1985) with modifications. Nuclei were extracted in a final volume of 1 ml/g flies with components added in the following order. First nuclei were resuspended in 1/10 final volume of a 10× inhibitor mix (20 μg/ml aprotinin, 5 μg/ml leupeptin, 7 μg/ml pepstatin) with 1 mM PMSF. Next was added 1/10 final volume of 10% Zwittergent 3-08 (Calbiochem) with gentle mixing then 1/10 final volume of 1 M Tris–HCl pH 9 with gentle mixing. An appropriate amount of $H_2O$ was added and NaCl to a final concentration of 0.35 M and PMSF to 1 mM final concentration. The extract was mixed gently on a rocking platform 1 h at 4°C, centrifuged as above and dialyzed against buffer A (no urea) with three changes.

To assay the amount of *zeste* present in the 100 000 *g* pellet during each extraction procedure, the pellets were sonicated in 10 mM Tris–HCl pH 7.6, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol (0.25 ml/g flies) and digested for 3 h with 100 μg/ml DNase I (Worthington). For comparison by Western blotting, appropriate volumes of digested pellets and 100 000 *g* supernatants were loaded to represent equivalent amounts of starting total lysate.

### *Nuclear scaffold extraction*

We used a procedure based on Cockerill and Gerrard (1986). Nuclei were isolated as above, washed once with buffer S: 0.25 M sucrose, 10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris−HCl pH 7.6, 0.5 mM PMSF plus inhibitors (see above) centrifuged at 750 *g*, resuspended in buffer S plus 1 mM $CaCl_2$ so that $A_{260}$ = 40, and digested with 100 μg/ml DNase (Worthington) for 1 h at 23°C. After centrifugation at 750 *g* for 10 min at 4°C, pellets were resuspended in 1/2 vol buffer S followed by the addition of an equal volume of cold solution containing 4 M NaCl, 20 mM EDTA, 20 mM Tris−HCl pH 7.6, 0.5 mM PMSF plus inhibitors and after 10 min at 0°C were centrifuged at 1500 *g* 15 min at 4°C (supernatant = S1 in Figure 5). The pellets were extracted twice by suspension in one volume cold 2 M NaCl, 10 mM EDTA, 10 mM Tris−HCl pH 7.4, 0.25 mg/ml BSA, 0.5 mM PMSF plus inhibitors and centrifuged at 4500 *g* 15 min at 4°C (supernatants S2 and S3). The final scaffold pellet was solubilized in 1 vol 4 M urea, 0.1% Zwittergent 3-08, 40 mM Tris−HCl pH 7.6, 25 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol and inhibitors, centrifuged 30 000 r.p.m. in a Beckman 42.2Ti rotor 30 min at 4°C and the supernatant stored at −20°C. For electrophoresis equal volumes of S1, S2 and S3 were TCA precipitated and compared with an equivalent volume of the solubilized scaffold pellet (P).

### *Antibodies and Western blots*

Rabbit antiserum was raised against a β-galactosidase fusion protein expressed in the pEX expression vector (Stanley and Luzio, 1984) containing the *zeste* *Ava*I−*Hin*dII fragment (1546−2493 in the genomic sequence, Pirrotta *et al.*, 1987) and was affinity purified as described in Pirrotta *et al.* (1988) against a β-galactosidase fusion protein containing a smaller *zeste* fragment (*Hae*III fragment from 1791 to 2042). This enabled us to prepare an antibody (N-Ab) specific for the N-terminal half of *zeste*. An affinity purified antibody specific for the last 105 amino acids (C-Ab) of *zeste* has been described previously (Pirrotta *et al.*, 1988).

For Western blots, proteins were solubilized in gel sample buffer: 4 M urea, 100 mM Tris−HCl pH 7.6, 2% SDS, 5% β-mercaptoethanol and 5% Ficoll, electrophoresed on a 10% acrylamide SDS gel (Dreyfuss *et al.*, 1984) and electroblotted onto Immobilon-P membrane (Millipore). Membranes were blocked, incubated with the appropriate antibodies and developed as described in Pirrotta *et al.* (1988).

For analysis of sedimentation properties of the different mutant *zeste* proteins, each mutant extract was adjusted to 5 mg/ml with buffer A/G (see above) and 40 μl centrifuged 10 min in an Eppendorf microfuge (15 000 *g*) at 4°C. The supernatant (S) was removed to a new tube and the pellet (P) washed with an equal volume of buffer A/G and recentrifuged 5 min. The pellet and supernatant were adjusted to equal volumes with gel sample buffer and equal volumes loaded in each lane.

### *Gel filtration*

Approximately 650 μg of total bacterial extract (200 μl) was prespun 10 min in a microfuge and loaded onto a Pharmacia Superose 6 HR 10/30 column equilibrated with 10 mM Tris−HCl pH 7.6, 50 mM NaCl, 1 mM EDTA, 1 mM DTT at a flow rate of 0.4 ml/min. 1.0 ml fractions were collected, TCA precipitated and 1/2 of each fraction analyzed by Western blotting. For calibration, Sigma Gel Filtration molecular weight standards were analyzed under the same conditions.

### *Construction of transposons*

P-transposons containing the *hsp70* promoter driving the *zeste* wild type or mutant genes were constructed in the pUChsneo vector (Steller and Pirrotta, 1985a). The *hs−zeste* gene was assembled with the *hsp70* promoter fragment previously used (Steller and Pirrotta, 1985b) ligated to a genomic *Bam*HI fragment containing the entire *zeste* gene which had been trimmed at its 5′ end to reduce the leader sequence to ~100 nucleotides before the translation start. Mutant *zeste* sequences were substituted into this construct by excising the bulk of the coding sequence and 3′ flanking sequence with *Bst*EII and *Bgl*II and reinserting the mutant coding sequences as *Bst*EII−*Hin*dIII or *Bst*EII−*Bam*HI fragments. The transposons were injected into *In(1)e(bx)* embryos at a concentration of 400 μg/ml together with 80 μg/ml of the helper phsπ plasmid (Steller and Pirrotta, 1986). The G1 progeny were selected on food containing 0.5 mg/ml G418 (Geneticin, Sigma).

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