Stepwise assembly of hyperaggregated forms of *Drosophila* Zeste mutant protein suppresses *white* gene expression *in vivo*

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The *zeste* gene is involved in two chromosome pairing-dependent phenomena: transvection and the suppression of *white* gene expression. Both require the ability of *zeste* protein to multimerize, dependent on three interlaced hydrophobic heptad repeats in the C-terminal domain. The first step is dimerization through a leucine zipper. Two other heptad repeats are then required to form higher multimers. The *z¹* mutation, which causes the pairing-dependent suppression of *white*, creates a new hydrophobic nucleus that allows the formation of a new and larger aggregate. The *z^{op6}* mutation, which suppresses even unpaired copies of *white*, makes even larger aggregates. The phenotypic suppression of *white* by a series of mutants is strictly correlated with hyperaggregation and the larger the hyperaggregates, the weaker the requirement for the pairing of *white*. Hyperaggregation of the *Z¹* protein *in vitro* is suppressed by co-translation with the C-terminal peptide of wild-type protein, lacking the DNA-binding domain. This C-*Z⁺* peptide also complements the *z¹* allele *in vivo* and restores normal eye color, demonstrating that *zeste* product also exists in a multimeric complex in the cell. Complementation *in vivo* is strictly correlated with the prevention of hyperaggregation of the *zeste* mutant products *in vitro*, supporting the interpretation that excessive association of *z¹* and *z^{op6}* proteins is responsible for their repression of *white* gene expression.

Key words: hydrophobic interactions/*in vitro* complementation/multimer assembly/transvection/*zeste*–*white* interaction

Introduction

The product of the *Drosophila zeste* gene is a nuclear, DNA-binding protein found associated with >60 specific sites on the polytenic chromosomes (Pirrotta *et al.*, 1988; Rastelli *et al.*, 1993). It is required for transvection effects at several genetic loci such as *Ultrabithorax* (*Ubx*), *decapentaplegic* (*dpp*) and *yellow* (*y*) (Lewis, 1954; Kaufman *et al.*, 1973; Gelbart, 1982; Geyer *et al.*, 1990). These phenomena are manifested by an interallelic complementation in which regulatory elements on one copy of a gene regulate the expression of a second copy homologously paired with it. Transvection effects at these loci fail to occur in *zeste* loss-

of-function mutants such as *z^a*, *z^{11G3}* or *In(1)e(bx)*. A distinct, *zeste*-dependent phenomenon occurs at the *white* locus, an X-linked gene necessary for the accumulation of pigment in the eye of the fly, as well as in ocelli, malpighian tubules and testis sheaths. The *zeste*–*white* interaction requires a particular allele of *zeste*, *z¹*, whose product is able to suppress the transcription of the wild-type *white* gene in the eye, resulting in a yellow eye color instead of wild-type red (Gans, 1953; Bingham and Zachar, 1985). This effect requires two copies of the *white* gene in close proximity produced either by homologous pairing or by tandem duplication (Jack and Judd, 1979, reviewed by Wu and Goldberg, 1989 and Pirrotta, 1990). Since the *white* gene is on the X chromosome, *z¹* females are yellow-eyed, but *z¹* males have wild-type red eyes. Although the pairing dependence is a striking feature of the *zeste*–*white* interaction, it is not intrinsic to it. Lifschytz and Green (1984) found that the *z¹* mutant gene could be further mutated to produce the *z^{op6}* allele which is now able to cause the *zeste*–*white* effect also on a single, unpaired copy of *white*. Male, as well as female, *z^{op6}* flies are yellow-eyed. The effect of the *z¹* mutation is strangely selective: it affects *white* expression only in the eye, not in other tissues in which the *white* gene is expressed. It does not interfere with transvection effects at the *Ubx* or *y* genes, but it prevents transvection interactions between *dpp* alleles (Kaufman *et al.*, 1973; Gelbart and Wu, 1982; Geyer *et al.*, 1990), suggesting that its action depends on the specific configuration of regulatory elements in each case.

The ability of Zeste protein to bind cooperatively and simultaneously to two DNA molecules, and the distribution of its binding sites in many genes, suggested that, by binding near distant regulatory elements and near the promoter, it might participate in mechanisms that bring the two in contact with one another (Benson and Pirrotta, 1988). The activating effect of Zeste protein bound in the vicinity of promoters has been seen *in vitro* (Biggin *et al.*, 1988), in tissue culture cells (P. Miller and V. Pirrotta, unpublished) and in embryos (Laney and Biggin, 1992), and is probably due to anti-repression by displacement of H1 histone (Pan *et al.*, 1991). Whether or not *zeste* plays an important role in normal gene regulation, it is not an essential gene since flies lacking *zeste* function entirely are viable and fertile (Goldberg *et al.*, 1989; V. Pirrotta, unpublished). These flies are sickly, however, and have a distinct brown eye phenotype similar to that caused by certain loss-of-function *zeste* alleles, suggesting that wild-type *zeste* function is necessary for full expression of *white* and perhaps other genes. The sequence of the *zeste* gene predicts a protein of 575 amino acids with an unusual structure (Pirrotta *et al.*, 1987; Mansukhani *et al.*, 1988b). The DNA-binding domain, which is of the helix-turn-helix type and bears resemblance to the recognition helix of homeodomain proteins, is contained in a 90 amino acid domain near the N-terminus of the protein (Mansukhani *et al.*, 1988a; Chen *et al.*, 1992). The C-terminal region is

Fig. 1. Gel-filtration and cross-linking analysis of *zeste* C-terminal domain mutants. (A) The predicted C-terminal helical region is shown diagrammatically with the three sets of hydrophobic heptad repeats indicated by gray stripes. The two major repeats, FLLLI and LLHLLL, are separated by two amino acids, and are on opposite faces as well as at opposite ends of the helix. (B) Gel-filtration analysis of some representative C-terminal domain mutants (black squares) compared with wild-type protein (open squares). The vertical axis indicates the percentage of radioactivity loaded. Note that only the supernatant fraction was applied to the column: hyperaggregates in the pellet fraction are therefore not represented. The column was calibrated with molecular weight markers that eluted as follows: blue dextran (2000 kDa) at 7.0 ml; thyroglobulin (669 kDa) at 11.5 ml; apoferritin (443 kDa) at 13.5 ml; amylase (200 kDa) at 14.5 ml; alcohol dehydrogenase (150 kDa) at 15 ml; albumin (66 kDa) at 16 ml; carbonic anhydrase (29 kDa) at 17 ml. Three distinct peaks are labeled 1, 2 and 3. (C) Dimerization of various *zeste* C-terminal mutants detected by chemical cross-linking. After treatment with EGS, the products were analyzed on 7.5% SDS-PAGE. The mutations and the amount of EGS used are indicated above each lane. The monomer (M) migrates as an 80 kDa protein; the dimer (D) migrates as an 160 kDa protein; tetramer or higher forms do not enter this gel and are retained at the top (Top), which corresponds to a mol. wt of ≥ 300 kDa.

predicted to form an extended α -helical structure including ~ 75 amino acids and containing hydrophobic heptad repeats. The central part of the protein contains extensive runs of glutamines, alanines and alternating glutamine–alanines whose secondary structure is not known and whose functional role, if any, is unclear.

Whether expressed in bacteria or in flies, the Zeste protein is found predominantly in fast-sedimenting complexes and its extraction generally requires denaturing or solubilizing agents like urea or detergents. Bickel and Pirrotta (1990) found that the protein undergoes extensive aggregation after the solubilizing agent is removed by dialysis, that the aggregated protein is particularly active in DNA binding and, most interestingly, that the ability to form aggregates *in vitro* was correlated with the ability to produce phenotypic effects *in vivo*. The aggregation property is due to the C-terminal helical domain, which is necessary and sufficient for the formation of fast-sedimenting complexes of Zeste protein expressed in bacteria or translated *in vitro* (Chen *et al.*, 1992). Coiled-coil interactions through the hydrophobic heptad repeats in the 75 amino acid sequence of the C-terminal domain might be responsible for the formation of chains of Zeste protein monomers. In this paper, we analyze the role of these heptad repeats and their configuration in the formation of multimers. We show that the assembly of Zeste protein into multimeric forms is an orderly, stepwise process which can be arrested at intermediate stages by mutations affecting the integrity or configuration of the heptad repeats. We also show that the *z^l* and *z^{op6}* mutations create a new hydrophobic nucleus in the region of the protein

preceding the C-terminal domain, resulting in a further stage of multimerization. The resulting formation of very large aggregates is strictly correlated with the ability to suppress *white* gene expression in the eye. Furthermore, we demonstrate that Zeste–Zeste interactions occur *in vivo* as well as *in vitro* by showing that the C-terminal domain of wild-type Zeste can rescue the hyperaggregation of *Z^l* protein *in vitro* and complement the *zeste* eye phenotype in *z^l* mutant flies.

Results

Modulation of *zeste* multimerization by C-terminal helix mutations

The aggregation ability of Zeste protein depends on its C-terminal region, a 75 amino acid domain predicted to form a stable α -helical configuration (Chen *et al.*, 1992). Three sets of heptad repeats in this region potentially serve as surfaces for hydrophobic protein–protein interactions (Figure 1A). First from the C-terminus is the heptad repeat LLHLLL, with strong similarity to C/EBP, *c-myc* and other typical leucine zippers (Landschulz *et al.*, 1988). A second heptad repeat, FLLLI, is at the N-terminal end of the helical domain and two residues out of phase with respect to the leucine zipper. This arrangement puts the two sets of heptads not only at opposite ends of the helix, but also on opposite faces. A third, shorter heptad repeat, YLM, is found within the leucine zipper region. To investigate further the relative importance of these hydrophobic repeats and their spatial arrangement for multimerization, we generated *in vitro* a

Fig. 2. Summary of the multimerization and dimerization properties of various *zeste* mutants. **(A)** Multimerization and dimerization abilities of various deletion mutants. The polypeptide is shown as a bar with the DNA-binding domain (DNA), acidic domain (Acidic), z^1 region (Z^1) and the C-terminal domain 11G3 and leucine zipper regions (Z^{11G3} and Zip) indicated. The abilities of these deletion mutants to form dimers or multimers are shown on the right. ND: not done. **(B)** Multimerization and dimerization properties of C-terminal point mutations. The C-terminal helical domain is divided into the 11G3 region, containing the hydrophobic ridge FFLLI, and the leucine zipper region containing the LLHLL and YLM ridges. Amino acids in a triangle indicate a deletion and those in a square box indicate a substitution. Multimer and dimer formation are shown on the right and formation of intermediate peak 2 (see Figure 1B) is indicated by +/-.

variety of deletion or substitution mutations (Figure 2) and tested the corresponding proteins produced by *in vitro* translation for their ability to form multimers. Different batches of reticulocyte lysate produced Zeste protein with comparable DNA-binding abilities, but with different degrees of aggregation, as determined by rapid sedimentation in a microfuge (data not shown). We suspect that this effect, like that reported for the *in vitro* translated p53 protein (Cook and Milner, 1990), is due to the presence of different amounts of chaperone-like proteins that assist the folding of nascent protein and may regulate the assembly of oligomers (Langer *et al.*, 1992). Regardless of the batch of lysate used, gel filtration chromatography of the soluble protein shows that *zeste* product forms multimeric species whose size depends on the salt concentration. At 50 mM NaCl, most of the protein elutes in the exclusion volume (mol. wt > 2000 kDa, not shown), but at high salt (0.5 M NaCl), this large aggregate dissociates into a smaller complex of ~ 1200 kDa (Figure 1B, peak 3). A broad low molecular weight peak (peak 1) is an unresolved mixture of monomer and lower

Fig. 3. Sedimentation properties of Z¹ and Z^{op6} mutant proteins. The *in vitro* translated ³⁵S-labeled proteins were diluted in low-salt elution buffer and centrifuged for 10 min at 10 000 g in the cold. The supernatant (S) and pellet (P) fractions were analyzed on SDS-PAGE. The majority of the wild-type protein remained in the supernatant fraction, while half of the Z¹ protein and more than half of the Z^{op6} product were in the pellet. The Z⁺Z^{op6} product, which contains the P456L and S496A mutations, but not the K425M mutation, behaves like the wild-type. The Z¹ and Z^{op6} hyperaggregates could not be appreciably dissociated in 0.5 M NaCl. The positions of molecular weight markers are shown on the right; marked in kDa.

oligomers, together with products of degradation or incomplete translation (visible in Figure 3). In this work, we used a single batch of lysate that produced predominantly soluble wild-type protein to study the effects of a number of mutations on Zeste multimerization. Figure 1B shows four representative examples of the high-salt gel filtration patterns of *zeste* proteins containing mutations in the C-terminal helix. Mutations L518D and Y510Δ (a change found in the natural z^{11G3} mutant, a revertant of z^1), which reduce the extent of the FFLI heptad repeat, abolish peak 3 completely and increase peak 2, which is usually barely visible in wild-type protein. Peak 3 is also abolished by the Leu 555 to Gln mutation (L555Q), which introduces a polar residue in the leucine zipper. Interestingly, peak 3 is significantly increased by the Lys 564 to Leu mutation (K564L) which extends the YLM hydrophobic ridge to YLML. Finally, a deletion of Ile 532 and Glu 533 (Δ IE), which fuses the two major hydrophobic ridges and brings them on the same face of the helix, also abolishes the formation of peak 3 completely.

Dimerization through the leucine zipper is the first step

To see the effect of the mutations on dimer formation, we used chemical cross-linking with ethyl glycol *bis*(succinimidylsuccinate) (EGS). Figure 1C shows that wild-type protein is cross-linked by 5 mM EGS to the dimer and to multimeric forms that are too large to enter the polyacrylamide gel. Formation of the wild-type dimer could be detected with as little as 0.5 mM EGS or with 100-fold diluted protein. A peptide consisting of the C-terminal half of the protein, cross-linked by the same treatment, displayed a ladder of products increasing in steps of dimers up to the octamer, as well as material too large to enter the gel (data not shown). Figure 1C shows that most of the mutations that disrupt multimerization do not abolish dimerization unless they affect the lucine zipper domain (L550P and L555P).

The multimerization and dimerization abilities of various *zeste* mutants are summarized in Figure 2. The deletions and point mutations show that only the C-terminal domain is involved in multimerization and that, within this domain,

Table I. z^1 -induced hyperaggregation in C-terminal domain mutants

C-terminal domain mutations	without z^1 mutation			with z^1 mutation		
	% pellet	peak 3	peak 2	% pellet	peak 3	peak 2
WT	5	+	—	49	—	—
K564L	4	++	—	67	+/-	—
H548L	5	+	+/-	22	—	+/-
L518Q	5	+	—	26	—	—
L518P	6	—	—	6	—	—
L518D	6	—	+	8	—	+
Y510Δ	4	—	+	6	—	+
L550Q	3	—	—	5	—	—
L555Q	5	—	—	5	—	—

The aggregation properties of the full-length proteins with the indicated mutations in the C-terminal domain in combination with the wild-type or z^1 sequences were analyzed by sedimentation and gel filtration. The percentage of pelletable aggregates (% pellet) in the range of 3–8% was considered as the background level. The presence of the multimeric form (peak 3) and an intermediate complex (peak 2) in the gel-filtration analysis are indicated qualitatively. The z^1 mutation cannot induce the formation of pelletable aggregates when combined with C-terminal domain mutations that do not allow peak 3 formation. Formation of pelletable aggregates occurs at the expense of peak 3.

both the leucine zipper and the 11G3 region are necessary. Abbreviation or disruption of the FFLI hydrophobic ridge in the 11G3 region prevents multimerization, but not dimer formation, while disruption of the leucine zipper abolishes both. Dimerization does not require an intact 11G3 region, but it is dependent on the leucine zipper and cannot be induced by the FFLI hydrophobic ridge alone. These results suggest that complex formation initiates through the leucine zipper, not the FFLI repeat, forming the dimer, presumably by a coiled-coil arrangement. The FFLI repeat is involved in further interactions that assemble the dimers into multimers. The short YLM repeat contributes to the interaction since changing the Leu to a Gln (L550Q) disrupts multimerization while its extension by one more heptad repeat (K564L) enhances it (see also Figure 1B). As in other leucine zippers, a histidine is accepted instead of a leucine in some positions (H548) and even a glutamine can sometimes replace a hydrophobic residue. The change Leu 518 to Gln (L518Q) in the FFLI repeat is tolerated with little adverse effect on multimerization and changing a Leu to Gln (L555Q) in the leucine zipper or in the YLM heptad repeat (L550Q) prevents multimerization, but not dimerization. Finally, when the two long hydrophobic ridges are fused into one by deletion of Ile 532 and Glu 533 (Δ IE), multimerization but not dimer formation is prevented, showing that the spatial relationship between the hydrophobic repeats is important for multimer formation.

Z^1 and Z^{op6} aggregate more than wild-type zeste protein

The natural mutant alleles z^1 and z^{op6} affect a part of the protein immediately preceding the C-terminal domain and suppress specifically the expression of the *white* gene in the adult eye (Bickel and Pirrotta, 1990). In the case of z^1 , this effect requires the physical proximity of two copies of the *white* gene, while z^{op6} has a repressive effect even on a single copy of *white*. To test whether these phenotypes might be associated with different degree of multimerization than wild-type *zeste* protein, we compared the Z^1 , Z^{op6} and wild-type products translated *in vitro*. Under conditions in which wild-type *zeste* protein remains predominantly in the supernatant, about half of the Z^1 product and more than half of the Z^{op6} product still form fast-sedimenting

aggregates that pellet in a microfuge (Figure 3). This result indicates that z^1 and z^{op6} mutations enhance the degree of aggregation and generate a new and larger type of aggregate than that formed by the wild-type protein. The protein sequence of the z^1 mutant differs from wild-type *Zeste* protein by two amino acid substitutions: a Lys 425 to Met (K425M) and Ser 496 to Ala (S496A). The latter is a polymorphism since it is also found in wild-type flies (Pirrotta *et al.* 1987). The z^{op6} mutant was generated by EMS mutagenesis of z^1 (Lifschytz and Green, 1984). In addition to the two changes found in z^1 , it contains an additional Pro 454 to Leu mutation (Pirrotta *et al.*, 1987). By themselves, the S496A and P454L mutations have no effect since a Z^+Z^{op6} hybrid, constructed by cutting the Z^{op6} cDNA with *Sac*II (at residue 450) and reconstituting the full-length cDNA with wild-type *zeste* gene, has properties identical to those of the wild-type gene both *in vitro* (Figure 3) and *in vivo* (not shown).

To determine whether hyperaggregation observed in the Z^1 protein has a component independent of the C-terminal domain, we constructed double mutants combining the z^1 mutation with various C-terminal mutations. Table I shows that, without the z^1 mutation, all the C-terminal changes alone give only aggregation comparable to that of wild-type protein. When combined with the z^1 K425M mutation, we observe hyperaggregation only with those C-terminal mutations that can form peak 3 multimers similar to wild-type. All the mutations that disrupt the wild-type degree of multimerization, either completely or partially (L518P, L518D, Y510D, L550Q, L555Q), prevent the z^1 mutation from inducing hyperaggregation. Table I also shows that the z^1 -induced formation of the pelletable form occurs at the expense of peak 3. These results indicate that the formation of the aggregates proceeds in a stepwise manner from monomer to dimer to the peak 2 form to the peak 3 multimer which is a necessary precursor for the assembly of the z^1 -induced hyperaggregates.

Hydrophobicity at position 425 induces hyperaggregation

How can the mutation at residue 425 produce such drastic changes in the physical and physiological properties of the protein? The amino acid sequence shows that Lys 425 is the

Fig. 4. Hydrophobicity at Lys 425 residue induces hyperaggregation. (A) The supernatant (S) and pellet (P) fractions of the C-terminal half peptides with different amino acid substitutions at Lys 425 were analyzed on SDS–PAGE. Each pair of S and P lanes is identified by WT, Z¹, or the single letter amino acid substituted at position 425. The K substitution is in principle the same as wild-type but was isolated after the mutagenesis procedure. Molecular weight markers in kDa are indicated on the right. The stacking gel (St) was left intact to allow detection of products not dissociated by boiling in 2% SDS. (B) Gel filtration analysis of the supernatant fractions of the Lys 425 mutations. Each panel is labeled with the name of the mutation whose elution profile (black squares) is compared with the wild-type profile (open squares). Peaks 1, 2 and 3 correspond to the peaks formed by the full-length proteins. The leucine substitution (K425L) forms an additional complex that elutes in the void volume (peak 4). Note that since only the supernatant fraction of each protein was applied to the column, those mutants that produce a high proportion of pelleting material (see Table I) appear over-represented in comparison with the wild-type, while in fact all their large aggregates have already been removed. All polar or charged amino acid substitutions (K425Q and others not shown) behave like wild-type.

only charged residue within a stretch of 166 amino acids constituting the central part of the protein, until the beginning of the C-terminal domain. Is the hydrophobic nature of the K425M substitution the critical feature responsible for the hyperaggregation or is the lysine residue specifically needed to prevent further aggregation? To answer this question we substituted the Lys 425 with a variety of amino acids. We used the C-terminal half of the Zeste protein (C-Z) instead of the full-length protein, which increases resolution in the gel filtration assay and reduces the amount of degradation or products of incomplete synthesis in the *in vitro* translation reaction. Figure 4A shows that the behavior of the C-

terminal half peptide is entirely comparable to that of the full-length protein: the wild-type peptide remains in solution while the Z¹ mutant forms pelletable aggregates which are partially resistant to boiling in sample buffer containing 2% SDS and are too large to enter the running or the stacking gel (Figure 4A). This is also true for the full-length protein, although it cannot be seen clearly in Figure 3 because the stacking gel has been removed. The results with the different substitutions at position 425 show that only hydrophobic residues like leucine or valine induce hyperaggregation. Leucine induces more pelletable aggregates than methionine, which in turn produces more than the valine substitution. None of the other amino acid substitutions could generate pelletable aggregates above the background level of the wild-type peptide.

Although they prevent hyperaggregation, these mutations do not interfere with the normal formation of multimers. Figure 4B shows the soluble fraction of the different mutant peptides analyzed by gel filtration, after the hyperaggregates have been removed by centrifugation. The C-terminal half of the protein behaves very much like the full-length protein also in this assay, except that peak 3 is more prominent and the intermediate peak 2 is more distinct. The soluble fraction of the Z¹ C-terminal peptide shows a clear reduction in the relative size of peak 3 as compared with the wild-type peptide, indicating that the hyperaggregate is formed at the expense of the multimer. Peaks 2 and 1 of the Z¹ peptide, although they appear larger in Figure 4B, contain about the same fraction of the total protein as the wild-type if we include the pelleted material instead of normalizing to the soluble counts loaded on the column. The K425L substitution causes an even stronger reduction in peak 3 and the appearance of another, more aggregated form eluting with the exclusion volume (peak 4). The valine substitution (K425V) also shows a significant reduction in peak 3, but much less pronounced than with the Leu or Met (Z¹) substitutions. These results confirm those obtained with the sedimentation test (Figure 4A), showing that the degree of hydrophobicity at residue 425 is correlated with the degree of hyperaggregation induced. The mutants bearing hydrophilic or neutral amino acid substitutions (K425G, K425E and K425Q) show no differences relative to the wild-type peptide (Figure 4B, K425Q and others not shown). We conclude that the charged side chain of the lysine residue is not specifically involved in regulating the degree of multimerization and that any one of a number of hydrophilic residues is sufficient to prevent the hyperaggregation.

Since the Z¹ hyperaggregation is induced by hydrophobicity at position 425, it might be possible to revert it by introducing a charged residue nearby. We converted the leucine at position 428 of the Z¹ protein to glutamate, resulting in a doubly mutant protein bearing the K425M and L428E mutations (Z¹L428E). The C-terminal half of this mutant was translated *in vitro* and analyzed by centrifugation and gel filtration. Figure 6A shows that the double-mutant product (Z¹L428E) is no longer fast-sedimenting, but remains in the supernatant like the wild-type protein. In contrast, a nearby threonine to alanine mutation does not revert the Z¹-induced hyperaggregation (Z¹T434A). Gel filtration of the soluble fraction confirms that the L428E substitution completely reverts the Z¹-induced hyperaggregation (compare Z¹L428E in Figure 6B with WT in Figure 4B), while the profile of the Z¹T434A peptide

shows little difference from that of the Z^1 peptide (not shown).

Z^{op6} aggregates more than Z^1

In most cases, Z^{op6} product forms more pelletable aggregate than Z^1 , both when the full-length protein is used (Figure 3) or the C-terminal half peptide (Figure 6A), suggesting that Z^{op6} aggregates more than Z^1 . When the supernatant fraction of the Z^1 and Z^{op6} peptides are analyzed by gel filtration we found that the multimer (peak 3) is even more suppressed in Z^{op6} than in Z^1 (Figure 6B) and, in addition, the Z^{op6} peptide generates a new higher order aggregate (peak 4) eluted in the void volume. Peak 4 was also seen with the K425L mutation, but in much smaller amounts (Figure 4B) and with the full-length Z^{op6} , but not Z^1 product (data not shown). We do not know whether the peak 4 complex is a specific form or a range of higher multimers, but it is clearly not simply a contamination with pellet material since it always occurs at the expense of peak 3 and is not seen in other mutants that also form hyperaggregates (Figure 6B). These results indicate that the Z^{op6} protein forms more and larger aggregates than Z^1 , probably differing from the Z^1 aggregates only in degree: enhancing the hydrophobicity at the 425 residue (K425L) apparently produces a similar result. To understand the cause of the enhanced hyperaggregation produced by Z^{op6} , we introduced different amino acid substitutions in the Z^{op6} region starting from a z^1 template (Figure 5). The site of the z^{op6} mutation, Pro 454, was changed to a charged (P454E) or to another hydrophobic residue (P454V). We also changed the nearby Pro 456 to Leu (P456L) to see if it mimicked the z^{op6} effect. Figure 6A shows that these mutations do not interfere with the Z^1 -induced hyperaggregation and the greater part of the translated products are pelleted in the sedimentation assay, as with the Z^1 protein. Gel filtration analysis of the soluble fractions shows that hydrophobic substitutions at position 454 or 456 produce similar amounts of peak 4 aggregates (Figure 6B, Z^1 P456L and Z^1 P454V not shown), while the Z^1 P454E mutant gives a pattern similar to that of the Z^1 peptide. These results suggest that the behavior of Z^{op6} is due to the increased hydrophobic nature of the z^1 - z^{op6} region rather than to specific changes at specific amino acids.

Hyperaggregation is correlated with white gene repression *in vivo*

Both the z^1 and the z^{op6} mutations repress the expression of the *white* gene, and both display degrees of hyperaggregation above the multimer typical of the wild-type protein. To further confirm the correlation between hyperaggregation and *zeste*-*white* interaction, we inserted the various *zeste* mutant genes into the pUCHsneo vector (Steller and Pirrotta, 1985), under the control of the hsp70 promoter, and introduced the construct into *In(1)e(bx)* flies by P-mediated germ line transformation. Figure 7 shows the eye color phenotypes of flies carrying some representative *zeste* mutant transgenes and the results, summarized in Table II, indicate a complete correlation between the ability of the mutants to form hyperaggregates and the ability to suppress eye pigmentation. The z^1 transgene, like the endogenous z^1 gene, suppresses *white* gene expression in the eyes of females but not of males (Figure 7, and Pirrotta *et al.*, 1987). The K425L mutant, which aggregates better than Z^1 , has a

Fig. 5. Amino acid sequence of the Z^1 and C-terminal domains showing the mutated residues. The mutations produced in this work are shown above the residue affected and the genetically isolated mutations z^1 , z^{op6} and z^{1163} are indicated by arrows. The z^{op6} and z^{1163} mutants are double mutants, containing also the z^1 mutation. The C-terminal helical domain is underlined with the three hydrophobic heptad repeats indicated below the sequence (FFLLI; LLHLLI; YLM). The numbering corresponds to that of the amino acid residues in the intact protein.

similar yellow eye phenotype in females, but it clearly has an effect also in males, reducing pigmentation to a reddish-brown. The valine substitution, which forms hyperaggregates to a lesser extent than Z^1 , reduces eye color to brown in females, but not in males (Table II). Hydrophilic substitutions at position 425, which behave *in vitro* like wild-type protein, produce wild-type eye color and are as capable as wild-type of complementing the z^1 or z^{op6} mutation in heterozygous combination. The L428E mutation, which reverts the Z^1 hyperaggregation back to wild-type, also reverts the z^1 phenotype back to wild-type eye color (Figure 7, Z^1 L428E) and complements the z^1 mutant gene as the wild-type does. The z^{op6} transgene (Z^1 P454L) reduces to yellow the eye color of both female and male flies just as the endogenous z^{op6} allele does (Figure 7). If the 454 residue is changed to glutamate, the transgene (Z^1 P454E) behaves just like z^1 and gives a yellow eye color in females, but not males, consistent with its Z^1 -like hyperaggregation. The change of Pro 454 to Val (Z^1 P454V) or the introduction of a leucine in a nearby position (Z^1 P456L) results in a phenotype similar to that of the z^{op6} mutation and in the appearance of peak 4 aggregates. In most of the independently transformed lines, the Z^1 P454V and Z^1 P456L transgenes show a stronger repression of eye pigmentation in the female than in the male, indicating that pairing of the *white* target gene enhances the repressive effect. The results indicate that the pairing-dependent suppression of *white* gene expression is closely associated with the Z^1 type of hyperaggregation, while the ability to suppress even single unpaired copies of *white* is accompanied by the appearance of still higher states of aggregation exemplified by peak 4 in the excluded volume of the gel filtration column.

The C-terminal peptide prevents hyperaggregation *in vitro*

In vivo, the z^+ allele can complement the yellow eye phenotype of the z^1 mutation. If the hyperaggregated form of the Zeste protein is actually responsible for *white* suppression, the Zeste forms that can complement the z^1

Fig. 6. The *z^{op6}* mutation enhances the *z¹*-induced hyperaggregation. (A) Sedimentation test of the C-terminal half peptides with mutations in the *Z^{op6}* region. The supernatant (S) and pellet (P) fractions for each mutant were analyzed by SDS–PAGE. St indicates the stacking gel where undissociated aggregates remain trapped. The wild-type (WT) and Z¹L428E mutants remain in the supernatant. Z¹ and Z¹P454E show similar levels of pelleted aggregate. Z^{op6}, Z¹P456L and Z¹P454V reproducibly give more pelleted aggregates than Z¹. (B) Gel filtration analysis of the supernatant fraction of these mutant peptides (black squares) compared with the Z¹ peptide (open square). Peaks 1, 2, 3 and 4 are indicated as in Figure 4B. The Z^{op6} (or Z¹P454L), Z¹P456L and Z¹P454V (not shown) have similar profiles with a prominent peak 4 eluting in the void volume. The L428E mutation reverts the Z¹ profile to wild-type and Z¹P454E and Z¹T434A (not shown) show little difference from Z¹.

phenotype *in vivo* should also be able to prevent the *z¹* protein from forming hyperaggregates *in vitro*. To test the ability of the C-terminal half peptide (C-Z⁺) to prevent hyperaggregation of the full-length *z¹* protein, we co-translated *in vitro* equal amounts of C-Z⁺ and *z¹* mRNA and compared the degree of aggregation of the product with that of C-Z⁺ and *z¹* proteins translated separately. Figure 8A shows that the C-Z⁺ by itself remains in the supernatant soluble fraction and, when co-translated with the full-length *z¹* protein, can decrease the amount of hyperaggregation. Little or no inhibition of hyperaggregation was observed when the two proteins were translated separately and then mixed together, suggesting that the Zeste multimers are fairly stable and not in a rapid equilibrium. This result indicates that the C-terminal half peptide alone is sufficient to prevent *z¹* from forming insoluble aggregates, but only when the two proteins have a chance

to interact as monomers or in the early stages of oligomer assembly. The percentage of pelleted aggregates and the proportion of *z¹* and C-Z⁺ peptides present in the pellet were quantitated using a PhosphorImager (Molecular Dynamics), and the relative molar ratios were calculated according to the number of methionine residues present in each methionine-labeled peptide. Figure 8B shows that the proportion of insoluble aggregates declines gradually as the input ratio of C-Z⁺ to Z¹ increases and a 4-fold excess of C-Z⁺ prevents hyperaggregation almost completely. However, the amount of C-Z⁺ found in the pellet remains a fairly constant percent of the input, suggesting that hyperaggregate formation is inhibited when the complex includes more than a small proportion of C-Z⁺. Inhibition of hyperaggregation of the Z^{op6} protein is much less efficient and requires ~5 times more C-Z⁺. This behavior parallels the *in vivo* complementation effects where *z⁺/z¹* results in a wild-type red eye color while *z⁺/z^{op6}* females still have yellow eyes (Lifschytz and Green, 1984). Entirely similar results were obtained using full-length Z⁺ protein. As expected, the inhibition of hyperaggregation is mediated by the hydrophobic ridges in the C-terminal domain. The C-Z¹IG3 half peptide (which carries both the *z¹* and the Y510Δ mutations) forms dimers but not multimers and does not hyperaggregate by itself, is ~8 times less efficient in inhibiting hyperaggregation of Z¹. Cross-linking with EGS shows that when C-Z⁺ and Z¹ are co-translated, heterodimer as well as homodimer forms are produced (not shown), demonstrating that the C-Z⁺ peptide is incorporated in Z¹ complexes.

Complementation of *zeste* phenotype by C-Z peptides

The prevention of the hyperaggregation of the *z¹* protein by the wild-type protein *in vitro* provides a molecular basis for the complementation effect observed *in vivo*. It is difficult to compare the relative concentrations of the different expected forms of Zeste that would be expected in heterozygous flies or to predict their relative binding affinity for DNA target sites. The resulting phenotypes would be even harder to predict since residual pure wild-type multimer might give sufficient expression of the *white* gene to produce significantly pigmented eyes regardless of the properties of the heteromultimer. To avoid such complications, instead of the full-length proteins, we tested the complementation of mutant *zeste* alleles by the C-Z⁺ peptide which cannot bind to DNA by itself since it lacks the DNA-binding domain. A transposon expressing C-Z⁺ under the control of the *hsp70* promoter was constructed and transformed into flies by P element-mediated germ line transformation. This transposon by itself neither stimulates nor suppresses *white* gene expression in either a *zeste* null (*z^{v77h}*) or in *In(1)e(bx)* flies even after heat shock induction (data not shown). If the hyperaggregation of *z¹* is responsible or necessary for its repressing effect *in vivo*, the ability of the C-Z⁺ peptide to prevent hyperaggregation *in vitro* leads us to expect that it would reverse the *z¹* phenotype *in vivo*. When the hs-C-Z⁺ transposon is introduced into the *z¹* background, the yellow eye color phenotype reverts to red in a dosage-dependent manner (Figure 9). With one copy of the hs-C-Z⁺ transposon, the yellow eye color of *z¹/z¹* females becomes reddish even at room temperature. With two copies of the transposon, the color becomes almost completely wild-type red. The same hs-C-Z⁺ transposon, introduced into a *z^{op6}* background, could not complement the yellow eye

Fig. 7. Eye color phenotype of flies carrying representative *zeste* mutant transgenes. The mutant transgenes are indicated above, the sex of the fly is indicated on the left. Note that the K425L transgene suppresses eye color in the female and, to some extent, also in the male. The z^1 L428E transgene has wild-type eye color and z^1 P454E gives a phenotype similar to that of z^1 . Z^{op6} and z^1 P456L both repress eye color in both female and male flies. Flies were photographed 1 day after eclosion.

phenotype of z^{op6}/z^{op6} females, but it was able to revert the eye color of z^{op6}/Y males to bright red. This behavior parallels its ability to reduce hyperaggregation of z^{op6} *in vitro* to the level of z^1 , when present in equimolar amounts. This ability of the C- Z^+ peptide to restore wild-type eye color only in combination with z^1 or z^{op6} proteins, but not by itself, suggests that it is interacting with the mutant protein and most likely preventing it from forming hyperaggregates much as it does *in vitro*. These results show that the C- Z^+ peptide behaves like the full-length wild-type protein in complementing the repression of *white* gene expression by the z^1 mutant protein. It is unlikely that this effect is due to a weakening of the DNA-binding ability of the z^1 protein by the C-terminal peptide because the eye color restored is bright red like wild type, not brown like the loss-of-function mutants.

We also tested the effect of the C-terminal half peptide of the z^{op6} protein (C- Z^{op6}) in various genetic backgrounds. In z^{v77h} or in *In(1)e(bx)* backgrounds this gene has no detectable effect, indicating that the C- Z^{op6} peptide cannot repress the *white* gene by itself. This result implies that the suppression of *white* gene expression is not due to sequestration of *white* enhancer factors, but that it requires binding of the mutant product to the eye enhancer. A very weak effect was observed when the C- Z^{op6} gene was introduced in z^{op6} males, but not in females, increasing the pale yellow eye color to orange. This might be due to a weakening of the DNA-binding ability of Zeste when the complex incorporates the C-terminal peptide, which cannot contribute to DNA binding. We observed no significant effect when C- Z^{op6} was introduced in a z^+ background, except for a weak eye color reduction in the females of one of the eight transformed lines, indicating that the C- Z^{op6} peptide might be able to recruit sufficient wild-type protein into a hyperaggregated form which retains enough DNA-binding ability to cause some *white* gene repression.

Discussion

Stepwise assembly of Zeste aggregates

The formation of large, fast-sedimenting aggregates was shown by Bickel and Pirrotta (1990) to be a property of Zeste

Table II. Correlation between aggregation and the eye color phenotype of various *zeste* mutations

Mutations	Complexes observed	Eye color phenotype	
		Female	Male
WT	Multimer	Red	Red
z^1	Aggregate	Yellow	Red
z^{op6}	Aggregate	Yellow	Yellow
z^{11G3}	Oligomer	Red	Red
$z^+ z^{op6}$	Multimer	Red	Red
Y510Δ	Oligomer	Red	Red
z^1 K564L	Aggregate	Yellow	Red
z^1 H548L	Aggregate	Yellow	Red
z^1 L518D	Oligomer	Red	Red
ΔBB	Multimer	Red	Red
ΔEP	Oligomer	Red	Red
ΔAD	Multimer	Red	Red
K425G	Multimer	Red	Red
K425L	Aggregate	Yellow	Dull red
K425V	Aggregate	Dark red	Red
K425E	Multimer	Red	Red
K425Q	Multimer	Red	Red
z^1 L428E	Multimer	Red	Red
z^1 P456L	Aggregate	Yellow	Yellow–orange
z^1 P454V	Aggregate	Yellow	Yellow–orange
z^1 P454E	Aggregate	Yellow	Red
z^1	Aggregate	Yellow	Red

The mutations are described in the text and the aggregation properties are observed in 500 mM NaCl. Aggregate indicates the formation of pelletable product by spinning for 10 min at 10 000 g. Multimer and oligomer indicate the formation of peak 3 and peak 2, respectively, in the gel-filtration analysis. The eye color phenotypes of flies carrying various transgenes was determined in a *In(1)e(bx)* genetic background. Transgenes that give weak reduction in the eye color give stronger suppressing effects when crossed into the null z^{v77h} genetic background. Transgenes that give wild-type eye color also complement the endogenous z^1 mutation.

protein expressed in bacteria or isolated from flies. However, extraction of the protein from cells required detergents or denaturing agents and inappropriate aggregation could result when these were removed by dialysis. Chen *et al.* (1992) showed that Zeste protein translated *in vitro* and never

Fig. 8. (A) The C-Z⁺ peptide prevents Z¹ hyperaggregation. The C-Z⁺ peptide was translated alone or co-translated with full-length Z¹ protein and the products were analyzed by the sedimentation test and SDS-PAGE. The Z¹ protein forms a large proportion of pelleted aggregates, while the C-Z⁺ remains largely soluble. Co-translation of Z¹ with C-Z⁺ significantly reduced the proportion of the Z¹ aggregates. When these two peptides were translated separately and then mixed, no significant effect was observed. (B) Titration of Z¹ and Z^{op6} hyperaggregation by C-terminal half peptides. The relative amounts of each peptide in the pellet and supernatant fraction were quantitated with a PhosphorImager and adjusted to the number of labeled methionines in each peptide. The percentages of the pelleted aggregates are plotted against the relative molar ratios of the C-Z peptide to full-length Z¹ or Z^{op6} protein. Closed circles: C-Z⁺ with Z¹; closed squares: C-Z^{11G3} with Z¹; open triangles: C-Z⁺ with Z^{op6}.

subjected to the denaturation or detergents displayed the same property, dependent on the C-terminal domain. As we show in this study, the multimerization that is observed with Zeste protein translated in most batches of reticulocyte lysate is not a disorderly aggregation, but a sequential assembly through a series of steps involving different parts of the protein.

The C-terminal domain responsible for the aggregation is predicted to form an extensive helical domain with three hydrophobic ridges whose arrangement on different faces and at different ends of the helical structure suggests that they allow simultaneous interaction of Zeste molecule with two or more other Zeste monomers to build multimeric chains (Chen *et al.*, 1992). Our results show that all three hydrophobic ridges are involved in this process. Dimer formation mediated by the leucine zipper is the first and prerequisite step in multimerization. The fact that the assembly begins with the most C-terminal part of the protein, which is therefore also the last part to be synthesized, argues against the possibility that the aggregation is an artefact of *in vitro* translation where nascent proteins may interact before they are completely folded. The fact that mutations that disrupt the hydrophobic heptad repeats within the 11G3

Fig. 9. Complementation of the eye color phenotypes of z¹ and z^{op6} flies by C-Z⁺. The C-Z⁺ peptide complements the z¹ yellow eye color phenotype in a dosage-dependent manner (A). The z¹ females normally are yellow-eyed (A, top left), but in the presence of one copy of C-Z⁺ transposon, the eye color become dull reddish (A, right). With two copies of C-Z⁺ transposon, the z¹ females are restored to a bright red eye color (A, bottom) similar to wild-type flies. The C-Z⁺ transposon complements the z^{op6} yellow eye phenotype in males, but not in females. With one copy of C-Z⁺, z^{op6} males are restored to bright red eye color (B, bottom). However, even with two copies of the transposon, the yellow eyes of the z^{op6} females remained unaffected (B, top right). The yellow eye color of the z^{op6} male is also shown in B (top left) for comparison. The C-Z⁺ transposon has no visible effect on the eye color of wild-type flies or of z^{v77h} or *In(1)e(bx)* flies which have respectively complete and partial loss of zeste function.

region do not interfere with dimer formation indicates that the leucine zipper-dependent dimerization is independent of the other hydrophobic interactions and implies that the leucine zipper region interacts homotypically with another leucine zipper region and not heterotypically with the 11G3 region. By analogy with the interaction of leucine zippers in GCN4 dimers (O'Shea *et al.*, 1991), we presume that in our case also the two helices align in parallel orientation. This arrangement leaves the FFLI heptad repeat on the opposite face of the helix available for further interaction with another Zeste molecule. The importance of this

arrangement, with the two major hydrophobic surfaces on opposite faces and at opposite ends of the helix, is underlined by the behavior of the ΔIE mutant. This mutant protein, in which the two hydrophobic ridges are brought together to form a single continuous hydrophobic surface, still forms dimers efficiently, but does not go on to form higher multimers.

If the interaction between two FFLI ridges cannot by itself form stable dimers, how can it stably link two or more dimers produced by the interaction between leucine zippers? We suppose that the formation of the coiled-coil structure in the leucine zipper region of the dimer stabilizes the interaction with a third molecule through the FFLI and the short YLM hydrophobic ridges. This might occur through an alteration of the helical structure or by favorably positioning other residues for additional interactions with the third molecule. We find, in fact, that certain mutations in the leucine zipper region, such as L555Q or L550Q, that do not interfere with dimer formation, still prevent multimerization. The effects of these mutations are consistent with the observations of many others, showing that substitution of a single leucine in a leucine zipper, even with a polar amino acid such as Gln, does not necessarily abolish dimerization (Hu *et al.*, 1990). In our case, however, Gln prevents further multimerization, possibly by distorting or weakening the coiled-coil structure of the dimer and preventing the interactions that stabilize further pairing through the FFLI ridge.

Hyperaggregation of Z^1 mutant protein

The z^1 mutation creates the potential to form still larger and more stable aggregates, so large that they can be sedimented at low speed and so stable that they are not dissociated by high salt and are partially resistant to boiling in 2% SDS. In z^1 , the K425M mutation generates instead a block of hydrophobic residues VMM that promotes further protein-protein association. The degree of hydrophobicity at this position is correlated with the extent of hyperaggregation but the lysine, as such, is not essential for normal *zeste* function since substitution with other polar or neutral amino acids does not affect the degree of aggregation. A hydrophobic amino acid in this position can be counteracted by the introduction of another charged group nearby, as in the mutant L428E, although we cannot be sure that this effect is due to the introduction of the charge rather than the loss of the hydrophobic leucine residue. If the presence of a charged group prevents hyperaggregation, it is interesting to speculate that, *in vivo*, phosphorylation of the many threonines and serines in this domain (9 out of 20 residues following the z^1 mutation site are Thr or Ser conserved in the *D. virilis zeste* gene) might regulate the degree of aggregate formation.

The mutations in the z^1 region do not have an effect on the properties of the protein unless accompanied by an intact C-terminal domain. Amino acid changes that interfere at any stage with the normal assembly of multimers prevent the z^1 mutation from inducing hyperaggregation, indicating that multimerization is a prerequisite for the z^1 -induced hyperaggregation. However, further stages of hyperaggregation can be induced by additional changes in the z^{op6} region. The z^{op6} P454L mutation apparently enhances the z^1 -induced hyperaggregation by increasing the hydrophobicity in the region. It is unlikely that this is due specifically to the loss

of the proline since substitution with non-hydrophobic amino acids does not produce the effect. Furthermore, a nearby proline to leucine substitution has the same result both with respect to the aggregation properties *in vitro* and the pairing independence *in vivo*, suggesting that this region of the polypeptide may regulate the degree of multimerization through a balance of hydrophilic and hydrophobic residues.

Is the *in vitro* aggregation significant for *in vivo* behavior?

Although our results demonstrate that the aggregation of Zeste monomers is an orderly and directed assembly, how do we know that this kind of aggregation takes place *in vivo*? We cannot demonstrate the molecular form of the Zeste protein in the cell, but upon gentle lysis of nuclei followed by high-salt extraction, Zeste is found in rapidly sedimenting complexes that behave just like the aggregates observed *in vitro* (Bickel and Pirrotta, 1990). This does not exclude the possibility that Zeste engages in interactions with other proteins rather than with itself, using the same C-terminal domain features that we have shown are physiologically important. The following arguments demonstrate that Zeste-Zeste interactions must occur *in vivo* both through the C-terminal domain and through the z^1 region. The ability of the z^1 allele to repress *white* gene expression can be competed *in vivo* by a z^+ allele, but also by a z^+ transgene or by a transgene expressing only the C-terminal domain of Z^+ , hence lacking the DNA-binding domain. The formation of Z^1 hyperaggregates *in vitro* is correspondingly suppressed by co-translation with C-terminal domain peptide. Both the *in vivo* and the *in vitro* effects depend on the ability to form multimers through an intact C-terminal domain. Therefore, whether or not the aggregates formed *in vivo* are as large as those observed *in vitro*, Zeste-Zeste interactions involving these surfaces must take place in the cell and are required for *zeste* function *in vivo*. Furthermore, they need to take place in the cell because only the oligomeric form of the protein is capable of binding efficiently to DNA (Chen and Pirrotta, 1993, accompanying paper). Binding of full-length Zeste protein to DNA is cooperative and requires the interaction of oligomeric *zeste* protein with multiple adjacent binding sites. Mutations in the C-terminal domain that reduce the ability to form multimers reduce binding affinity and mutations that prevent dimerization cause loss of binding activity.

Hyperaggregation and suppression of *white* expression

The ability to form aggregates is important for both phenotypic effects associated with the *zeste* gene: the pairing-dependent interallelic complementation referred to as transvection and the repressive effect of the z^1 mutation on the expression of the *white* gene in the eye (Bickel and Pirrotta, 1990). When we tested some of our C-terminal domain mutants for the ability to support transvection effects in the *Ubx* gene, we found that mutants like H548L and K564L, which aggregate efficiently, also support *Ubx* transvection, while mutants that do not aggregate are also deficient in transvection. However, none of these mutations, by itself, affects the expression of the *white* gene. The *zeste-white* interaction requires the z^1 mutation, and our results show a direct and detailed correspondence between the effects of mutations or combinations of mutations on

hyperaggregation and the ability to suppress the expression of the *white* gene *in vivo*. Furthermore, the ability to suppress a single or an unpaired copy of *white* is not due to specific mutations near position 454, the site of the *z^{op6}* mutation, but occurs along a continuum of hyperaggregation correlated rather with the appearance of peak 4 in our gel filtration assay. Even the single K425L change, which induces stronger hyperaggregation than the *z^l* K425M mutation, results in a small amount of peak 4 in parallel with a significant suppression of *white* gene expression in male flies (Figures 4 and 7).

These results all suggest that it is the size of Zeste protein aggregates that is responsible for *white* gene suppression and the larger the aggregate, the less it is dependent on pairing of the *white* gene to have a repressive effect. Although we could not measure it directly, it is likely that the size of the *Z^{op6}* aggregate is greater than that of the *Z^l* aggregate since more pelletable material is formed and a larger intermediate is visible in the soluble fraction. Furthermore, substitutions at L425 that cause greater aggregation are less dependent on pairing than the *z^l* mutation, while mutations at P454 that decrease aggregation are more dependent on pairing than *z^{op6}*. The correlation between the ability of C-*Z⁺* to inhibit hyperaggregation of *Z^l* *in vitro* and its ability to suppress the *z^l* phenotype *in vivo* strongly implies that it is the additional Zeste–Zeste interactions that result in hyperaggregates that are responsible for the *z^l* phenotype. Other possible explanations in terms of titration of a nuclear factor do not seem tenable and are excluded by the fact that C-*Z^{op6}* expression *in vivo* does not have appreciable effects on eye pigmentation. Wild-type *zeste* normally acts as a stimulator of *white* expression, as shown by the fact that in flies homozygous for a *zeste* deletion or for a *zeste* null mutation like *z^{v77h}*, the eye pigmentation becomes dull brown and uneven (Goldberg *et al.*, 1989; V.Pirrotta, unpublished observations). Overexpression of the full-length *zeste* protein or of the C-*Z⁺* peptide does not have the significant effect on eye color that would be expected if the C-terminal region could sequester a positive transcription factor. It is interesting to note that incorporation of the C-*Z⁺* peptide in a complex with the *Z^l* or *Z^{op6}* proteins both limits their aggregation and converts them from repressors to positive activators. These experiments do not exclude the possibility that Zeste might interact with other proteins through the C-terminal domain or other part of the protein. We have found, for example, that there exist *Drosophila* proteins that interact specifically with the Zeste leucine zipper in a yeast two-hybrid screen (Fields and Song, 1989; J.D.Chen, unpublished experiments). Such interactions, however, are either not strong enough or not exclusive or not limiting enough to produce a phenotype when the C-*Z⁺* domain is overexpressed.

How could a large Zeste aggregate interfere with gene expression? Qian *et al.* (1992) showed that the suppression of *white* gene expression is most likely due to interference with the activity of the eye-specific enhancer or its interaction with the promoter. This enhancer contains five strong Zeste binding sites embedded in sequences responsible for enhancer activity, providing an explanation for the fact that the *z^l* mutation interferes only with expression of *white* in the eye and not in the testes, ocelli or malpighian tubules. Hyperaggregated forms of Zeste protein might interfere with eye enhancer action in at least two ways. The simplest

explanation is that they block the assembly of enhancer factors on the eye enhancer. This is unlikely because various experiments show that, in many chromosomal contexts, the eye enhancer works perfectly well even in the presence of *z^l* or *z^{op6}*. Furthermore, Qian *et al.* (1992) showed that the ability to produce the *zeste* effect requires a short sequence immediately upstream of the promoter, suggesting that what is involved is the interaction of the enhancer with the promoter. Current models of enhancer action envision a looping mechanism through which the factors bound at the enhancer are brought in contact with the promoter complex. This loop formation frequently involves other proteins that mediate interaction between promoter-proximal regions and the enhancer in a geometry that is likely to be affected by the chromatin structure of the region. We suppose that a very large Zeste protein aggregate bound to the eye enhancer might prevent the interaction of enhancer-bound factors with the promoter complex. Finally, the demonstration that the *z^l* effect on the *white* gene is dependent on the formation of hyperaggregates brings us back to the most curious aspect of the *zeste*–*white* interaction: its dependence on the physical pairing of the target gene. This paper shows that large aggregates of Zeste bound to the eye enhancer of *white*, prevent its function and that the greater the tendency to form aggregates, the less dependent on chromosome pairing the effect. The simplest interpretation of this behavior is that the pairing of two target regions enhances the formation of large aggregates and the consequent interference with the eye enhancer. In the case of the *z^l* mutant, the size of the aggregate is large, but not sufficient to cause this interference. The pairing of two copies of the gene brings together two eye enhancers, resulting in the formation of a larger Zeste protein aggregate which is now able to block activation of the promoter. Mutations that enhance hyperaggregation achieve this result without need of pairing. The assembly of large protein complexes involved in repressing gene expression is a feature that has also been attributed to the *Polycomb* (*Pc*) group of genes, necessary to maintain the repressed state of homeotic loci like *Ubx* and *Antp* (Jürgens, 1985; Paro, 1990). It is very suggestive therefore that several members of the *Pc* group are also modifiers of *zeste*. Both suppressor and enhancer of *zeste* alleles have been found at loci such as *E(z)*, *Psc* and *Scm*, members of the *Pc* group, or at *Su(z)2*, a closely related gene (Wu *et al.*, 1989). These modifier alleles suggest that the suppression of *white* expression by *z^l* requires at least some members of the *Pc* group and may involve the assembly of multiprotein complexes as well as Zeste multimers. We do not know whether these proteins are normally associated with *white* gene chromatin or whether they are induced to assemble there by the formation of large *Z^l* hyperaggregates bound to the eye enhancer.

Materials and methods

Site-directed mutagenesis

Site-directed mutagenesis was performed as specified by the Muta-gene M13 *in vitro* mutagenesis kit (Bio-Rad). The sequences of the oligonucleotides used are listed below in the 5' to 3' direction: GAAGGTAAACGCGGCGTGGCG for the T434A mutation; CCACAGGCACT(T/A)CAACCGCCGCG for the P454V and P454E mutations; CAGTGGCCACAAGCACTGGAACC for the P456L mutation; GGCGGCGGTTT(T/C)TTGCATCTTAAC for the L428E mutation;

GGCGTTAGTTGCATC(A/C/T)(C/G)AACCGCCGCCGCC for the K425V, K425L, K425G, K425Q and K425E mutations; CCTGCGCCTTGTTGAG()GCGCTTGGCTTCTG for the Δ IE mutation; CCGTATCTTCATTGTC(G/T)(G/C)CTCCTCGACTCG for the L555P and L555Q mutations; TCCTTGACAGCGC(G/T)(G/C)TTCAGCCTCGC for L518P and L518Q mutations; CTCCTCGACTCGC(G/T)(G/C)TCGATGCACCTCC for the L550P and L550Q mutations.

The bases underlined represent mismatches; / indicates degenerate bases at that position; the empty parentheses indicate a deletion. The mutagenized clones were confirmed by sequencing using primers CCTCCAGACC-TCATGAAGTG and GACCGCCACTGAG.

Plasmid constructions

DNA templates used for site-directed mutagenesis were constructed by cloning a suitable fragment of the wild-type *zeste* cDNA in an M13 vector. The mutated sequences replaced the wild-type sequences in a pET3c vector (Studier and Moffatt, 1986) containing a full-length *zeste* cDNA (as described by Chen *et al.*, 1992) or were cloned directly into the pET3c vector in the case of the C-terminal half peptide (C-Z). In this case, the C-Z peptide produced includes a 13 amino acid leader (MASMTGGNNMGRI) encoded by the pET vector, followed by the *zeste* sequence from residue Gly324 to the end of the protein. The Y510A clone was constructed by replacing the *Bst*BI–*Nco*I fragment of the wild-type cDNA with that from the z^{11G3} sequence. The Z^+Z^{op6} clone was constructed by replacing the *Sac*II–*Nco*I fragment with that of the z^{op6} sequence. The peptide produced by the N-Z clone contains the N-terminal part of the protein until Ala 324, followed by the peptide sequence GDPAANKARKEAELAAATAEQ encoded by the vector. The Δ OPA construct was made by an internal deletion from Ala 324 to Val 411. The Δ AD clone was constructed by P. Miller using PCR with appropriate primers to delete amino acids between Gln 158 and Met 242, and insert in their place the sequence LGASTL. The Δ EP clone was constructed by an internal deletion from Ala 410 to Leu 525 (between *Eag*I and *Pvu*II sites). The Δ BB construct was made by deleting the C-terminal sequence from Phe 504 (cutting at the *Bst*BI site) and the peptide produced ends with GSGC encoded by the vector. The C-Bst clone was constructed by an N-terminal deletion up to Ser 485 (the *Bst*NI site). The Δ BB clone was made by an internal deletion between the *Ban*I and *Bst*NI sites, removing amino acids 157–485 and replacing them with a leucine.

In vitro transcription and translation

The DNA templates were linearized by appropriate restriction enzyme and transcribed *in vitro* with T7 RNA polymerase. The RNA was extracted with phenol–chloroform and translated *in vitro* with nuclease-treated rabbit reticulocyte lysate (Promega) in the presence of 10 mCi [35 S]methionine. All the proteins used in this report were translated from the same batch of lysate and reagents. Repeated freezing and thawing did not change the degree of aggregation or the gel-filtration pattern of the *zeste* protein. However, different batches of lysate produced protein with significantly different degrees of aggregation. For titration experiments, constant amounts of the RNA for the full-length protein were mixed with increasing amounts of C-Z RNA.

Sedimentation test for aggregation

Two microliters of the *in vitro* translated 35 S-labeled protein were diluted into 8 μ l low-salt gel filtration buffer (50 mM NaCl, 20 mM Tris, pH 7.8). The aggregated and soluble fractions were separated by spinning for 10 min at 10 000 g and 4°C. The pellet was washed once with 10 μ l buffer. The pooled supernatant fraction and the pellet fraction were adjusted to equal final volumes in 2 $\times$ SDS sample buffer (0.12 M Tris–HCl, 2% SDS, 2% β -mercaptoethanol, 20% glycerol, 0.001% bromophenol blue, pH 6.8), boiled for 5 min and analyzed on an SDS–polyacrylamide gel (Laemmli, 1970). The gel was stained in 0.25% Coomassie Blue, destained in 40% methanol/7% acetic acid and equilibrated in 1 M sodium salicylate enhancing solution for 30 min. After drying, the gels were exposed at –70°C overnight with an intensifying screen. In some cases, the stacking gel was left intact to detect aggregates that resist SDS dissociation. The relative amounts of each peptide in the titration experiments were quantitated with a PhosphorImager (Molecular Dynamic) and adjusted for the number of labeled methionines present in the peptide. The full-length *zeste* protein contains 12 methionines, the z^1 , z^{op6} and z^{11G3} proteins have 13 methionines. The C-Z $^+$ peptide has six methionine residues while, C-Z 1 , C-Z op6 and C-Z 11G3 have seven.

Gel filtration analysis

Gel filtration was performed in a Pharmacia FPLC system, using a Superose 6/HR column equilibrated with elution buffer (20 mM Tris–HCl, pH 7.8,

500 mM NaCl). Five microliters of *in vitro* translated products were diluted into 25 μ l elution buffer. After spinning for 10 min at 10 000 g and 4°C in a microfuge, the supernatant and pellet were carefully separated and the pellet was washed once with 30 μ l elution buffer. The supernatant and wash were pooled (total 60 μ l) and loaded onto the gel filtration column using a 50 μ l loop. The column was eluted at room temperature at a flow rate of 0.5 ml/min and 0.5 ml fractions were collected. The fractions were treated with 1 M NaOH and 2% H₂O₂ at 37°C for 10 min, precipitated with 20% TCA and bovine serum albumin, and their radioactivity determined by scintillation counting.

Chemical cross-linking

Chemical cross-linking was performed using ethyl glycol bis(succinimidylsuccinate) (EGS, Pierce) as described by Abdella *et al.* (1979). The *in vitro* translated protein was diluted into cross-linking buffer (100 mM triethanolamine, 2 mM EDTA, 5% glycerol, pH 8) and cross-linked for 20 min at room temperature with 5 mM EGS. The reaction was stopped with an equal volume of sample buffer containing 20 mM lysine and 2% SDS. The samples were boiled for 5 min and analyzed by SDS–PAGE.

Transposon constructs and germ line transformation

The mutagenized DNA fragments were put under the control of the *hsp70* promoter and subcloned into the pUChsneo vector (Steller and Pirrotta, 1985). The transposons expressing the C-Z $^+$ or C-Z op6 peptides were constructed using the 3'-end sequences of the corresponding cDNA from the *Nae*I site at amino acid residue Gly324. The C-Z transposons direct the expression of the C-terminal half of the protein with four amino acids (MARI) at the N-terminus encoded by the vector. The resulting constructs, together with helper plasmid, were injected into *ln(1)e(bx)* embryos. The G₀ flies were mated with partners of the same genotype and the transformed G₁ progeny were selected on food containing 0.5 mg/ml G418 (Geneticin, Sigma). More than three independently transformed lines were obtained for each construct except for the Δ AD mutant, which gave only one line and Δ BB, which gave two. The inversion in the *ln(1)e(bx)* host flies has a breakpoint in the middle of the *zeste* coding region (Pirrotta *et al.*, 1987). As a result, this mutation does not complement z^1 and cannot support transvection effects at *Ubx*. However, the *ln(1)e(bx)* allele is not completely null for *zeste* function: it can produce a peptide that includes the *zeste* DNA-binding domain and can modify the phenotype of weak *zeste* alleles. To confirm the phenotype of constructs that showed weak eye color effects (i.e. the K425V mutant), the transposon was also crossed into a z^{v77h} background, which behaves as a true null. The eye color in females homozygous for the endogenous z^{v77h} allele and carrying one copy of the transgene under the control of the *hsp70* promoter is orange, indicating even greater *white* suppression than in the *ln(1)e(bx)* background. We also tested transposons that show wild-type eye color in the *ln(1)e(bx)* background for ability to complement the endogenous z^1 allele. Heterozygous z^1 / *ln(1)e(bx)* females are normally yellow-eyed, but become wild-type red if they carry one copy of a complementing transgene. To introduce transposons into different genetic backgrounds, including z^{v77h} , z^1 and z^{op6} , we crossed transformed males with virgin females of the desired genetic background and selected the progeny on G418.

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