The EMBO Journal vol.12 no.5 pp.2075–2083, 1993

Multimerization of the *Drosophila zeste* protein is required for efficient DNA binding

J.Don Chen[1] and Vincenzo Pirrotta[2,3]

Department of Cell Biology, Baylor College of Medicine, Texas Medical Center, Houston, TX 77030, USA
[1]Present address: Gene Expression Laboratory, Howard Hughes Medical Institute, The Salk Institute for Biological Studies, PO Box 85800, San Diego, CA 92186–5800, USA
[2]Present address: Department of Zoology, University of Geneva, 30 quai E.Ansermet, CH 121 Geneva, Switzerland
[3]Corresponding author

Communicated by V.Pirrotta

The *Drosophila* zeste protein forms multimeric species *in vitro* through its C-terminal domain. Multimerization is required for efficient binding to DNA containing multiple recognition sequences and increasing the number of binding sites stimulates binding in a cooperative manner. Mutants that can only form dimers still bind to a dimeric site, but with lower affinity. Mutations or progressive deletions from the C-terminal show that when even dimer formation is prevented, DNA-binding activity is lost. Surprisingly, binding activity is regained with larger deletions that leave only the DNA-binding domain. Additional protein sequences apparently inhibit DNA binding unless they permit multimerization. The DNA-binding domain peptides bind strongly even to isolated recognition sequences and they bind as monomers. The ability of various *zeste* peptides to stimulate *white* gene expression *in vivo* shows that multimeric forms are the functional species of the *zeste* product in vivo. The DNA-binding domain peptide binds well to DNA *in vitro*, but it cannot stimulate *white* gene expression in vivo. This failure may reflect the need for an activation domain or it may be caused by indiscriminate binding of this peptide to non-functional isolated sites. Multimerization increases binding specificity, selecting only sites with multiple recognition sequences.

Key words: binding specificity/cooperative binding/enhancement of expression/inhibitory domain/multimerization domain

Introduction

The product of the *Drosophila zeste* gene 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). Transvection is a kind of interallelic complementation in which regulatory elements on one copy of a gene act *in trans* to regulate the expression of its homologously paired copy. Loss of function *zeste* alleles such as z^a, z^{11G3} or *In(1)e(bx)* fail to support transvection effects, but there exists a gain of function allele of *zeste*, z^1, that produces a pairing-dependent repressive effect on the expression of *white*, a gene responsible for the accumulation of pigments in the eye. In the presence of the z^1 mutation, the transcription of the wild-type *white* gene in the eye is strongly repressed, 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). Since the *white* gene is on the X chromosome, z^1 females are yellow-eyed, but z^1 males have wild-type red eyes. The suppression of *white* gene expression by the *zeste* mutant product does not strictly require chromosome pairing. Lifschytz and Green (1984) found that the z^1 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*. However, chromosome pairing clearly enhances the suppressive effect because the z^{op6} effect is recessive in the presence of a single copy of *white* (e.g. in males), but dominant over z^+ if two paired copies of *white* are present (e.g. in females).

The *zeste* gene encodes a nuclear, DNA-binding protein found associated with >60 specific sites on the polytenic chromosomes (Pirrotta *et al.*, 1988). It binds to the promoter and to enhancer regions of the *white* gene as well as those of many developmentally important genes such as *Ubx*, *dpp* and *twist* (Benson and Pirrotta, 1988; Pan *et al.*, 1991). DNA binding is strongly cooperative and requires the presence of multiple recognition sequences. Furthermore, the DNA-binding form of the protein is able to bind simultaneously to two different DNA molecules, suggesting an oligomeric structure (Benson and Pirrotta, 1988). In fact, the *zeste* protein expressed in bacteria or translated *in vitro* aggregates to form multimeric structures held together through interactions of the C-terminal domain of the protein (Bickel and Pirrotta, 1990; Chen *et al.*, 1992). This region is predicted to form an extended α-helical structure including ~75 amino acids which is necessary and sufficient to cause the formation of fast-sedimenting complexes with *zeste* protein expressed in bacteria or translated *in vitro* (Chen *et al.*, 1992). The C-terminal domain contains heptad repeats which, in an α-helical structure, would form prominent hydrophobic ridges on opposite faces of the helix. The integrity of these hydrophobic ridges is essential for the ability of *zeste* monomers to multimerize, probably through coiled-coil interactions.

Benson and Pirrotta (1987) used a co-immunoprecipitation assay to show that *zeste* protein expressed in bacteria binds specifically to DNA. Footprinting assays showed that the recognition sequence is T/CGAGT/CG, but that binding *in vitro* requires two or more target sites spaced at distances varying from 18 to 50–60 nucleotides (Benson and Pirrotta, 1988). The DNA-binding domain of *zeste* was mapped near the N-terminus by Mansukhani *et al.* (1988) who used an immunoprecipitation assay and constructs fusing various *zeste* peptides to β-galactosidase. Although the cooperative binding property and the ability to bind to different DNA molecules simultaneously (Benson and Pirrotta, 1988)

suggested that *zeste* protein binds to DNA in an oligomeric form, immunoprecipitation assays revealed no important contribution to DNA-binding activity by other parts of the protein sequence, including the C-terminal multimerization domain (Mansukhani *et al.*, 1988). Chen *et al.* (1992) showed that *zeste*−DNA interactions could be visualized by the band shift electrophoretic method, using *in vitro* translated protein, and demonstrated that mutations in the DNA-binding domain (amino acids 47−136) abolish DNA binding *in vitro* (Chen *et al.*, 1992).

In this report, we further characterize the DNA-binding property of the *Drosophila* zeste protein using this method. We show that *zeste* protein binds to DNA in a highly cooperative manner which depends on its ability to form multimers which can interact simultaneously with multiple recognition sequences. Dimerization through the C-terminal leucine zipper is absolutely required for binding of the full-length protein to a probe containing at least two binding sites; however, the DNA-binding domain alone binds as a monomer to a single binding site. The protein sequence outside of the DNA-binding domain has an inhibitory effect on binding such that DNA affinity is restored only if multimerization, and therefore cooperative binding, can occur and if the binding site contains two or more recognition sequences. Furthermore, we show that the DNA-binding domain alone is not sufficient for *white* gene stimulation and suggest that another region of the protein is required for proper function *in vivo*.

Results

Titration of the DNA-binding affinity of zeste

The *in vitro* translated *zeste* protein has been shown to bind well to a DNA probe containing four *zeste* binding sites from the *Ubx* promoter (Chen *et al.*, 1992). The gel mobility shift assay revealed a large protein−DNA complex remaining at the top of the gel only in the presence of a probe containing multiple *zeste* recognition sequences. By quantitating the percentage of the bound probe versus total probe in the reaction, we determined the binding curve with increasing concentrations of the *zeste* protein. The concentration of the full-length protein in the *in vitro* translation reaction was estimated to be ~0.1 nM by quantitating the radioactivity of the [^{35}S]methionine in the protein separated by SDS−PAGE. Figure 1A shows the binding obtained with a serial dilution of *zeste* protein and a constant amount of probe (10 000 c.p.m. or ~0.1 ng) containing four *zeste* binding sites from the *Ubx* promoter. From the percentages of bound probe plotted against the concentration of *zeste* protein in each reaction (Figure 1B), we estimated that the protein concentration of *zeste* required for 50% binding under these conditions is about 6.4×10^{-12} M.

DNA-binding cooperativity of the zeste protein

Co-immunoprecipitation assays have shown that the binding of the *zeste* protein to the 5-fold binding site at the *Ubx* promoter is greatly reduced by the progressive deletion of consensus sequences, becoming virtually undetectable when three of the five footprinting sites are removed from either end of the array (Biggin *et al.*, 1988). We have shown that *zeste* protein binds well to a DNA probe containing four *zeste* binding sites from the *Ubx* promoter (probe Ubx4). *Hae*III cleavage of this DNA fragment produces two other probes, one with three sites (Ubx3) and one with a single binding site (Ubx1). When the three probes were used in the mobility shift assay, we found that, consistent with the immunoprecipitation results, *zeste* protein has no detectable binding activity for the single-site probe although, according to the footprinting results, this is a strong binding site in the intact fragment. Significant binding was detected with the probe containing three sites and the amount bound increased ~7-fold when all four sites were present (Figure 2A, left).

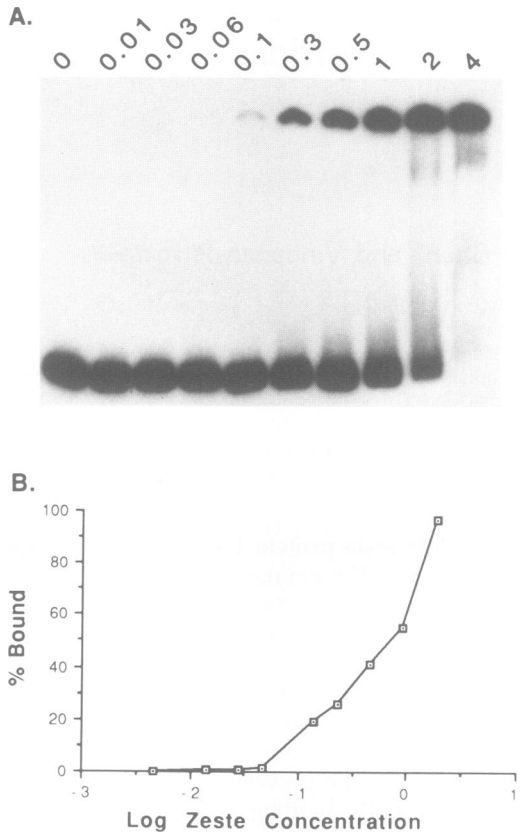

Fig. 1. Dosage-dependent DNA-binding activity of the wild-type *zeste* protein. (**A**) The *in vitro* translated wild-type *zeste* was serially diluted and added into the DNA-binding reactions with a probe from the *Ubx* promoter containing four *zeste* binding sites (probe Ubx4). The products were analyzed on a 5% native polyacrylamide gel and autoradiographed. A single specific protein−DNA complex was observed at the top of the gel. (**B**) The percentages of probe shifted are plotted against the concentration of *zeste* protein (expressed as 10^{-11} M) in each reaction.

The dependence of *zeste* DNA-binding ability on the number of binding sites was further tested using synthetic oligonucleotide probes containing different numbers of copies of a single footprinting site (see Materials and methods). The double-stranded synthetic monomer was ligated to produce probes containing 1−5 copies (probes Z1−Z5) which were tested for binding to full-length *zeste* protein (Figure 2A, right). We detected no binding activity with one or two binding sites, but specific binding was observed with increasing intensity as the number of binding sites increased from three to five. No binding activity was observed with a probe containing three copies of a binding site in which the recognition sequence had been mutated (mZ3). The increase in binding activity is not due to the increased DNA length of the oligomeric probes, since competition assays with DNA fragments of equal length, but with different numbers of binding sites, shows similar results (see

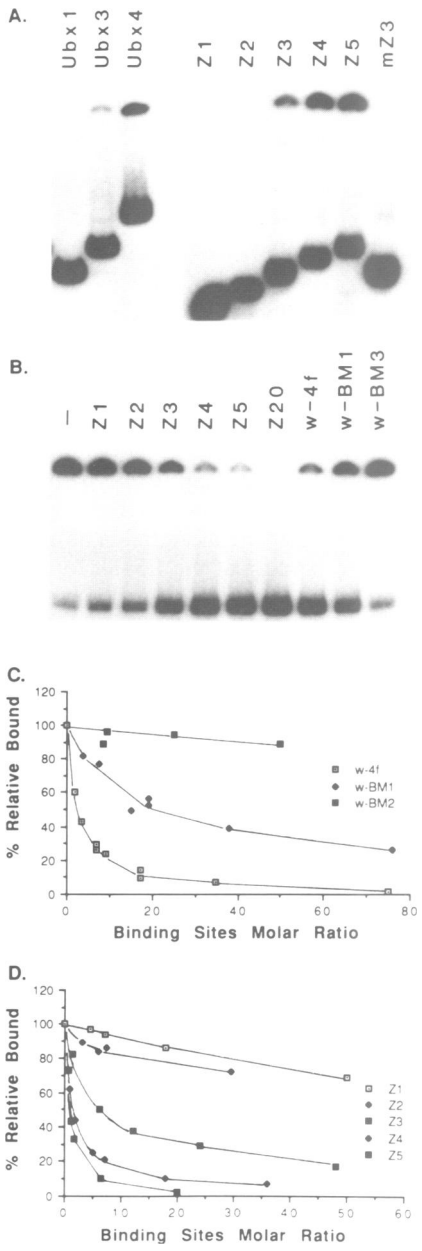

Fig. 2. Cooperative binding of the *zeste* protein to DNA. (**A, left**) The DNA binding of wild-type *zeste* to a *Ubx* promoter fragment containing one (Ubx1), three (Ubx3) or four (Ubx4) *zeste* footprinting sites, analyzed by the mobility shift assay. A constant amount (1 μl) of *in vitro* translated *zeste* protein was used in each reaction and the protein–DNA complex was analyzed on a 5% polyacrylamide gel. (**A, right**) DNA binding of the wild-type *zeste* protein to DNA probes containing 1–5 copies of a synthetic oligonucleotide representing a *zeste* footprinting site (Z1–Z5). mZ3 is a similar probe containing three copies of a mutated site. (**B**) Competition for binding to probe Ubx4 by a 10-fold binding site excess of the competitor indicated above each lane. (**C**) Binding of *zeste* to the Ubx4 probe in the presence of competing DNA fragments from the *white* gene eye enhancer containing three binding sites (w-4f), with one (w-BM1) or two (w-BM2) binding sites mutated, plotted against the molar ratio of binding sites. (**D**) The degree of competition by DNA fragments containing different numbers of copies of a synthetic binding site.

Figure 2B). These results clearly indicate that efficient binding of *zeste* to DNA requires the recognition of multiple adjacent sites, implying a strong contribution from cooperative interactions.

The contribution of cooperativity for binding to targets containing 1–5 binding sites was further examined by a series of competition experiments. The binding of *zeste* protein to the Ubx4 probe was challenged with various amount of competitor DNA fragments containing 1–20 *zeste* binding sites prepared by concatenating a synthetic oligonucleotide (competitors Z1–Z20) or using a fragment from the eye enhancer of the *white* gene in which different numbers of binding sites were mutated (competitors w-4f, w-BM1 and w-BM2 from Qian *et al.*, 1992). Figure 2B shows the competing ability of different competitors present in a 10-fold molar excess of binding sites over the probe. It is clear that the same number of binding sites in higher oligomeric form is a much more effective competitor than in monomeric or lower oligomeric form. The degree of competition displayed by different competitors is shown by plotting the percent binding against the molar ratio of total competitor binding sites to probe binding sites in Figure 2C and D. Taking the amount necessary to give 50% competition, we estimate that the dimer is about twice as efficient as the monomer. The trimer is ~6 times better than the dimer, the tetramer three times better than the trimer and the pentamer two times better than the tetramer. Higher oligomers give only slight further improvement.

The C-terminal leucine zipper is required for DNA binding

zeste protein produced *in vitro* or isolated from the fly tends to form large multimeric complexes mediated by the C-terminal domain of the protein (Bickel and Pirrotta, 1990; Chen *et al.*, 1992; Chen and Pirrotta, 1993). The cooperativity of *zeste*–DNA interactions and the ability of *zeste* to bind simultaneously to two DNA molecules suggest that the preferred binding species is a multimer. To examine this possibility, we compared the DNA-binding ability of a number of *zeste* mutants defective in multimerization (Chen *et al.*, 1992; Chen and Pirrotta, 1993). Approximately equal amounts of *in vitro* translated full-length proteins, quantitated by SDS–PAGE, were used for binding to a DNA probe containing four copies of synthetic binding site. We first tested the effects of mutations that disrupt the FFLLI hydrophobic ridge, producing proteins which dimerize but are defective in multimerization. These mutant proteins bind to a DNA probe containing four *zeste* binding sites less well than wild-type protein does under the same conditions. The DNA–protein complex formed migrates faster than that formed by wild-type protein (Figure 3, L518P, ΔIE and L555Q mutants). Another set of mutant proteins (L550P and L555P), in which the mutations disrupt the leucine zipper required for dimerization (the first and obligatory step in the formation of multimers), show no detectable DNA-binding activity. On the other hand, mutants that display hyperaggregation properties (Z^1 and Z^{op6}) bind to DNA as well as the wild-type protein and form a large DNA–protein complex retained at the top of the gel. The involvement of the leucine zipper in DNA binding was further elucidated by constructing deletions of the C-terminal domain (see below).

Multimerization of zeste protein is required for cooperativity

Our results, which point out the importance of the conserved C-terminal domain for DNA binding, agree with the finding that *zeste* protein exists in solution as a stable multimer and binds to DNA in a multimeric form (Benson and Pirrotta,

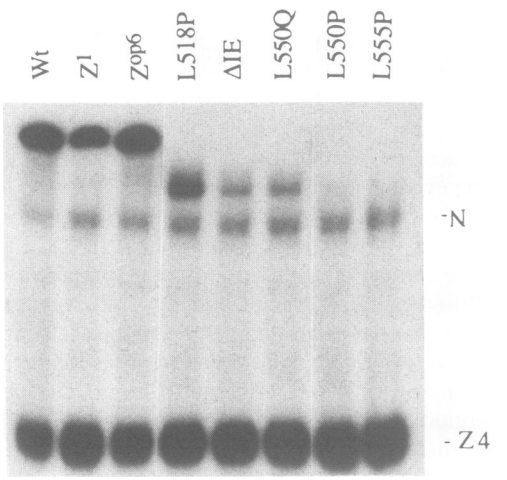

Fig. 3. The C-terminal leucine zipper is required for DNA binding of the full-length *zeste*. The DNA-binding abilities of *zeste* containing various point mutations in the C-terminal domain were analyzed by mobility shift assay as described in Figure 1. Approximately equal amounts of the *in vitro* translated products were used to bind to a synthetic DNA probe containing four binding sites (probe Z4). A non-specific complex (N) is observed in all binding reactions using the synthetic probe and is due to proteins in the reticulocyte lysate. The multimerization-proficient wild-type (Wt), z^1 and z^{op6} proteins form specific complexes that remain at the top of the acrylamide gel. The multimerization-defective, but dimerization-proficient, mutants (L518P, ΔIE, and L555Q) form a specific complex migrating between the non-specific complex and the top of the gel. No specific binding was observed with the mutations that disrupt the leucine zipper (L550P and L555P).

1988; Bickel and Pirrotta, 1990). Mutations that disrupt dimerization block the formation of higher oligomers (Chen and Pirrotta, 1993) and prevent binding to DNA, suggesting that *zeste* binds in the form of a dimer or higher oligomer. Our results show that interactions of higher order also contribute to stabilizing the protein−DNA complex since mutations that allow dimer formation, but prevent further oligomerization, permit DNA binding but (i) less efficiently than wild-type and (ii) resulting in a faster-migrating complex than the wild-type *zeste*−DNA complex (Figure 3).

To examine the role of these higher order interactions in DNA binding, we compared in greater detail the binding of wild-type protein, of the z^1 protein, which makes hyperaggregates, and of the L518P mutant protein, which dimerizes normally but is defective in multimerization. As DNA targets we used a series of oligomers containing different numbers of copies of the *zeste* recognition sequence and we analyzed the protein−DNA complex formed in a 1% agarose gel, which gives better resolution of the slow-migrating complexes. Figure 4 shows that the wild-type protein binds to the probe forming a specific complex capable of entering the 1% agarose gel. Such a complex is formed only with probes containing three or more *zeste* binding sites, while a probe containing three mutated consensus sequences (mZ3) does not bind. As before (Figure 2A), the binding efficiency increases dramatically with probes containing more than three binding sites. In the agarose gel, however, it is possible to distinguish different complexes: probe Z3 gives a single band, but probes Z4 and Z5 give doublet bands which we interpret as due to binding of *zeste* multimers of different sizes. The z^1 protein gives very similar results but, with probes Z4 and Z5, the slower component migrates even slower, suggesting that this is the complex formed by the larger z^1 aggregates. The L518P mutant protein also binds to DNA, but with three clear differences from the wild-type protein: (i) significant binding is obtained to the probe containing two binding sites (Z2), which does not bind detectably to wild-type protein; (ii) the DNA-binding ability does not increase as dramatically with increasing numbers of binding sites and (iii) the mobility of the protein−DNA complexes increases as the number of binding sites increases (compare Z2−Z5 in the L518P complex). Similar results were obtained with other *zeste* mutants that were dimerization proficient, but multimerization defective (not shown).

The higher binding ability of the non-multimerizing mutants to the probe containing two binding sites could be explained by the fact that a given amount of protein that stops at the dimer generates a greater number of dimeric molecules capable of binding to DNA than wild-type protein, which would form fewer molecules in a higher oligomeric state. We suppose that the higher concentration of binding species permits detectable binding of the L518P to the Z2 probe. However, being unable to form multimers, the mutant protein cannot interact with the larger probes much better than with Z2. The increase in mobility of the complexes formed with the larger probes can probably be explained if we suppose that the protein makes the main contribution to the drag while the probe makes the main contribution to the charge, hence to the force driving the migration. Since a larger probe carries a greater number of charges, the resulting complex would have a higher mobility. Although we cannot exclude that complex formation induces changes of shape in the DNA, we do not expect to detect the effect of bending DNA molecules of 50−150 bp in a 1% agarose gel (Levene and Zimm, 1989).

The fact that mutants that can form dimers, but not multimers, bind well to a dimeric target site, but not to a monomeric one, suggests that the affinity for a single site is low but the binding is stabilized by simultaneous interaction of the dimeric protein to two adjacent binding sites. The inability to form higher multimers means that larger arrays of binding sites bind little better because they do not profit from additional cooperativity. These results imply that the monomeric *zeste* protein has very low affinity for DNA. Efficient binding results only when the protein can multimerize and interact simultaneously with a cluster of binding sites.

The DNA-binding inhibitory domain

Although the DNA-binding domain of *zeste* has been mapped to amino acids 48−138 at the N-terminal region (Mansukhani *et al.*, 1988), the preceding results indicate that the C-terminal domain is indirectly important for DNA binding by mediating *zeste*−*zeste* association. Further analysis of its role was carried out by constructing serial deletions of the C-terminal region of the gene and monitoring their effect on DNA binding by the mobility shift assay. The serial deletion mutant genes, represented in Figure 5A, were transcribed and translated *in vitro* and the products analyzed on an SDS acrylamide gel (Figure 5B). The relative amounts of each peptide were quantitated using a Betascope analyzer and adjusting for the number of labeled methionines present in the peptide. Equivalent amounts of each peptide were used for DNA-binding assays with a synthetic DNA probe containing three copies of the *zeste* binding site. Figure 6

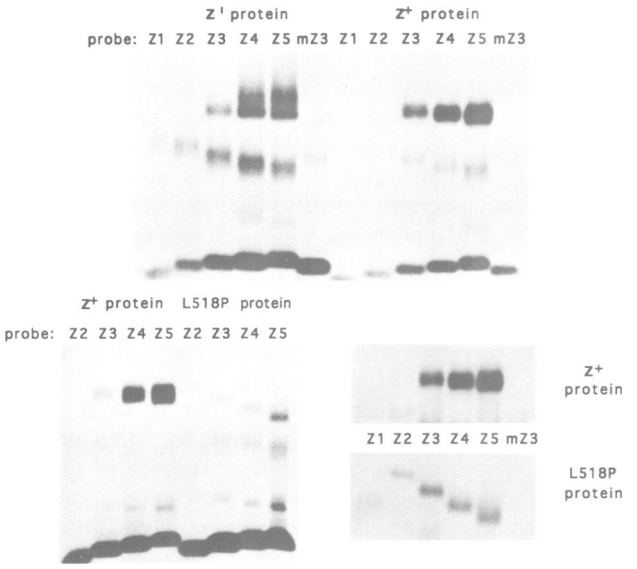

Fig. 4. The protein−DNA complexes formed by z^+, z^1 and L518P mutant proteins. Equivalent amounts of the *in vitro* translated proteins were incubated with DNA probes containing different numbers of *zeste* binding sites (probes Z1−Z5) or with a probe containing three copies of the mutated binding site (mZ3). The complexes formed were analyzed on a 1% agarose gel. As in Figure 2, equimolar amounts of each probe were used but, in the agarose gels, the shorter free probes are partially lost while drying. The diffuse band running in the middle of the gel is caused by non-specific binding of proteins from the reticulocyte lysate. The z^1 or z^+ proteins do not bind detectably to Z1 or Z2 probes, but bind increasingly well to probes Z3 to Z5. The complexes formed with the z^+ and L518P mutant proteins are shown in greater detail in the inserts at bottom right. Note the increase in mobility as the size of the probe increases, particularly with the L518P protein, which can only form dimers. In addition, the z^1 and z^+ proteins form slower-migrating complexes with probes Z4 and Z5 (the heavy bands formed when z^+ protein binds to these probes are actually double bands).

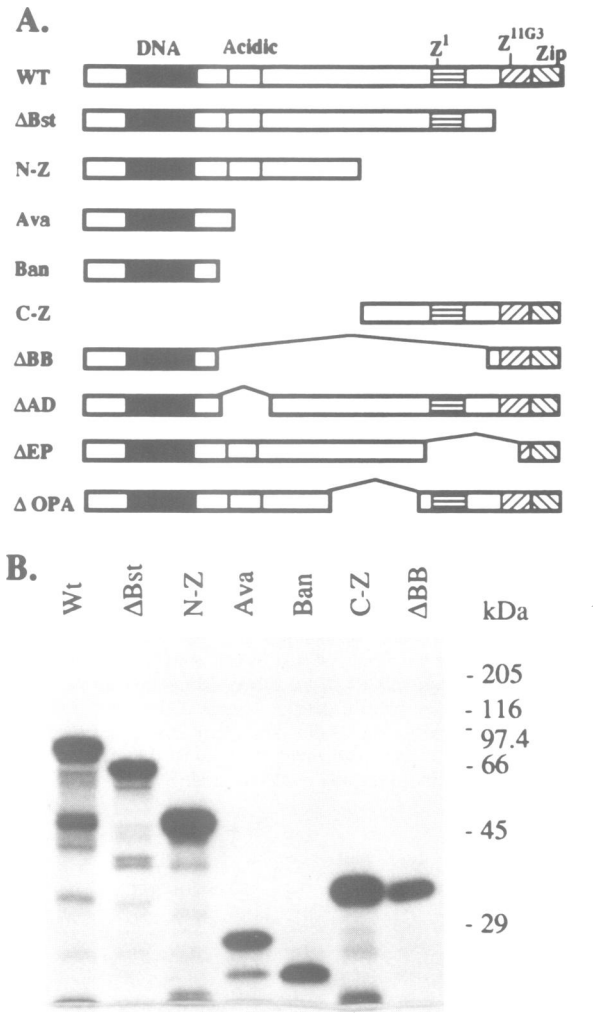

Fig. 5. Serial deletion mutants of the *zeste* protein. (**A**) Schematic map for the serial deletion mutants of *zeste*. The DNA-binding domain (DNA), acidic region (Acidic) and the z^{11G3} region (Z^{11G3}), as well as the leucine zipper repeats, are indicated above. (**B**) Autoradiograph of a 10% SDS−PAGE showing the *in vitro* translated mutant products. The mutant peptides were quantitated with a Betascope blot analyzer and the relative amounts were adjusted according to the numbers of labeled methionine residues in each peptide. The molecular size markers are shown at the right in kDa.

shows that the wild-type protein (Wt) binds well to this probe, but deletion of the C-terminal multimerization domain (ΔBst) or C-terminal half of the sequence (N-Z), abrogates the DNA-binding ability even though these mutations do not affect the DNA-binding domain. The loss of DNA-binding activity is unlikely to be due to the presence of extraneous amino acid sequences at the C-terminal of the mutant peptides since they are short (e.g. only four residues, GSGC, in the ΔBst mutant) and since point mutations in the leucine zipper have the same effects (Figure 3). These results demonstrate that the C-terminal multimerization domain is important for DNA binding.

Surprisingly, the DNA-binding ability was restored when more amino acids were deleted from the C-terminus. The Ava and Ban peptides, which contain little more than the DNA-binding domain, bind even more efficiently than the full-length protein to a probe containing three binding sites (Figure 6). The protein−DNA complexes migrated much faster than those formed with the wild-type protein and more than one complex is observed, probably due to the presence of multiple binding sites. The C-terminal half of *zeste* protein, which multimerizes as extensively as the full-length protein (Chen and Pirrotta, 1993), has no DNA-binding ability while an internal deletion mutant, ΔBB, in which the central part of the protein is removed leaving only the DNA-binding domain and the multimerization domain, binds to DNA well. Other internal deletion mutants including ΔAD (deletion of acidic domain, residues 158−242) and ΔOPA (deletion of the central *opa* repeat region, residues 325−410) bind well to DNA with three or more sites (data not shown) while ΔEP (deletion of residues 410−525, including the z^1 region and the FFLLI hydrophobic ridge) behaves like point mutations that can dimerize but not multimerize and binds to DNA containing two or more sites. The specificity of these DNA interactions is shown by the failure to bind to DNA containing three concatenated mutated binding sites (mZ3). These results indicate that sequences outside the DNA-binding domain have an inhibitory effect on DNA binding. The presence of residues 176−324 is sufficient for this inhibitory effect because deletion of this region restores binding activity (compare binding of the Ava and N-Z peptides). Whether the other C-terminal sequences make any contribution to the inhibitory effect is not known. The inhibitory effect seems to be overcome or compensated for by the cooperative interactions mediated by the C-terminal

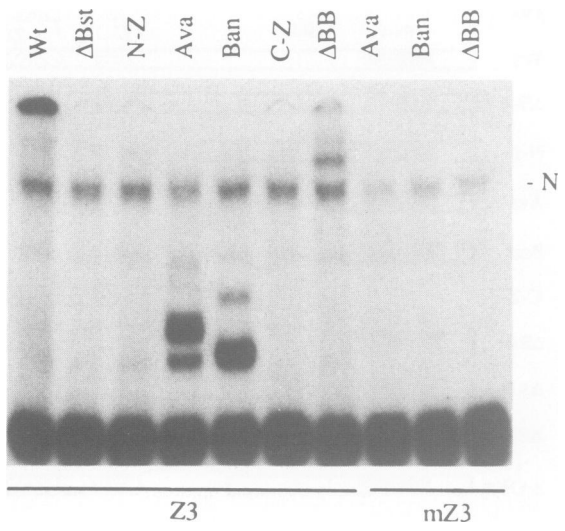

Fig. 6. The domain of *zeste* protein that inhibits DNA binding. Equal amounts of the *in vitro* translated deletion mutants were used to bind to a synthetic DNA probe containing three *zeste* binding sites (probe Z3) and the complexes were analyzed by the mobility shift assay. Only the wild-type protein (Wt), the DNA-binding domain peptides Ava and Ban, and the internal deletion mutant ΔBB show specific DNA-binding activities. The binding specificity is shown by their inability to bind to a probe containing three mutated *zeste* binding sites (mZ3). A non-specific band was observed in all reactions and is indicated by N.

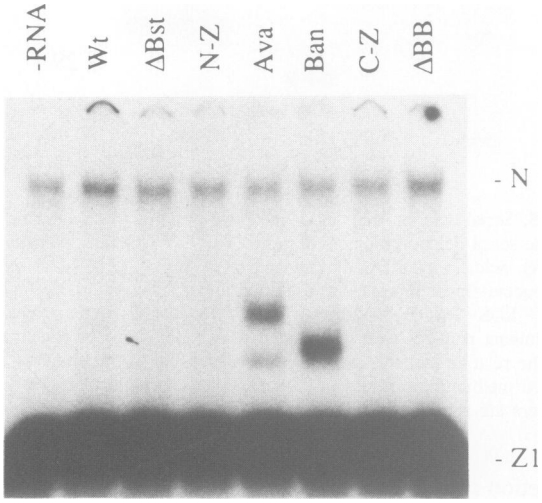

Fig. 7. The DNA-binding domain peptides bind to DNA containing single *zeste* binding site. The DNA-binding abilities of the serial deletion mutants to a DNA probe containing a single binding site (probe Z1) were analyzed by the mobility shift assay. Equal amounts of the *in vitro* translated products, adjusted as described in Figure 5, were used for the binding reactions. N is the non-specific complex.

multimerization domain with DNA targets containing three or more binding sites.

The DNA-binding domain peptide binds strongly to a single site

Since the results described above suggest that the DNA-binding domain peptide regains efficient binding ability though lacking the multimerization domain, we tested its ability to bind to DNA containing a single binding site (probe Z1), to which full-length *zeste* does not bind (Figures 2 and 4). Figure 7 shows that the DNA-binding domain peptides Ava and Ban are able to bind to the single-site probe, while no specific binding was observed with the wild-type, ΔBst, N-Z, C-Z and ΔBB peptides. No binding was observed with multimerization-defectve but dimerization proficient mutants (Figure 4, L518P; also ΔEP, z^{1G3}, L518Δ, not shown) or with dimerization-defective mutants (L555P and L550P, not shown). The ΔBB mutant, in which the inhibitory region is removed, still fails to bind to the single-site probe. This might mean that the C-terminal domain also inhibits single-site binding or it might be a consequence of multimerization, which decreases the effective concentration of binding species for a given amount of protein.

Although the Ava peptide seems to produce two protein−DNA complexes with the single-site DNA probe (Figure 7), this does not indicate the existence of dimer peptides. The two bands are due to the presence of a shorter component in the Ava peptide preparation, approximately the size of the Ban peptide. This component can be seen in Figure 5B and is caused either by a strong pause in the *in vitro* transcription reaction or by a proteolytic activity in the reticulocyte lysate. Partial proteinase K digestion of the protein−DNA complex with the Ava peptide (not shown) increases, in fact, the proportion of the faster component and favors the latter explanation. Furthermore, the ratio of the slower- and faster-migrating complex does not change with increasing amounts of Ava peptide, as would be expected for a monomer−dimer relationship (not shown).

The DNA-binding domain peptide binds as a monomer

The discovery that, unlike the full-length protein, the DNA-binding domain peptide is able to bind to a DNA target containing a single site gave us the possibility to determine the molecular species that binds to a single footprinting site. We co-translated *in vitro* the Ava peptide (197 amino acids, 21.4 kDa) and the Ban peptide (160 amino acids, 16.9 kDa) to allow the possible formation of heterodimers. We found that when the products bind to the Z1 probe, containing a single recognition sequence, are analyzed by the mobility shift assay, they give rise only to the complexes seen with the two peptides separately (not shown). No intermediate complexes were formed, other than those resulting from the degradation products discussed above, indicating that the two peptides can only bind separately as monomers.

Although not able to mediate dimer formation, amphipathic helical structures within the DNA-binding domain might be involved in protein−protein interactions and contribute to cooperative binding. To test whether such interactions between two monomers stabilize binding to two adjacent sites, we analyzed the DNA-binding cooperativity of the DNA-binding domain peptide to the Z2 probe. Figure 8A shows that the second site starts to be occupied only after the first binding site is >30% saturated. The percentage of shifted complexes representing single and double occupation is plotted against the amount of the Ban peptide used in each reaction (Figure 8B). When ~35% of the probe is singly occupied, only 5% is doubly occupied, indicating that the DNA-binding domain peptide does not bind to two adjacent sites in a significantly cooperative manner.

DNA binding and promoter stimulation in vivo

The wild-type *zeste* product has a general stimulatory effect on the expression of the *white* gene since null mutants such

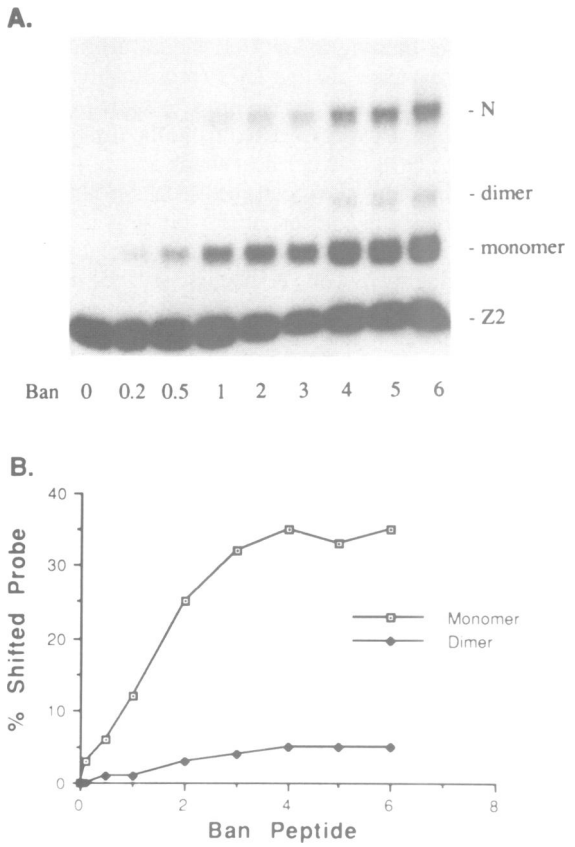

Fig. 8. The DNA-binding domain peptide does not bind cooperatively. (**A**) Mobility shift assay with a synthetic probe containing two *zeste* binding sites and increasing amounts of the Ban DNA-binding domain peptide (indicated in μl). Monomer indicates the complex with one site occupied. Dimer indicates the complex with two molecules of Ban. N indicates a non-specific complex whose intensity increases with increasing amounts of lysate. (**B**) Relative percentage of the dimer versus monomer complex plotted against the amount of the Ban peptide used.

as z^{v77h} or *zeste* deletion mutants have a dull brown eye color. We have used this effect as an assay for *in vivo* activity of different deletion mutants of the *zeste* gene introduced in a z^{v77h} background by means of the pUChsneo transposon vector (Steller and Pirrotta, 1985a) and expressed under the control of the *hsp70* promoter. These experiments, summarized in Table I, show that the normal bright red eye pigmentation is restored by a transposon containing the wild-type *zeste* gene. A similar stimulatory effect on *white* gene expression is also obtained with transposons expressing the Z^1L518Δ, ΔEP or ΔAD products, all of which form at least dimers and have DNA-binding activity. However, a transposon carrying the L555P mutation that disrupts the leucine zipper and DNA-binding activity does not restore wild-type color and the eye remains brown even after a heat shock treatment in the early pupal stage (Steller and Pirrotta, 1985b). The *zeste* N-terminal peptide N-Z, which has no detectable binding activity to DNA containing one or multiple *zeste* binding sites, also fails to stimulate *white* expression with or without heat induction.

We have shown that the DNA-binding domain peptide binds to a single *zeste* binding site as a monomer and with higher affinity than the full-length wild-type protein (Figure 7). Since the promoter-activating effect of *zeste*, according to the *in vitro* transcription assays of Pan *et al.* (1991), is due to relief of H1 histone repression, it is possible that the DNA-binding domain peptide might be sufficient to antagonize H1 histone. When we expressed the Ban DNA-binding domain peptide in z^{v77h} flies, none of the six independently transformed lines showed enhanced eye pigmentation either at room temperature or after heat shock treatment. Although we cannot exclude the possibility that this peptide is unstable *in vivo*, these results suggest that additional *zeste* sequences lying between the DNA-binding domain and the C-terminus may be necessary for the transactivation of *white in vivo*.

Table I. The eye color phenotypes of DNA-binding mutants of *zeste*

Transgenes	Eye color in z^{v77h} background
None	Dull brown
Wild-type	Bright red
L555P	Dull brown
Z^1L518Δ	Bright red
ΔEP	Bright red
ΔAD	Bright red
Ban	Dull brown
In(1)e(bx)	Dull brown

Eye color phenotypes of flies carrying the indicated transgenes under the control of hsp70 promoter. The transposon constructs were first injected into the *In(1)e(bx)* flies and the progeny was selected with G418. Transformed males homozygous for the transposon were crossed to z^{v77h} females and the eye color of the resulting males which have one copy of the transgene with a z^{v77h} allele was recorded. All flies were raised at room temperature and eye color phenotypes were compared at different ages with those of z^{v77h} flies.

An inconsistency with the picture presented here is created by the *In(1)e(bx)* allele of *zeste*, an inversion with a breakpoint in the middle of the *zeste* coding region, which is expected to produce a protein lacking the C-terminal half and corresponding approximately to that produced by our N-Z construct (Bickel and Pirrotta, 1990). Flies carrying this mutation are defective in *zeste* function with respect to transvection or to complementation of the z^1 phenotype but, surprisingly, they have bright red eyes like the wild-type. To confirm this observation, we constructed a transposon expressing the *zeste* gene sequence cloned from *In(1)e(bx)* flies and provided with the 3′ untranslated sequence of the normal *zeste* gene to ensure proper RNA processing. This construct fails to restore red eye pigmentation to z^{v77h} flies and the protein it produces fails to bind to DNA *in vitro*, consistent with our other observations, but in disagreement with the behavior of the endogenous allele. In explanation, we can only suppose that, *in vivo*, a readthrough product may form a *zeste* hybrid protein with greater DNA-binding activity.

Discussion

Multimerization and DNA-binding cooperativity of zeste

Natural DNA-binding sites for many regulatory proteins frequently contain multiple consensus sequences recognized by the protein. Binding of enhancer factors to such multiple sites tends to enhance strongly the promoter-activating effect produced by a single site. Natural *zeste* binding sites, such as those found at the *Ubx* promoter, the *dpp* promoter, the *white* enhancer (Benson and Pirrotta, 1988) or the *twist* promoter (Pan *et al.*, 1991), also contain multiple consensus

sequences, spaced at uneven intervals that vary from 18 to >50 nucleotides. In the case of *zeste*, the multiplicity of recognition sequences at a site is essential for binding since reducing their number has drastic effects on the binding affinity. This suggests that strong binding results only when a *zeste* protein complex interacts simultaneously with more than one recognition sequence and is consistent with the fact that *zeste* forms stable multimers in solution. Our results show that multimerization of *zeste* protein is required for DNA binding. Deletion of the C-terminal multimerization domain or single point mutations within it have dramatic effects on the DNA-binding ability. Full-length *zeste* protein must form at least dimers in order to bind to DNA and the target must contain at least two consensus sequences. Multimerization, however, allows greater binding affinity to targets containing three or more binding sites and reduces the ability to bind efficiently to targets containing fewer sites. Deletion of the C-terminal half of the *zeste* protein or of the C-terminal domain or of the leucine zipper dimerization domain alone abolished the DNA-binding affinity toward any DNA probe, containing single or multiple sites, used in this experiment.

Such an essential contribution of the C-terminal domain to DNA-binding activity was not revealed by previous immunoprecipitation experiments (Mansukhani *et al.*, 1988; Bickel and Pirrotta, 1990). The reason is likely to be that, in those experiments, the antibody contributed a linkage between *zeste* proteins and substituted for the function of the C-terminal domain. In the experiments of Mansukhani *et al.* (1988), the binding domain was mapped using fusions of small *zeste* peptides to β-galactosidase and assaying DNA binding by immunoprecipitation. In this case, an additional contribution to the formation of oligomeric forms probably came from the β-galactosidase moiety which is itself able to form tetramers.

The inhibitory domain and binding specificity

Our results show that although loss of the capacity to form multimers causes loss of DNA-binding activity, larger deletions that leave little more than the DNA-binding domain reacquire ability to bind to DNA. The small DNA-binding domain peptides bind in fact even more strongly than the full-length protein and bind equally well to single as to multiple binding sites. This seemingly paradoxical effect has interesting implications. The *zeste* DNA-binding domain has strong affinity for DNA containing the consensus sequence T/C GAG T/C G, a very short nucleotide sequence that contains two degeneracies. Such a sequence is found in the genome roughly every 1000 nucleotides, much too frequently for a protein that binds to and affects the expression of specific genes. Binding sites for the *zeste* protein are in fact far less frequent *in vivo*, where <100 sites are detected on polytene chromosomes (Pirrotta *et al.*, 1988; Rastelli *et al.*, 1993), and even *in vitro* they are found at a frequency at least 50 times lower (Benson and Pirrotta, 1988). This low frequency and therefore high specificity results from the presence of an inhibitory domain that severely decreases the affinity of larger *zeste* peptides for DNA. Multimeric forms of *zeste*, on the other hand, bind strongly to DNA, but only if it contains multiple sites with which the multimer can interact simultaneously. The requirement for three or more consensus sequences raises the specificity of binding by several orders of magnitude while it multiplies the number of contacts formed by the multimer with the DNA target and hence the binding affinity. Residual affinity of *zeste* multimer for weaker binding sites (presumably containing one or two consensus sequences) is detectable *in vivo* when the *zeste* protein is overexpressed from the *hsp70* promoter (Pirrotta *et al.*, 1988). After heat shock activation, myriads of weaker binding sites can be seen on the polytene chromosomes. Such indiscriminate binding is apparently deleterious to the fly and overexpression of *zeste* causes extensive lethality when it occurs at the larva−pupa transition (Bickel and Pirrotta, 1990; Pirrotta, 1991). The general principle of handicapping the DNA-binding activity of the monomer in order to increase the binding specificity of the oligomer (either homo- or hetero-) might be widely applied in DNA-binding proteins.

DNA binding and eye color effect in vivo

The involvement of multimerization, and therefore of the C-terminal domain, in DNA binding further confirms the functional nature of the *zeste* interactions that occur *in vitro*. Whether or not *zeste* forms as large aggregates *in vivo* as it does *in vitro*, we have shown that the ability of *zeste* protein to support transvection effects and to complement the z^1 allele *in vivo* depends on its ability to form multimers (Bickel and Pirrotta, 1990; Chen *et al.*, 1992; Chen and Pirrotta, 1993). The present results show that the ability to multimerize is also needed for *zeste* function in stimulating *white* gene expression. Crickmore and Goldberg have observed that deleting of 30 amino acids from the C-terminus of *zeste* abrogates its ability to stimulate *Ubx* expression (cited in Wu and Goldberg, 1989). Our results suggest that this is due to the loss of DNA-binding activity.

Wild-type *zeste* protein has been shown to act as a promoter proximal transcription factor in an *in vitro* transcription assay (Biggin *et al.*, 1988), as well as in activating a reporter construct in the fly (Laney and Biggin, 1992). Pan *et al.* (1991) have shown that *zeste*-dependent transactivation is probably effected by antagonizing H1 histone-mediated repression instead of by actively enhancing transcription. This is a well-documented gene regulation mechanism (Croston *et al.*, 1991), but specific protein domains required for anti-repression have not yet been identified. The *zeste* sequence contains several potential transactivation domains, including an acidic region, a glutamine−alanine-rich domain and a proline-rich domain, but none of these sequences seem to function as transactivation domains in *zeste*. Deletion of the acidic region (ΔAD) or of the z^1 region and the first heptad repeat in the C-terminal domain (ΔEP), or a mutation disrupting the first hydrophobic ridge of the C-terminal domain (Z^1L518Δ), do not prevent the stimulation of *white*. Co-transfection experiments in cultured cells had similar results and showed that deletion of the *opa* repeats (ΔOPA) also has little effect on transactivation (Miller, 1991). These results suggest that there may not be a domain of *zeste* specifically required for transactivation unless it is the leucine zipper region itself, which is required for cooperative binding. We have not tested the internal deletion construct, ΔBB, which leaves only the DNA-binding domain and the C-terminal domain, to see if the protein region reponsible for *white* stimulation is located within the leucine zipper region. The results with the DNA-binding domain peptides suggest that DNA-binding ability can be separated from the activating effect of *zeste* since

DNA-binding domain peptides can bind but do not stimulate *white* expression *in vivo*. An explanation for this failure is that the DNA-binding peptide might be unstable *in vivo*, although we have detected no instability in this or other peptides in the reticulocyte lysate. The simplest explanation, however, is that although it binds to DNA very well, the decreased specificity of the DNA-binding domain peptide causes it to bind to too many sites in the genome and hence not enough protein might be available to bind to functional regulatory targets.

Materials and methods

Plasmid constructon

The *zeste* cDNA sequence was mutagenized to generate a *Nde*I site at the initiation codon and subcloned into the pET vector for *in vitro* transcription and translation (Chen *et al.*, 1992). Serial deletion constructs from the C-terminus were generated using appropriate restriction endonucleases. The ΔBst construct deletes the sequence from the *Bst*BI site (at position Phe-504) to the C-terminus and adds on four terminal amino acids, GSGC, from the vector. The N-Z construct deletes the sequence from the *Nae*I site (at position Ala-324) to the C-terminus and adds on the terminal peptide sequence GDPAANKARKEAELAAATAEQ from the vector. The Ava peptide deletes the sequence from the *Ava*I site (at position Glu-176) to the C-terminus and adds on the terminal sequence IRLLTKPERKLSWLLPPLSNN from the vector. The Ban peptide deletes sequence from the *Ban*I site (at position Val-156) to the *zeste* C-terminus and adds on RSGC from the vector. The C-Z construct deletes from the *Nae*I site (at position Gly-324) to the N-terminus and adds on the initial peptide MASMTGGNNMGRI at the N-terminus from the vector. The ΔBB construct has an internal deletion from the *Ban*I site (at position 157) to the *Bst*NI site (at position 485) and adds on a Leu residue at the joint. The ΔEP mutation has an internal in-frame deletion from amino acid 410 to 525. The ΔIE, ΔAD and ΔOPA mutants are described in Chen and Pirrotta (1993).

In vitro transcription and translation

In vitro transcription and translation were performed by using T7 RNA polymerase and Promega rabbit reticulocyte lysate as described previously (Chen *et al.*, 1992). *In vitro* translation was performed in the presence of [^{35}S]methionine (ICN) and the products were analyzed on SDS–polyacrylamide gels. The amounts of full-length peptide translated were quantitated using a Betascope blot analyzer, assuming a counting efficiency of 15% and adjusting for the number of methionines present in each peptide.

Electrophoretic mobility shift assay

DNA-binding reactions were performed in 10 mM Tris (pH 7.5), 20 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol and 5% glycerol in the presence of 1 μg poly(dI-dC) in a total volume of 20 μl as previously described (Chen *et al.*, 1992), using a 5% acrylamide or a 1% agarose gel as indicated. DNA probes were generated from *Ubx* promoter or from synthetic oligonucleotides encompassing one of the best available *zeste* footprinting sites with the following sequences for both strands: ACC-TGGGTTTTCCACTCGT-TTTTACC and AGGTGGTA AAAACG-AGTGGAAAACCC in the direction of 5′ to 3′. Mutated oligonucleotides for the same site were also made with two point mutations in the consensus sequences to give the following sequences: ACCTGGGTTTTCCAA-GCGTTTTTACC and AGGTGGT AAAAACGCTTGGAAAACCC in the direction of 5′ to 3′. Synthetic oligonucleotides were phosphorylated by T4 DNA kinase, annealed, and ligated with T4 DNA ligase overnight. The ligation mixture was labeled with [α-^{32}P]dATP using the Klenow fragment of DNA polymerase. The labeled mixture was separated on a 5% acrylamide gel and probes with one to five copies of *zeste* binding sites were excised and eluted in TE buffer. The position of the single binding site probe was confirmed by labeling the annealed phosphorylated oligonucleotide without ligation.

Transposon constructs and germ line transformation

Appropriate DNA fragments corresponding to the deletion mutants Ban, ΔEP, ΔBB, ΔAD, and Z^{1}L518Δ were subcloned into the pUChsneo vector (Steller and Pirrotta, 1985a). The resulting constructs, together with helper plasmid, were injected into *In(1)e(bx)* embryos. The G_0 flies were mated with partners of the same genotype and the transformed G_1 progeny were selected on food containing 0.5 mg/ml G418 (Geneticin, Sigma). The transformed flies, made homozygous for the transposon, were crossed to z^{v77h} females and the eye color phenotype assessed in males. All flies were raised at room temperature unless specifically indicated in the text.

References

Benson,M. and Pirrotta,V. (1987) *EMBO J.*, **6**, 1387–1392.
Benson,M. and Pirrotta,V. (1988) *EMBO J.*, **12**, 3907–3915.
Bickel,S. and Pirrotta,V. (1990) *EMBO J.*, **9**, 2959–2967.
Biggin,M.D., Bickel,S., Benson,M., Pirrotta,V. and Tjian,R. (1988) *Cell*, **53**, 713–722.
Bingham,P.M. and Zachar,Z. (1985) *Cell*, **40**, 819–825.
Chen,J.D. and Pirrotta,V. (1993) *EMBO J.*, **12**, 2063–2074.
Chen,J.D., Chan,C.S. and Pirrotta,V. (1992) *Mol. Cell. Biol.*, **12**, 598–608.
Croston,G.E., Kerrigan,L.A., Lira,L.M., Marshak,D.R. and Kadonaga,J.T. (1991) *Science*, **251**, 643–649.
Gans,M. (1953) *Bull. Biol. Fr. Belg. Suppl.*, **38**, 1–90.
Gelbart,W.M. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 2636–2640.
Geyer,P.K., Green,M.M. and Corces,V.G. (1990) *EMBO J.*, **9**, 2247–2256.
Jack,J.W. and Judd,B.H. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 1368–1372.
Kaufman,T.C., Tasaka,S.E. and Suzuki,D.T. (1973) *Genetics*, **75**, 299–321.
Laney,J.D. and Biggin,M.D. (1992) *Genes Dev.*, **6**, 1531–1541.
Levene,S.D. and Zimm,B.H. (1989) *Science*, **245**, 396–399.
Lewis,E.B. (1954) *Am. Nat.*, **88**, 225–239.
Lifschytz,E. and Green,M.M. (1984) *EMBO J.*, **3**, 999–1002.
Mansukhani,A., Crickmore,A., Sherwood,P.W. and Goldberg,M.L. (1988) *Mol. Cell. Biol.*, **8**, 615–623.
Miller,P. (1991) PhD Dissertation, Baylor College of Medicine, Houston, TX.
Pan, D., Huang,J.D. and Courey,A.J. (1991) *Genes Dev.*, **5**, 1892–1901.
Pirrotta,V. (1991) *Adv. Genet.*, **29**, 301–348.
Pirrotta,V., Bickel,S. and Mariani,C. (1988) *Genes Dev.*, **2**, 1839–1850.
Qian,S., Varjavand,B. and Pirrotta,V. (1992) *Genetics*, **131**, 79–90.
Rastelli,L., Chan,C.S. and Pirrotta,V. (1993) *EMBO J.*, **12**, 1513–1522.
Steller,H. and Pirrotta,V. (1985a) *EMBO J.*, **4**, 167–171.
Steller,H. and Pirrotta,V. (1985b) *EMBO J.*, **4**, 3765–3772.
Wu,C.-t. and Goldberg,M.L. (1989) *Trends Genet.*, **5**, 189–194.

Received on December 16, 1992; revised on February 8, 1993