

Developmental expression of the *Drosophila zeste* gene and localization of *zeste* protein on polytene chromosomes

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The expression of the *zeste* gene varies through the life cycle of the fly. Its transcription is most abundant in maternal RNA, declines to very low levels during larval growth, but rises again in late third instar larvae and pupae. Using transposons containing a *zeste-lacZ* gene, we found a corresponding variation in the tissue distribution of *zeste* from stage to stage. Nearly ubiquitous expression of the *zeste-lacZ* gene is found in late embryos and first instar larvae, but disappears almost completely except in brain and gonads by third instar larva. Shortly before pupation expression rises again in imaginal discs, Malpighian tubules, and salivary glands and again becomes nearly ubiquitous in pupae. *zeste* continues to be expressed in adult brain and gonads. We constructed flies carrying a *zeste* gene controlled by the heat shock promoter and studied the distribution of *zeste* protein in their polytene chromosomes as well as those of wild-type flies. Using affinity-purified anti-*zeste* antibodies, we find that wild-type salivary gland chromosomes contain about 60 strong bands of *zeste* immunofluorescence at specific cytological locations. After heat induction of larvae containing the *hs-zeste* gene, many hundreds of bands appear. These results suggest the involvement of *zeste* in the expression of a wide variety of genes at different developmental stages.

[Key Words: Transvection, *zeste*; developmental regulation; immunofluorescence; polytene chromosomes]

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The *zeste* gene of *Drosophila* was identified genetically on the basis of two apparently distinct effects that it exercises on certain other genes. One of these is the ability to mediate transvection, the effect of regulatory elements of one copy of a target gene on the expression of a second copy of the gene on a homologously paired chromosome. Such transvection effects were first observed at the *Ultrabithorax* (*Ubx*) gene by Lewis (1954), who showed that they were dependent on the activity of a gene later identified as *zeste* (Kaufman et al. 1972). *zeste*-dependent transvection effects since have been observed at two other loci, *white* and *decapentaplegic* (*dpp*) (Babu and Bhat 1980; Gelbart and Wu 1982). The second, perhaps more dramatic, effect of *zeste* is that produced by a particular *zeste* mutant, *z*¹, on the expression of the *white* gene. Gans (1953) found that in this mutant, the *white* gene is strongly repressed if it is present in two copies brought in close physical proximity by somatic chromosome pairing or by tandem duplication (Judd 1961; Green 1963). Bingham and Zachar (1985) showed that this effect was due to a strong suppression of transcription of the *white* gene in the eye, where it is re-

quired for pigment deposition. The effect is eye specific and it is not observed in other tissues that have *white*-dependent pigmentation. These observations strongly suggest an interaction of *zeste* with control elements that govern the transcription of its target genes, but the genetic evidence has so far not revealed any requirement for *zeste* in normal gene expression. Mutations in the *zeste* gene that prevent transvection have no detectable effect on the functioning of wild-type *Ubx* or *dpp* genes, although some of them may alter the expression of *white*.

The *zeste* gene has been cloned (Mariani et al. 1985; Gunaratne et al. 1986) and the sequences of the wild type and some of its mutants have been determined (Pirrotta et al. 1987; Mansukhani et al. 1988). Using *zeste* protein expressed in bacteria, Benson and Pirrotta (1987, 1988) have shown that the *zeste* product is a DNA-binding protein that recognizes specific sites in the *white*, *Ubx*, and other genes. One of these sites at *Ubx* is in the immediate vicinity of the transcription start site (nucleotides -40 to -150). *zeste* protein bound to this site acts in vitro as a transcription factor stimulating the synthesis of *Ubx* RNA (Biggin et al. 1988). Evidence of this sort raises the question of whether *zeste* might have a more important role in gene expression, a role not substantially affected by any of the known *zeste* mutations.

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If these mutations only interfere with a higher-order *zeste* effect such as transvection, they would uncover only a small part of the involvement of *zeste* in gene expression. To assess the extent of this involvement, it is important therefore to determine when and where the *zeste* gene is expressed. Furthermore, the three genes known to give transvection effects appear to have very little in common. *zeste* may well interact also with other genes a knowledge of which may provide a better basis for understanding the role of *zeste*.

In this paper, we explore the activity of the *zeste* gene during development of the fly, its distribution in different tissues, and the localization of the *zeste* product on polytene chromosomes.

Results

Developmental profile of zeste transcription

The three genes that have given us genetic evidence of *zeste* function are all expressed at various stages from embryo to adult but, in particular, they are active in the late larva and early pupa. The phenotypes affected by *zeste*-dependent transvection effects are all visible in the adult as defects in imaginal disc derivatives. We do not know whether *zeste* is active at any other stage since no transvection effects have been reported on the embryonic phenotypes of *dpp* or *Ubx* mutants. Gunaratne et al. (1986) have in fact found *zeste* mRNA expressed at earlier developmental stages. To examine the changes in activity of the *zeste* gene during development, we made poly(A)⁺ RNA preparations from all developmental stages, taken at 1-day intervals, and hybridized them to a *zeste* probe (Fig. 1). Surprisingly, the highest accumulation of *zeste* RNA was found in unfertilized eggs, corresponding therefore, to maternal RNA. *zeste* transcript

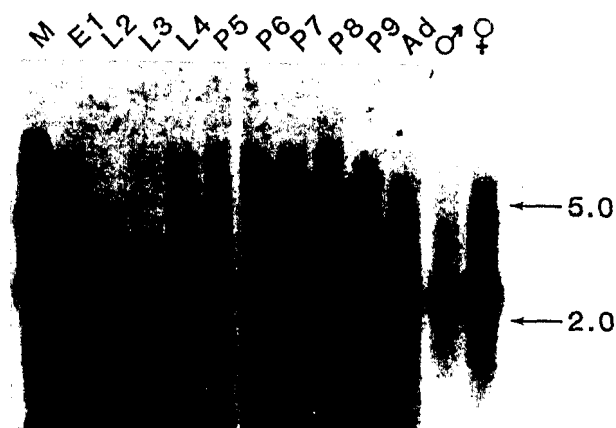


Figure 1. Northern blot hybridization to *zeste* RNA. Each lane contained 5 μ g of poly(A)⁺ RNA from different developmental stages except lane P8, which contained 2 μ g. The blot was hybridized with a nick-translated probe containing the entire *zeste* gene. (Lane M) Maternal RNA; (lanes E1–P9) developmental stages at 24-hr intervals at 25°C; (lane Ad) adult RNA; (lanes ♂ and ♀) male and female adult RNAs. The *zeste* mRNA is the major band of 2.5 kb. Arrows indicate the position of the 2- and 5-kb *copia* RNAs, determined on the same filter.

decreases in abundance during embryonic development and reaches a minimum, but still detectable, level in first and second instar larvae. It climbs again in third instar larvae and reaches a second peak in the pupa. Moderate levels are still found in both male and female adults.

zeste-lacZ fusions

To examine in more detail the tissue and developmental distribution of *zeste* activity, we resorted to the use of the bacterial *lacZ* gene as a reporter. We had previously shown that a functional *zeste* gene was contained in a 3.9-kb *Bam*HI fragment (Pirrotta et al. 1987). Therefore, we used a DNA fragment containing the *zeste* 5'-flanking region starting from the *Bam*HI site and including the promoter and the nucleotides encoding the first 49 amino acid residues of the *zeste* polypeptide. This was fused in-frame to a *lacZ* fragment encoding an active β -galactosidase polypeptide. The construction was assembled in the pUCHsneo vector for P-mediated germ line transformation (Steller and Pirrotta 1985a) and injected into embryos. Transformed lines, selected for their G418 resistance, were made homozygous and then examined for expression of the *zeste*- β -galactosidase gene at different developmental stages.

Despite the presence of abundant *zeste* RNA in unfertilized eggs, early embryos gave no detectable staining for β -galactosidase activity. Staining was first detected during germ band extension; it increased to a maximum around stage 16 of Campos-Ortega and Hartenstein (1985) in all tissues including the epidermis. Newly hatched larvae were still fairly uniformly stained, but the activity and its distribution continued to decrease during larval development (Fig. 2). In early third instar larvae, staining persisted only in the gonads, central nervous system, sections of the gut, and the tissues surrounding the head skeleton. As the larvae reached the climbing stage, a wider pattern of staining reappeared. Malpighian tubules, salivary glands, ring gland, and imaginal discs became progressively and sequentially stainable. By the beginning of pupation all these tissues were expressing the hybrid gene fully. During metamorphosis, this analysis was complicated by the higher levels of endogenous β -galactosidase that accumulate in the wild-type pupa. These could be reduced but not completely eliminated by performing the staining reaction at pH 8. It was clear, however, that expression of the hybrid gene continued in all imaginal disc derivatives, as well as in the abdomen of the developing imago. The intensity and distribution of staining receded in the pharate adult but was still detectable in parts of the appendages, particularly the antennae and in the thoracic musculature. Continued expression was found in the adult brain and gonads. A very similar pattern of expression was found in three independent lines transformed with the same transposon.

Of particular interest was the appearance of staining in the salivary glands of late third instar larvae. The earliest signs of activity occurred in the distal, posterior end

of the glands as accumulations of blue stain in the polytenic nuclei and especially at or near the nuclear membrane, suggesting that the *zeste*- β -galactosidase hybrid protein might partition preferentially in the nucleus. Later the entire gland acquired a blue color.

Heat shock-zeste transposons

The observation that the *zeste*- β -galactosidase gene was activated in the salivary glands of the third instar larva raised the possibility of detecting *zeste* protein on the

salivary gland polytenic chromosomes. Because its normal levels of expression are rather low, we decided first to overexpress the *zeste* gene by transcribing its coding sequence from the *hsp70* promoter. To do this, we used a nearly full-length *zeste* cDNA, trimmed its untranslated leader sequence by cutting it at the *KpnI* site, removed about 25 bp with *Bal31* exonuclease, and ligated it, together with a 456-bp fragment containing the *hsp70* promoter, into the pUCHsneo vector (Steller and Pirrotta 1985a). The resulting transposon was injected into *z^a* embryos, which produce a mutant *zeste*

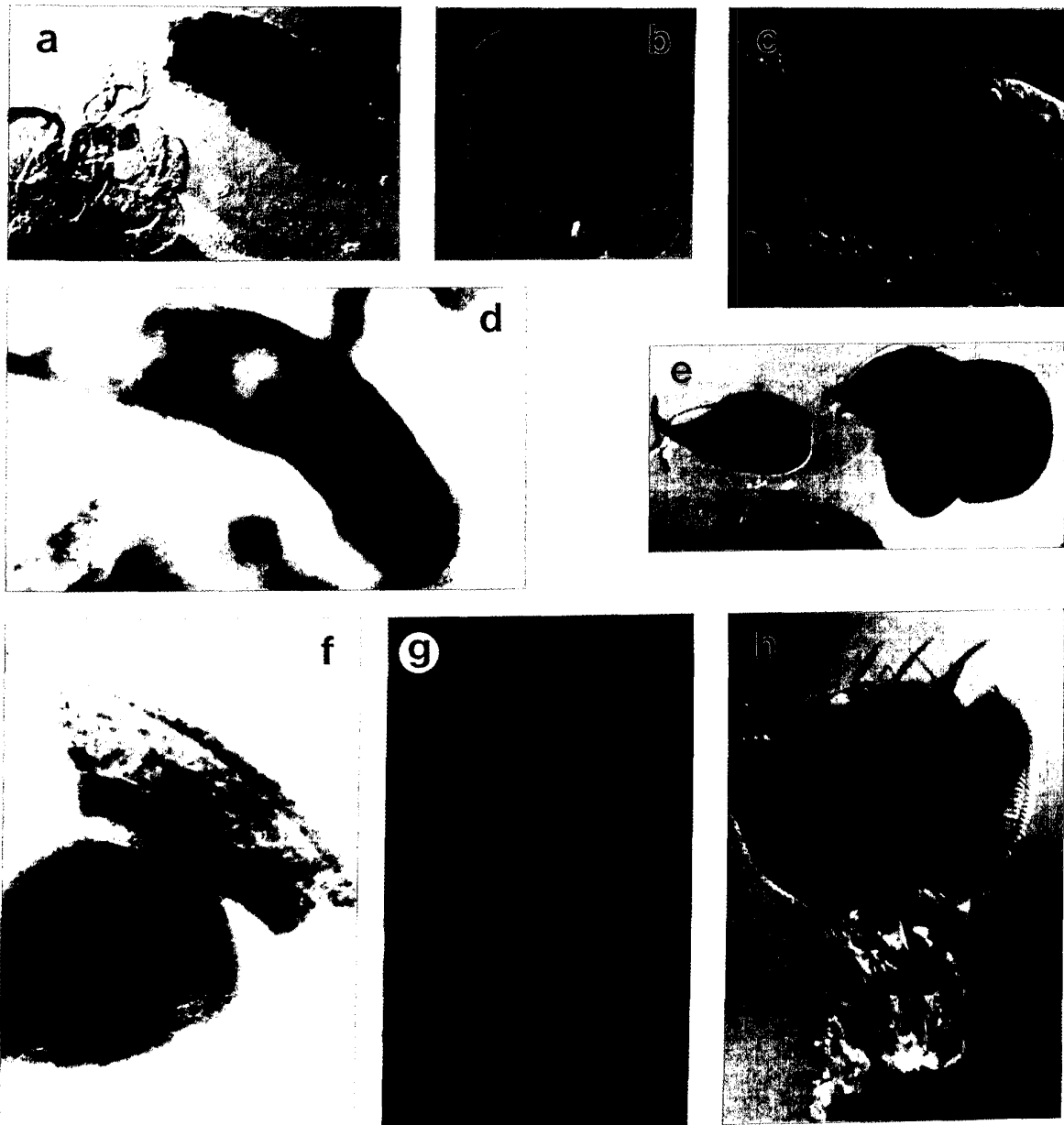


Figure 2. Expression of a *zeste*- β -galactosidase hybrid gene. Animals homozygous for a transposon containing the *zeste* gene fused in frame to the *lacZ* gene were dissected and stained for β -galactosidase activity. First instar larval cuticle (a) and central nervous system (b); late third instar larval testis (c), salivary gland (d), and imaginal discs (e), early pupa optic lobe and eye (f), adult ovaries (g), and head (h). The enlargement varies from case to case.

product unable to complement the z^1 mutation. Transformed flies were detected by crossing the G_0 flies to z^1 mutant partners and selecting individuals able to survive G418 food. All surviving females were also red-eyed when raised at room temperature, indicating that a functional *zeste* gene was present and that the basal level of the heat shock promoter in the *hs-zeste* gene sufficed to complement the z^1 mutant product. Two independent lines, *hs-z 2* and *hs-z 3*, made homozygous for the *hs-zeste* transposon, were selected for further study.

To verify that the transformed flies actually produce higher levels of *zeste* protein after heat shock induction, we compared the amounts of protein detected on Western blots with or without heat treatment. We used an affinity-purified antiserum directed against the carboxy-terminal region of the *zeste* protein. As Figure 3 shows, fly extracts made after heat shock contain at least 10 times more immunoreactive *zeste* protein than extracts from wild-type flies with or without heat shock. In the absence of heat shock, the uninduced level of *zeste* protein in flies carrying the *hs-zeste* transposon is still approximately two- or threefold higher than that found in wild-type flies.

We tested the ability of the *hs-zeste* gene to promote transvection at *Ubx* using the *Cbx Ubx/+ +* configuration in a z^a background (Gelbart and Wu 1982). In *Cbx Ubx/+ +* flies, *zeste*-dependent transvection causes the *Cbx* regulatory mutation on one chromosome to direct inappropriate expression of the wild-type gene copy on the other chromosome, resulting in a partial transformation of posterior wing to haltere. When such flies carrying one copy of the *hs-zeste* transposon in a z^a background were raised at room temperature transvection effects were minimal. Stronger transvection phenotypes, but still substantially weaker than those observed in the presence of a normal *zeste* gene, were obtained by heat-shocking the flies for 1 hr daily during larval growth.

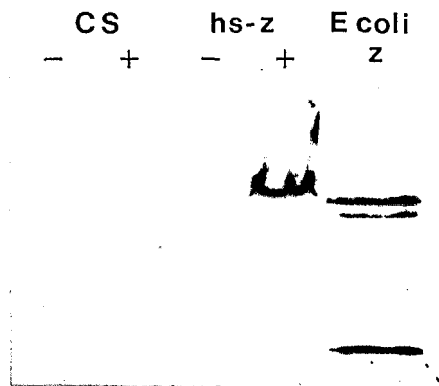


Figure 3. Western blot analysis of the *hs-zeste* gene expression. Nuclear extract equivalents of 50 flies were applied to each lane. *zeste* protein was detected after blotting with affinity-purified anti-*zeste* antibody. The two lanes on the left represent control Canton S flies without or with heat shock. The middle two lanes represent flies homozygous for the *hs-zeste* transposon, without and with heat shock. The lane on the right contains *zeste* protein expressed in *E. coli*.

Such short heat treatment regimes did not harm these flies appreciably and gave rise to no obvious abnormalities.

We tested the *hs-z 2* line homozygous for the *hs-zeste* transposon on the second chromosome for the effects of heat shock in two ways. In the first experiment we collected staged embryos, mounted them on a slide, timed their arrival to the cellular blastoderm stage, and then heat-shocked them at 37°C for 15 min. As a result, these embryos received a heat treatment at various stages between blastoderm and germ band extension. Their development was then monitored until hatching. We observed no lethality attributable to the heat shock nor any detectable segmental abnormality in the resulting first instar larvae. In the second experiment we prepared a series of vials by transferring the parental flies approximately every 12 hr. These vials, containing larvae at developmental stages ranging from first instar to pupae, were then heat-shocked at 37°C for 2.5 hr. After allowing development to continue, the vials were examined for pupal mortality or for the emergence of adults with abnormal phenotypes. By these criteria, heat treatment during larval development had no detectable effect until late third instar. Animals heat-shocked from this time on became increasingly sensitive, frequently dying as pupae or eclosing with generalized abnormalities usually affecting the wings or legs. The highest sensitivity was observed in animals that had just completed pupariation at the time of heat shock, in which case 16 out of 17 failed to eclose. Entirely analogous results were obtained with an independent line, *hs-z 3*, which carries the transposon on the third chromosome. Control wild-type flies in parallel experiments showed minimal effects. In the *hs-z* lines, the chromosome bearing the transposon is derived from the z^a stock originally injected. Heat treatment of the z^a flies at the same stage resulted in a mortality rate of only 5% (7 of 144).

In situ detection of *zeste* protein

To visualize *zeste* protein in salivary gland cells we first used animals carrying the *hs-zeste* gene under the assumption that this would maximize the chances of detection. Third instar larvae were placed at 37°C for 1 hr and allowed to recover for another hour at room temperature before dissecting and fixing the salivary glands. Figure 4 shows cells and chromosome spreads stained by indirect immunofluorescence using an affinity-purified antibody directed against the carboxy-terminal quarter of the *zeste* polypeptide. Under these conditions, the cytoplasm is not appreciably stained but the nuclei show intense fluorescence associated with the chromosomes. In chromosome spreads, the fluorescence is distributed clearly in a very large number of discrete bands found in all the chromosome arms. Comparison of the fluorescence and phase-contrast photographs makes it clear that the fluorescence bands do not correspond to the normal chromomeric banding pattern. We note the following: (1) Bands of fluorescence are found preferentially in interband regions or at least in regions devoid of

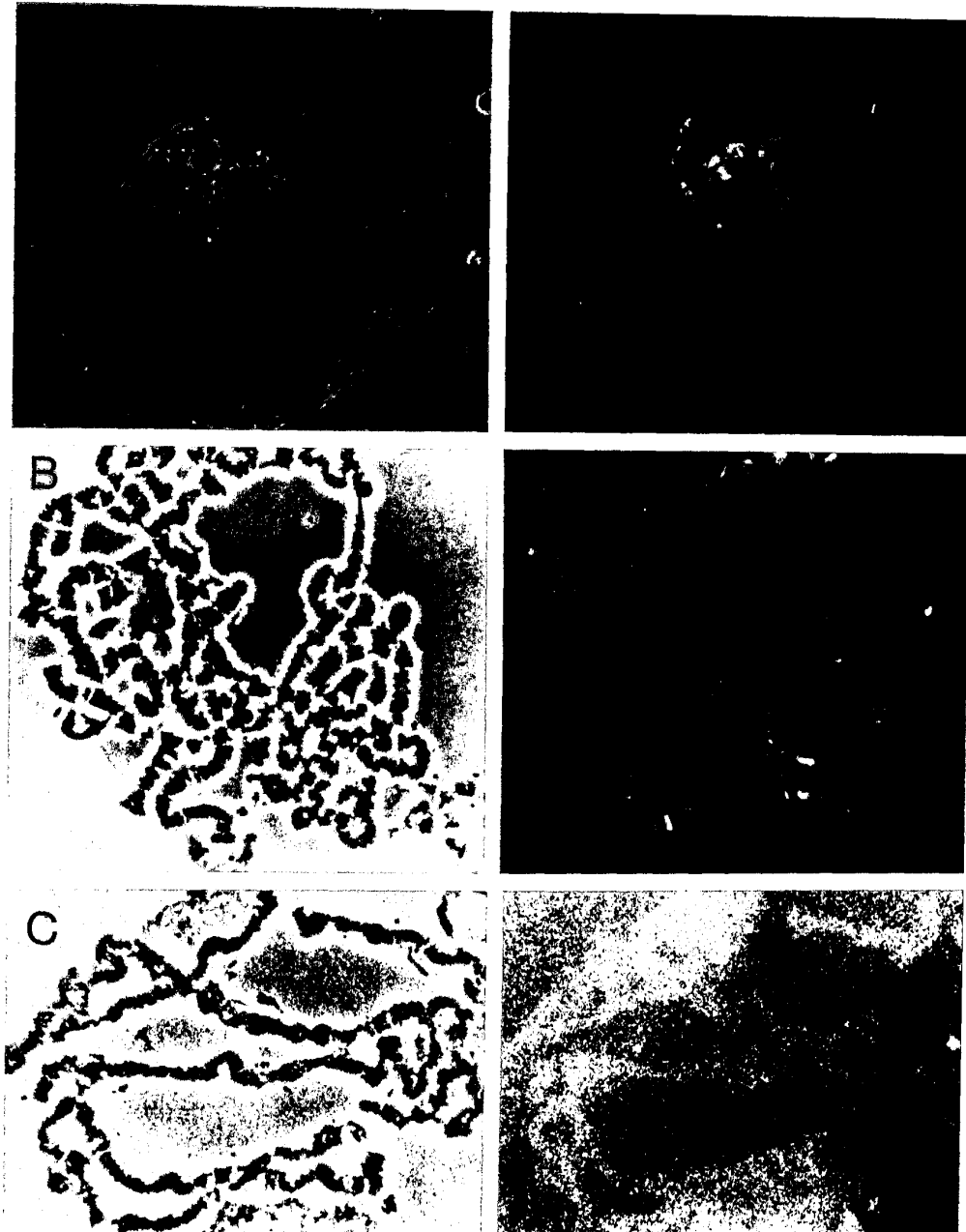


Figure 4. *zeste* immunofluorescence. In each case the panel on the left shows the phase-contrast picture, and the panel on the right, the immunofluorescence. (A) Salivary gland from larvae homozygous for the *hs-zeste* transposon, after heat shock. (B) Chromosome squash from cells such as in A. (C) Chromosome squash from salivary glands of *In(1) e(bx)* larvae; the panel on the right is overexposed greatly here to show the absence of fluorescence.

strong bands under phase-contrast optics. (2) Not all interband regions fluoresce. (3) The chromocenter does not stain or only very faintly. (4) No staining is found in the nucleolus except possibly very faintly at its edges. (5) Non-heat-shocked larvae lack this massive immunofluorescence as do wild-type larvae that have been heat-shocked.

Reassured of our capacity to detect *zeste* on the polytene chromosomes, we then investigated the pattern of

fluorescence attributable to the endogenous *zeste* gene in wild-type salivary gland chromosomes. Figure 5 shows representative chromosome spreads and individual chromosomes. Bands of immunofluorescence are still detectable but in much lower numbers, distributed in all chromosome arms in a highly characteristic and reproducible pattern that is not affected appreciably by heat shock treatment. Approximately 60 strong or medium strong immunofluorescing sites can be counted, as

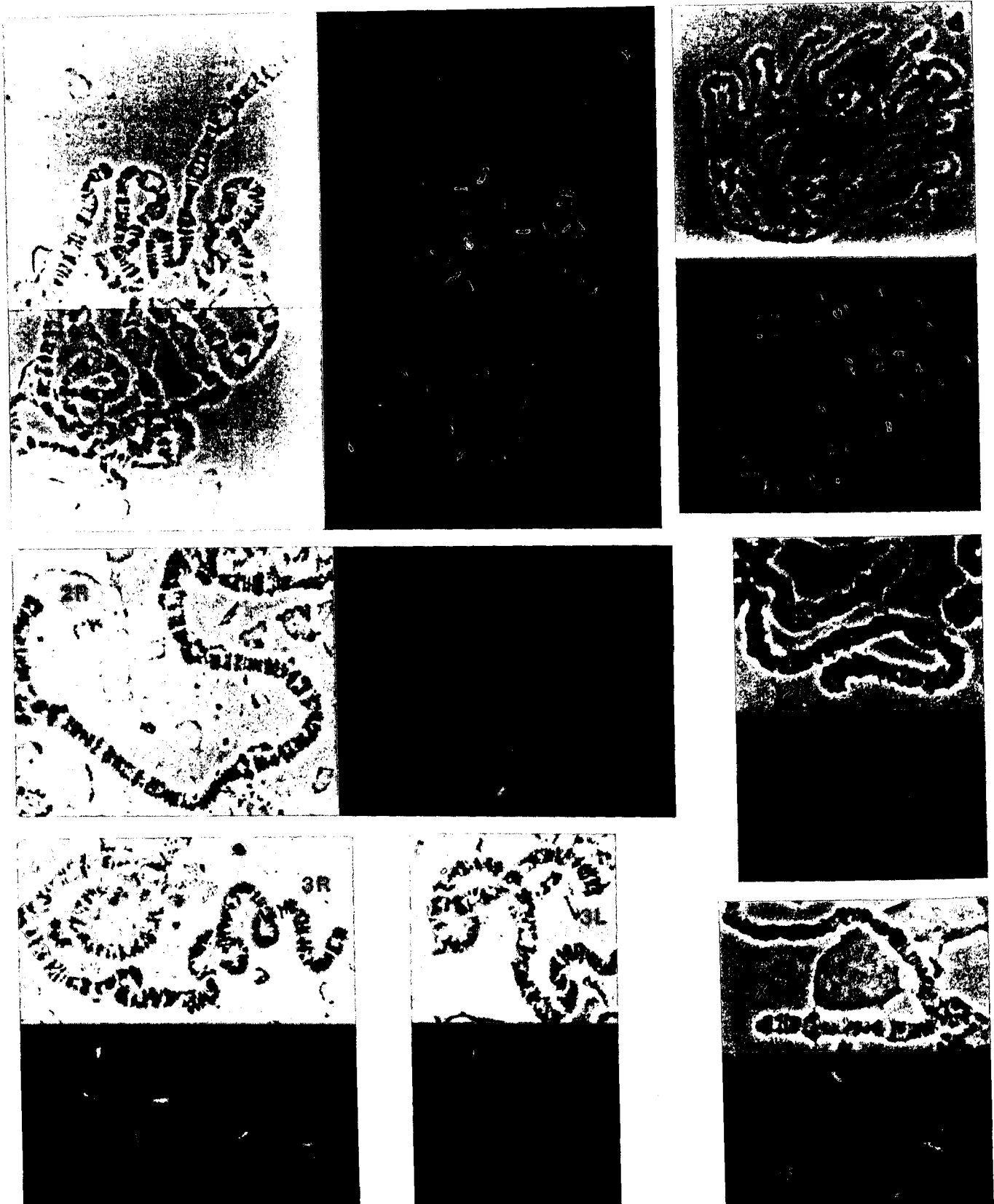


Figure 5. *zeste* immunofluorescence. Salivary gland chromosome squashes from wild-type larvae. The distal parts of individual chromosomes are shown.

before, associated not with phase-contrast bands but rather with interbands or regions containing only faint bands. Puffs are conspicuously devoid of immunofluorescence but several chromosome regions give a very faint, punctate background instead of bands. Occasionally, bands of immunofluorescence are associated with chromosome sites involved in ectopic bridges but this is not a regular association because many more ectopic threads do not fluoresce.

In comparison with the wild type, the chromosomes of non-heat-shocked larvae carrying the *hs-zeste* gene in addition to a normal, endogenous copy of *zeste* give the impression of having a somewhat higher number of immunofluorescent bands (Fig. 6). A detailed comparison between the two shows that the actual pattern of bands is approximately the same but the overall intensity is higher in the *hs-zeste* case, allowing normally weakly fluorescing sites to appear relatively strong. However, some new bands not visible in wild-type flies, clearly do appear in these chromosomes; for example, note a third band near the tip of the X chromosome at 1F-2A in addition to the two normally present at 1C and 1D (Fig. 7).

The effect of heat shock on the immunofluorescence observed with the *hs-zeste* larvae makes it clear that our antibody is truly detecting *zeste* protein. As an additional control, to ensure that it is not detecting anything other than *zeste* protein, we used chromosome spreads

prepared from the *In(1) e(bx)* strain. In these flies, the *zeste* gene is split by an inversion breakpoint approximately in the middle of the coding region (Pirrota et al. 1987). The resulting gene would make a polypeptide lacking the carboxy-terminal half of the *zeste* protein and therefore unable to react with the antibody. Figure 4C shows that these chromosomes, treated with the carboxy-terminal antibody exactly in parallel with wild-type chromosomes, fail to exhibit any appreciable immunofluorescence. An examination of the chromosomes from *z¹* mutant larvae showed that they immunofluoresce, as far as could be determined, in the same pattern and to a similar extent as the wild type.

Given the reproducibility of the banding pattern detected by the antibody, we next attempted to attribute some of the bands to loci known to interact with *zeste*, that is, *white*, *dpp*, and *Ubx*. In brief, a faint band of fluorescence was detectable at approximately 89E, the cytological location of the bithorax complex, and a distinct band was present at 22F, the approximate site of *dpp*. That this fluorescence corresponds to the *Ubx* and *dpp* loci cannot, of course, be established from these experiments. In contrast, no appreciable fluorescence could be detected at 3C1,2 where the *white* gene is known to reside (Fig. 8). Although this site is flanked by strong bands of fluorescence, 3C1,2 itself remains clearly unlabeled. Interestingly, the neighboring bands

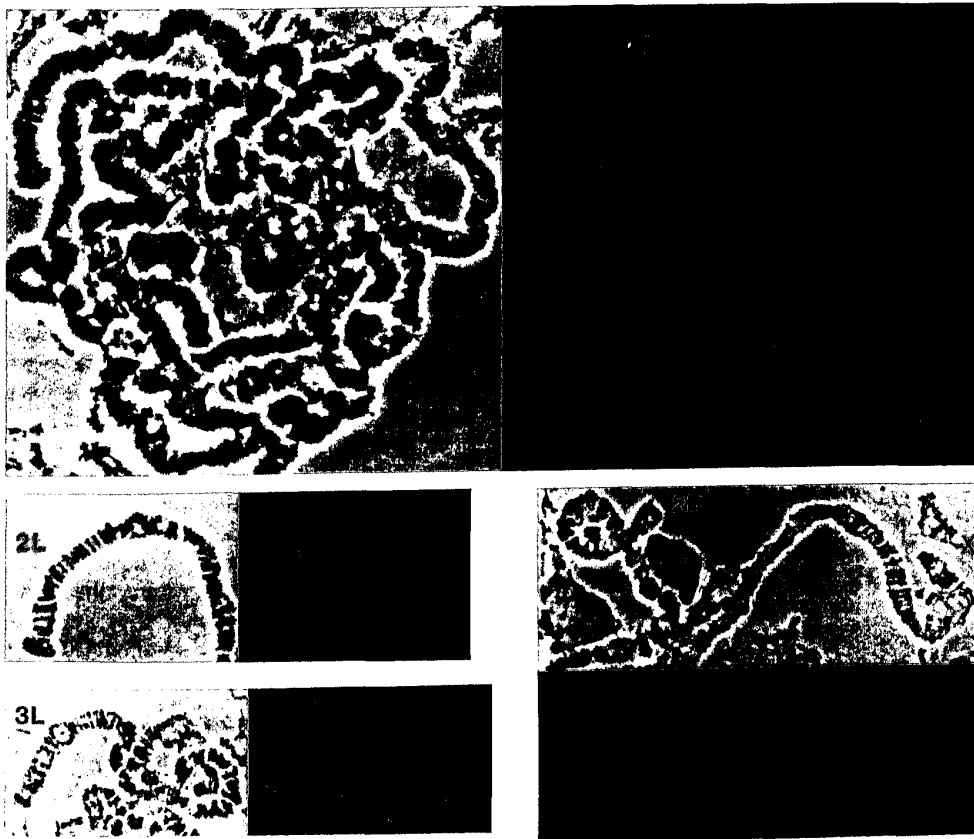


Figure 6. *zeste* immunofluorescence. Salivary gland chromosome squashes from larvae homozygous for the *hs-zeste* transposon with no heat shock. Note the appearance of bands that were weak or undetectable in corresponding chromosomal sites in Fig. 5.

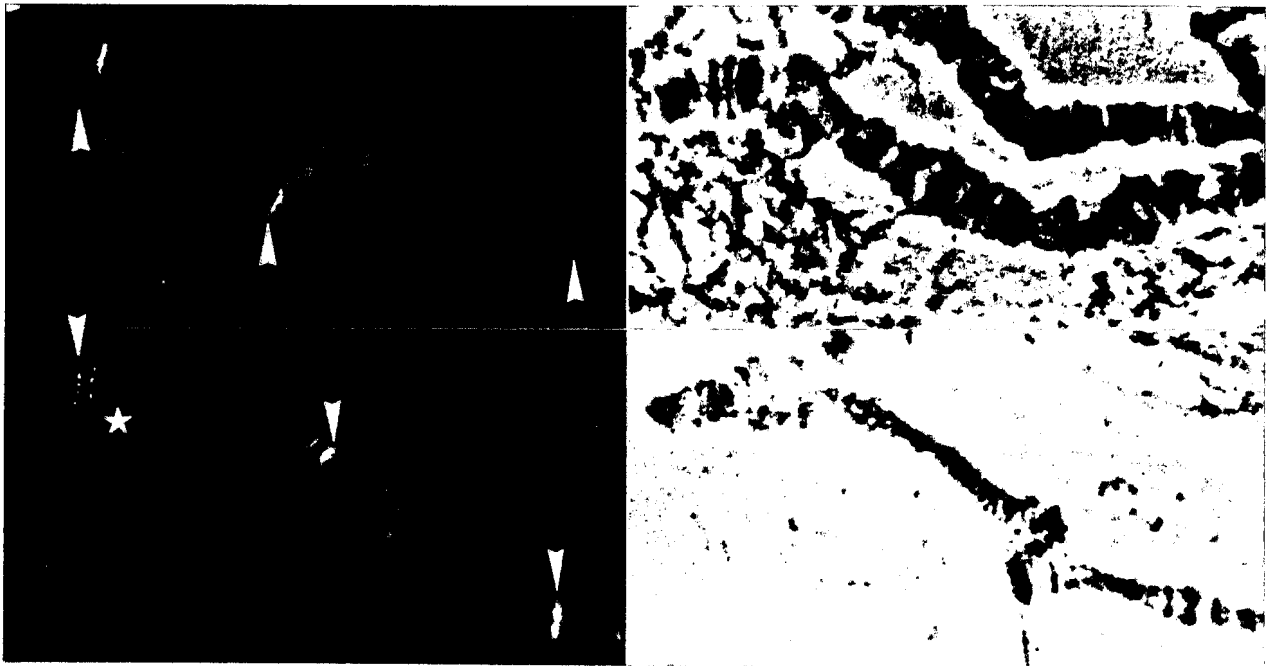


Figure 7. Reproducibility of *zeste* banding pattern. The banding pattern is highly reproducible in chromosomes from larvae at the same stage (white prepupae). This is illustrated by comparing the wild-type distal X chromosome shown here (upper panel) with the X chromosomes in Figs. 5 and 6 and with the X chromosome from non-heat-shocked larvae carrying the *hs-zeste* transposon (lower panel). In these larvae, containing two- to threefold more *zeste*, immunofluorescence is stronger and one new band appears at 1F-2E (marked by a star), which is not detected in the wild type. The arrowheads serve to align corresponding bands.

of fluorescence correspond as nearly as can be determined to 3D3,4 (strong band), to 3C11,12 (weaker band), the site of the *sgs-4* gene, and to 3A3, the site of the *zeste* gene itself.

Finally, we examined the salivary gland chromosomes of another *Drosophila* species, *D. virilis*, believed to have diverged from the branch that evolved into *D. melanogaster* about 45 million years ago (Beverley and Wilson 1984). Figure 9 shows that our antibody detects *zeste*-like bands in *D. virilis* salivary gland chromosomes but fewer in number and, with two or three exceptions, much lower in intensity than those observed in *D. melanogaster*.

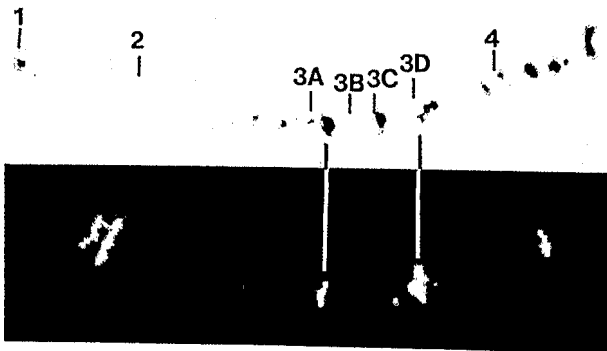


Figure 8. *zeste* immunofluorescence near the tip of the X chromosome. Although fluorescent bands are present at 3A2-3, 3C11-12, and 3D3-4, no band is visible at 3C1-2, the site of the *white* gene.

Discussion

In contrast to the known genetic effects involving *zeste*, which are so far limited to three loci and to phenotypes affecting imaginal derivatives, the results reported here indicate that *zeste* mRNA is present throughout development but principally in the embryo. After virtually disappearing in early larval stages, a second, strong peak of transcription occurs in the late third instar larvae and early pupa. This is confirmed by the experiments using *zeste-lacZ* hybrid gene. Surprisingly, despite the abundance of *zeste* mRNA in unfertilized embryos and the expression of the *zeste-lacZ* gene in ovaries, the early embryo does not stain for β -galactosidase activity until the extended germ band stage, suggesting some mechanism of translation control. Staining activity, which declines in the early larval stages, revives in intensity and distribution shortly before pupation and becomes nearly ubiquitous in the pupa. In contrast to the genetic data, these results indicate a much more widespread activity of the *zeste* gene in a large variety of tissues and developmental stages. In particular, they suggest the involvement of *zeste* in the expression of embryonic genes.

Effects of *zeste* overexpression

The *hs-zeste* construction was made with two basic purposes in mind. One was to overexpress the gene to produce higher amounts of *zeste* protein for biochemical and cytological studies and the other was to determine whether abnormally high levels of *zeste* in inappropriate tissues or developmental stages had deleterious effects

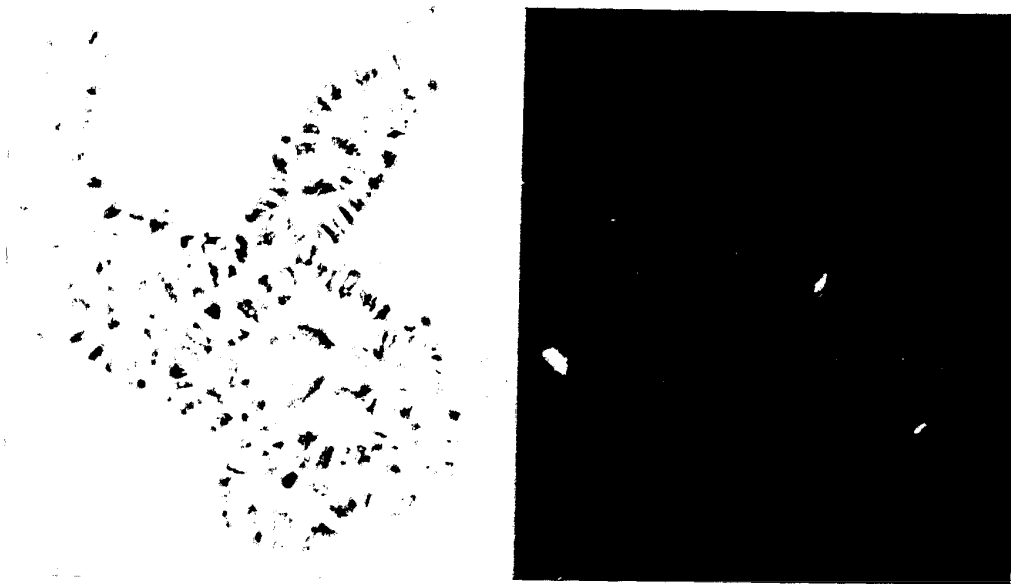


Figure 9. *zeste* immunofluorescence in salivary gland chromosome squashes from *Drosophila virilis*. Note that although the photograph was overexposed, the fluorescence is much weaker than in the *D. melanogaster* chromosomes.

on gene expression. With respect to the second question, activation of the *hs-zeste* gene at early embryonic stages caused no detectable lethality or segmental abnormalities. On the other hand, heat shocks administered to late third instar larvae or early pupae resulted in abnormalities and widespread pupal lethality in two different lines carrying the transposon on different chromosomes. It is unlikely that this heat sensitivity is caused by some preexisting mutation in the parent z^a strain, since the z^a flies have a much lower rate of pupal mortality. Furthermore, repeated backcrosses for both transformed lines have replaced all the chromosomes except for the one carrying the transposon. Therefore, it is likely that *zeste* overexpression in the late larva-early pupa has deleterious effects, possibly causing overexpression of some target genes. This would not be surprising in view of the effect of *zeste* on transcriptional activation in vitro (Biggin et al. 1988) and in tissue culture cells (P. Miller and V. Pirrotta, unpubl.). What is puzzling is that *zeste* overexpression did not appear to affect earlier stages. A possible explanation is that, at these stages, *zeste* is not the limiting factor governing the expression of critical genes like *Ubx* and *dpp*. Their transcription might depend on other factors present in limiting amounts or it might be suppressed by negative regulators.

The basal level of expression of the heat shock promoter at room temperature is adequate to provide enough *zeste* product to complement two endogenous copies of *zeste* gene carrying the z^1 mutation. This leakiness of the *hsp70* promoter, previously observed in the case of *hs-white* (Steller and Pirrotta 1985b) and of *hs-P* transposase gene (Steller and Pirrotta 1986) confirms the conclusion reached from the levels of *zeste* mRNA that the normal amounts of *zeste* protein present in cells (or at least in the eye disc) are rather low. The basal level of

the *hs-zeste* gene is not adequate to promote transvection effects in a z^a background. When the level of expression was boosted by the administration of short heat shocks during larval development, transvection effects became visible though less pronounced than those caused by an endogenous *zeste* gene or by a transposon carrying a wild-type *zeste* gene. A possible explanation for this result might be that higher or sustained local levels of *zeste* are required for transvection effects. The *zeste* protein produced after a pulse of activation might have a short half-life and thus be insufficient over a more extended time period, while later heat shocks cause pupal lethality.

zeste on polytene chromosomes

Larvae carrying the *hs-zeste* gene show clearly a massive increase in *zeste* product after heat shock, as detected by Western blots or by immunofluorescence. As far as can be detected in salivary gland cells, the immunofluorescence is associated exclusively with the nucleus and specifically with the polytenic chromosomes. Even after heat shock-induced overproduction, *zeste* is found only on the chromosomes, in a pattern of discrete bands not corresponding to bands of higher DNA concentration, indicating that it is associated with specific regions. In wild-type chromosomes, *zeste* is detected at about 60 sites. This is consistent with our previous observation that *zeste* protein binds in vitro to specific DNA sequences from the *white* and *Ubx* genes (Benson and Pirrotta 1987) and argues against a role of *zeste* as a generalized glue holding together homologous chromosomes. The vastly greater number of bands observed when *zeste* is overproduced suggests that at higher concentrations *zeste* can occupy lower affinity sites or, possibly, that under these conditions, it is able to displace

other proteins blocking access to many more potential binding sites.

Needless to say, we have no evidence that the presence of *zeste* protein at many or all of the chromosomal sites is functional or necessary. It could be fortuitous. Nevertheless, the number of sites in salivary gland chromosomes and the wide distribution of *zeste* activity indicated by the *zeste-lacZ* fusion gene, suggest that *zeste* can interact potentially with a wide range of genes. At least some of the genes correspond to complex, developmentally important loci such as *Ubx* and *dpp*, as intimated by the genetic results, as well as *engrailed* or *Antennapedia*, as shown by in vitro binding studies (Benson and Pirrotta 1988). The immunofluorescence observations indicate that *zeste* is not found in transcriptionally inactive regions of the chromosomes, such as the chromocenter or highly condensed bands, but rather in interband regions or at least in regions that do not contain strong Bridges bands. Experiments using incorporation of [³H]uridine or immunolocalization of RNA polymerase II have shown that it is in such decondensed regions that transcriptional activity is found in salivary gland chromosomes (Jamrich et al. 1977; Semshin et al. 1979). Clearly, however, not all transcriptionally active regions give *zeste* immunofluorescence in our experiments. In particular, even strong puffs show no detectable presence of *zeste*.

While it is possible that bands of immunofluorescence are associated with the *dpp* and *Ubx* loci, which we know interact with *zeste* from both genetics and in vitro binding studies (Gelbart and Wu 1982; Benson and Pirrotta 1987, 1988), we have not detected *zeste* protein at the *white* locus. Using calibrated, fluorescein-labeled beads, we estimated that about 1000 fluorescein molecules/ μm^2 would be detected easily over a dark background. A squashed chromosome is 2–4 μm wide and a band is $\sim 0.2\text{-}\mu\text{m}$ thick. Assuming that each binding sequence becomes labeled with one fluorochrome, with a level of polyteny of 1000 a single binding site would be easily visible. However, we do not know the efficiency of antibody binding to chromosome squashes, the number of *zeste* polypeptides associated with each binding site, or the number of antibody molecules able to bind to each *zeste* molecule. The *white* gene is not known to be active in the salivary glands. Therefore, it is possible that the accessibility of potential *zeste* binding sites is correlated with gene activity. Preliminary observations suggest in fact that changes in the pattern of immunofluorescing bands can be detected between younger third instar larvae and prepupae. An alternative explanation is that detection of *zeste* is much less efficient than our estimate and that the chromosomal sites revealed by immunofluorescence are sites of exceptional affinity or able to bind an unusual number of *zeste* molecules. We can rule out this possibility at least in the case of the band of immunofluorescence at 3A on the X chromosome. Clones representing a 220-kb chromosomal walk covering the 3A region (Mariani et al. 1985) were tested for *zeste* binding sites in vitro. Only a small number of *zeste* binding sites of intermediate strength

were observed, fewer and weaker than those found at the *white* or *Ubx* loci. Comparison of the immunofluorescence obtained with two deletion mutants allowed the localization of the fluorescing site to an 8-kb interval containing the *zeste* gene itself, raising the possibility that *zeste* is autoregulating (S. Qian and V. Pirrotta, unpubl.). The band of fluorescence detected near 3C11-12 is also of interest because it is at or near the cytological locus of *sgs-4*. This gene is active in third instar larvae, and transvection effects between some of its alleles have been reported (Korge 1981; Kornher and Brutlag 1986), although they have not been shown to be dependent on *zeste*.

Although our experiments localize *zeste* on the chromosomes, they leave unresolved the question of its possible association with other nuclear structures such as the nuclear envelope. It has been suggested, for example, that *zeste* might anchor certain chromosomal regions to nuclear structures where the transcriptional machinery resides (Pirrotta et al. 1985; Zachar et al. 1985). Because the chromosomes are so enormously hypertrophied in polytenic nuclei, they are likely to dwarf and obscure most other nuclear structures. Unless other possible *zeste* binding structures are magnified to a similar degree, their contribution to *zeste* immunofluorescence therefore is not likely to be detected.

zeste mutants

The pattern of fluorescence detected in salivary gland chromosomes of the *z¹* mutant did not differ appreciably from the wild type, and, in particular, no new band of fluorescence appeared over the *white* locus. This is in agreement with previous observations that indicated that this mutation does not cause overexpression of the gene (Mariani et al. 1985), that the mutant gene contains a single codon change near the carboxyl terminal of the molecule (Pirrotta et al. 1987), and that the mutant protein has the same DNA-binding properties as the wild-type protein (S. Bickel and V. Pirrotta, unpubl.). The lack of immunofluorescence observed with the *In(1) e(bx)* mutant confirms the conclusion that the antigen detected in wild-type flies is the product of the *zeste* gene. The *In(1) e(bx)* mutant has an inversion breakpoint in the middle of the protein coding region which removes the 3' half of the gene. Hence, its product would not be recognized by our antibody which is directed to the carboxy-terminal portion of the protein. We have shown (S. Bickel and V. Pirrotta, unpubl.) that a *zeste* mutant produced in vitro, deleted at approximately the same position, produces a protein that is still able to bind specifically to DNA and may have at least part of the *zeste* functions, although it cannot be detected by the antibody directed to the carboxy-terminal region.

Materials and methods

Fly strains and mutants

Oregon R (Heidelberg) flies were used as a source of RNA for the developmental Northern blots. *zeste* mutants used in this

work were $y z^a$, obtained from Gif-sur-Yvette and carrying an apparent point mutation in the *zeste* gene unable to complement z^1 or to support transvection and *In(1) e(bx)*, containing a small inversion with a breakpoint in the middle of the *zeste* coding region (Pirrota et al. 1987), obtained from E. Lewis. Transvection effects were tested using *Cbx Ubx g1³/T[2,3] ap^{x^a}*, obtained from C.-T. Wu. The *D. virilis* stock was kindly provided by A. Spradling.

Northern blot hybridization

RNA was isolated from unfertilized embryos or from embryos, larvae, pupae, and adults staged at 1-day intervals. The RNA was extracted as described by Pirrota et al. (1983) and the poly(A)⁺ component was isolated by two cycles of binding and elution from a poly(U) Sepharose column in buffer containing 10 mM Tris (pH 7.5), 10 mM EDTA, 0.2% SDS, and 0.2 M NaCl. The column was eluted with 10 mM Tris (pH 7.5), 10 mM EDTA, and 50% formamide. The RNA was analyzed on agarose-formaldehyde gels (Maniatis et al. 1982), blotted on a nitrocellulose filter, and hybridized in 50% formamide, 10× Denhardt's solution, 100 μm/ml denatured calf thymus DNA, and 2× SCC at 43°C. After hybridization the filter was washed in 2× SCC and then in 0.2× SCC at 65°C.

Construction of transposons and P-mediated transformation

Transposons were constructed in the pUChsneo vector (Steller and Pirrota 1985a). The *zeste-lacZ* gene was assembled using the *zeste* upstream sequence from the *Bam*HI site to the *Pvu*II site at position 1050 (Pirrota et al. 1987) and ligating it in phase to a *lacZ* gene bearing the SV40 polyadenylation site constructed by C. Thummel and D. Hogness. The *hs-zeste* gene was assembled with the same *hsp70* promoter fragment used previously (Steller and Pirrota 1985a) ligated to a nearly full-length *zeste* cDNA trimmed to reduce the leader sequence to ~120 nucleotides before the translation start. The transposons were injected into early embryos at a concentration of 400 μg/ml together with 80 μg/ml of the helper *phsπ* plasmid (Steller and Pirrota 1986). G₂ progeny were selected by growth on instant food 4-24 (Carolina Biological Supply Co.) containing 800 μg/ml G418 (Geneticin, Sigma).

In situ detection of β-galactosidase

The procedure was adapted from that of Glaser et al. (1986). Larvae and pupae of adults were dissected in Insect Ringer's solution, and then fixed for 15 min in 2% glutaraldehyde in 50 mM sodium cacodylate (pH 7.5). The dissected animals were washed twice with either citrate-phosphate buffer [0.2 M Na₂HPO₄, 0.003 M citric acid (pH 8.2)] or buffer A [10 mM sodium phosphate (pH 7.0), 150 mM NaCl, 1 mM MgCl₂] (Glaser et al. 1986) and then stained with the same buffer containing 3 mM K₃Fe(CN)₆ and 3 mM K₄Fe(CN)₆·3H₂O and 0.2% 5-bromo-4-chloro 3-indolyl-β-D-galactopyranoside (X-Gal). Incubation in the staining solution generally was allowed to continue for 8–14 hr. At the lower pH [buffer A], staining was more vigorous but endogenous activity was also higher, particularly in the pupa. At the higher pH, the endogenous activity was suppressed almost entirely at the cost of longer incubation and paler color development. The dissected organs were mounted on slides in 30% glycerol and 70% ethanol and photographed with Kodachrome 64.

Affinity purification of zeste antibody

β-Galactosidase or a β-galactosidase fusion protein containing the last 105 amino acids from the carboxy-terminal of the *zeste*

protein made in *Escherichia coli* (Benson and Pirrota 1987) were electroeluted from preparative acrylamide gels and coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia) at a concentration of 1.3–1.5 mg/ml of resin. After coupling, the columns were equilibrated with buffer containing 100 mM H₃BO₃, 25 mM sodium borate, 75 mM NaCl (pH 8.4). Serum from immunized rabbits (30 ml) was first depleted of anti-β-galactosidase antibodies by passing it three times through the β-galactosidase column. The flowthrough was applied to the *zeste*-β-galactosidase column. After washing, specifically bound antibodies were eluted using 200 mM glycine, 0.8% NaCl (pH 2.7). Fractions of 0.5 ml were collected and neutralized immediately with 25 μl 2 M Trizma base. Peak fractions were pooled, dialyzed against PBS buffer (pH 7.4), aliquoted, and frozen at -70°C.

Western blotting

One hundred flies (~0.1 gram) were either left at room temperature or heat-shocked 1 hr at 37°C followed by 1 hr recovery at room temperature. Each set was homogenized in 1 ml of buffer containing 0.15 M NaCl, 0.01 M Tris (pH 8), 5 mM EDTA, 0.2% NP-40, 2 mM phenyl methylsulfonyl fluoride (PMSF) in a 7-ml Dounce homogenizer with an 'A' pestle. The homogenate was filtered through glass wool, layered on a two-layer sucrose cushion composed of 1 ml of 1.6 M sucrose and 1 ml of 0.8 M sucrose and centrifuged for 20 min at 10,000 rpm in a JA-20 rotor at 4°C. Lipid material adhering to the walls was wiped off and the nuclear pellets were resuspended in 8 M urea, 100 mM Tris (pH 7.6), 2% SDS, 5% β-mercaptoethanol, 5% Ficoll and sonicated extensively. Nuclear extract corresponding to 50 flies was loaded in each lane of a 7.5% acrylamide-SDS gel (Dreyfuss et al. 1984). The gel was electroblotted onto a nitrocellulose filter using a Sartorius semidry electroblotter. The filter was blocked using 3% milk solids in TBS [10 mM Tris (pH 7.4), 0.9% NaCl] and then incubated for 1 hr at room temperature in affinity-purified antibody solution (0.8 μg/ml in TBS plus milk solids). After washing, alkaline phosphatase-conjugated anti-rabbit IgG (Promega Biotec) was added at a 1 : 7500 dilution in TBS plus milk solids. The blot was developed using the Promega AP substrate kit.

Immunofluorescence on polytene chromosomes

The procedure used is derived partly from that of Silver and Elgin (1976) and partly from Jamrich et al. (1977). Late third instar larvae and prepupae were dissected in Insect Ringer's solution and the salivary glands were transferred to a drop of PBS buffer containing 3.6% formaldehyde for 45 sec on a subbed slide. Lengthening the prefixing time or shortening it to 5 sec did not affect the results appreciably, except that with longer prefixing times the chromosomes become increasingly more difficult to spread. The glands were then transferred to a drop of 45% acetic acid, 3.6% formaldehyde and the chromosomes were spread under a siliconized cover slip by gently rotating the cover slip with the tip of a dissecting needle. After squashing, the slide was dipped in liquid nitrogen and the cover slip was flicked off with a razor blade. The slide was then washed in 95% ethanol for 1–24 hr, then in PBS for 1 hr. The squash was rinsed briefly with PBS containing 10% goat serum before shaking off the excess liquid and applying the affinity-purified antiserum at 3.3 μg/ml in 30 μl of the same buffer. After incubating for 30 min at room temperature under a cover slip in a humidified petri dish, the slide was rinsed and allowed to wash for 30 min in PBS. FITC-conjugated goat anti-rabbit second antibody (Cappel) that had been preadsorbed by incubating with permeabilized, formaldehyde-fixed *Drosophila* embryos was

then applied at a 1 : 200 dilution in PBS and allowed to incubate for 30 min. The slides were then washed 30 min in PBS and mounted under a cover slip in 50% glycerol containing 1 × PBS (pH 8.8) and 10 mg/ml *p*-phenylenediamine to retard photobleaching. Bleaching was also decreased by sealing the edges of the cover slip with nail polish to exclude oxygen. For extra sharp photomicrographic images, some of the squashes were prepared on 24 × 60-nm coverslips instead of normal microscope slides. In this case, after the antibody treatments the cover slip bearing the chromosomes was cut with a diamond pencil and mounted upside down on a cover slip. The chromosomes were viewed under epifluorescence and phase-contrast optics in a Zeiss Axiophot and photographed with Kodak Tri-X film.

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