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Developmental timing and tissue specificity of heterochromatin-mediated silencing

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Heterochromatic position-effect variegation (PEV) describes the mosaic phenotype of a euchromatic gene placed next to heterochromatin. Heterochromatin-mediated silencing has been studied extensively in *Drosophila*, but the lack of a ubiquitous reporter gene detectable at any stage has prevented a direct developmental characterization of this phenomenon. Current models attribute variegation to the establishment of a heritable silent state in a subset of the cells and invoke differences in the timing of silencing to explain differences in the patch size of various mosaic patterns. In order to follow the course of heterochromatic silencing directly, we have generated *Drosophila* lines variegating for a *lacZ* reporter that can be induced in virtually all cells at any developmental stage. Our data indicate that silencing begins in embryogenesis and persists in both somatic and germline lineages. A heterogeneity in the extent of silencing is also revealed; silencing is suppressed in differentiated tissues but remains widespread in larval imaginal discs containing precursor cells for adult structures. Using eye development as an example, we propose that the mosaic phenotype is determined during differentiation by a variegated relaxation in heterochromatic silencing. Though unpredicted by prevailing models, this mechanism is evident in other analogous systems.

Keywords: development/epigenetic silencing/germline/heterochromatin/position-effect

Introduction

In the nucleus of higher eukaryotes, heterochromatin represents chromosomal areas that replicate late and stay condensed throughout the cell cycle. This compact chromatin structure is known to inactivate genes, and one example is the random heterochromatinization of one of the two X chromosomes in mammals. Moreover, a normally euchromatic gene juxtaposed to heterochromatin can become silenced in a fraction of the cells, and the mosaic expression of this gene is termed heterochromatic position-effect variegation (PEV). Heterochromatic PEV is used to describe the variable lineage-specific expression of autosomal loci translocated to the X chromosome in mice (Cattanach, 1974) and genes adjoining the Xq27 site in fragile-X syndrome (Laird, 1987). In *Drosophila*, examples of variegated expression are numerous (Spofford, 1976) and, among factors identified to participate in heterochromatic silencing, homologous counterparts in other species including human have been reported (Singh *et al.*, 1991; Saunders *et al.*, 1993; Thompson *et al.*, 1993; Lorentz *et al.*, 1994). Even though heterochromatin in yeast is not cytologically visible, it also silences genes and exhibits many properties similar to those in higher organisms (Laurenson and Rine, 1992).

To understand heterochromatin-mediated silencing, PEV in *Drosophila* has been studied extensively. Silencing has been proposed to be a heritable, epigenetic event that involves no alteration in the DNA content; genes are either packaged into a higher chromatin structure, inaccessible by transcriptional activators (Locke *et al.*, 1988; Grigliatti, 1991; Wallrath and Elgin, 1995) or localized to a special nuclear compartment that confers transcriptional repression (Dorer and Henikoff, 1994; Karpen, 1994). Once the transcriptional state is determined, it can be stably maintained through mitosis resulting in clones of expressing and non-expressing cells. Such propagation of epigenetically established transcriptional states is also reflected in the repression of the silent mating-type loci in yeast (Nasmyth and Shore, 1987) and the inactivation of X chromosome (Lyon, 1992). An alternative mechanism explains loss of gene expression by DNA loss rather than transcriptional regulation (Karpen and Spradling, 1990). It argues that underrepresentation of the variegating sequences, apparent in certain polytene tissues, implicates a somatic elimination of DNA, which also occurs in other dipterans (Pimpinelli and Goday, 1989). A demonstration of the reversibility of a heterochromatic silencing should distinguish between transcriptional repression and irreversible DNA loss.

Another intriguing aspect of PEV concerns the developmental programing of variegated silencing. It has been assumed that variegated silencing involves the imposition of a mitotically transmissible silent state in a fraction of the cells, and the onset of such silencing is early in *Drosophila* lines displaying sectored or 'large patch' mosaicism but delayed in those with a fine-grained or 'salt and pepper' phenotype (Spofford, 1976; Tartof *et al.*, 1984; Pirrotta and Rastelli, 1994). A different but not mutually exclusive explanation for 'salt and pepper' mosaicism postulates that, regardless of the timing of gene suppression, switches in the transcriptional state can occur with cell proliferation and, thereby, contribute the mottled phenotype. Examples of such mitotically metastable silencing can also be found in the maintenance of repression at the silent mating-type loci (Pillus and Rine, 1989) and telomeric PEV in yeast (Gottschling *et al.*, 1990).

To derive the timing of a determinitive or heritable gene suppression, silencing is believed to occur at develop-

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mental stages in which an environmental or genetic perturbation can change the adult phenotype. Thus, silencing in 'large patch' variegation is assumed to occur as early as blastoderm because of a coincident temperature-sensitive period (Chen, 1948; Hartmann-Goldstein, 1967; Spofford, 1976), and fine-grained mosaicism is explained by a later temperature-sensitive period spanning the pupal stage (Spofford, 1976; Tartof and Bremer, 1990). In contrast to these discrete periods, a study based on dosage alteration of a heterochromatin-associated protein has suggested that variegation can be modified at any time within a continuous interval from blastoderm to late larval development (Eissenberg and Hartnett, 1993). Alternatively, another method to deduce the timing of variegated silencing is to determine how early in development variegated expression can be observed, and, by using PEV markers that can be expressed prior to adulthood, mosaic larval tissues have been reported (Spofford, 1976; Kornher and Kauffman, 1986; Spradling 1993).

However, as a means to study the course of heterochromatic silencing, the PEV reporter genes employed in these studies are usually stage and tissue specific or exposed to the euchromatic position-effect on transgenes. These limitations preclude a direct and sensitive characterization of PEV in various tissues throughout development. To circumvent this difficulty, we have devised a novel reporter construct, containing a gene not only inducible in different tissues at different developmental stages but also insulated from euchromatic position-effect. In flies exhibiting variegation of this reporter construct, we have tracked visually the course of PEV throughout development, and demonstrated directly that heterochromatic silencing is established during embryogenesis and maintained in both somatic and germline lineages. Tissue-specific and developmental differences in the extent of silencing are also perceived; differentiated tissues display variegated transgene expression whereas widespread silencing prevails in their precursors. These observations argue that differentiation and a concomitant relaxation of heterochromatic silencing underlie the variegated patterns seen in adult tissues.

Results

Creation of an autonomous but ubiquitously inducible reporter gene that can be silenced by heterochromatin

In order to visualize the extent of gene silencing in any tissue at any stage of development, we devised a P-element cassette containing a *Hsp70* promoter-driven *lacZ* reporter (Figure 1), which is sensitive to silencing by heterochromatin (Henikoff, 1981) but is otherwise fully inducible in virtually all cells (Bonner *et al.*, 1984) at any time (except pre-blastoderm embryos, which do not have a heat-shock response; Dura, 1981). Adjacent to the *lacZ* reporter is a mini-*white* gene regulated by an eye-specific enhancer. The eye-specific enhancer confers expression in the developing eye, where *white* is essential for red eye pigment production, and, thus, allows transformed flies to be visually identified. To ensure that any induced *lacZ* expression can accurately reflect the extent of heterochromatic silencing, the P-element cassette is flanked by scs′ and scs elements, which are specialized chromatin structures that delineate a domain of independent gene activity insulated from euchromatic but not heterochromatic position-effect (Kellum and Schedl, 1991).

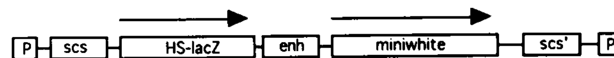

Fig. 1. Schematic representation of the transgene used in these studies. 'miniwhite' refers to the *white* gene coding sequences and minimal promoter sequences found in the pCaSpeR transformation vector (Pirrotta, 1988). 'enh' refers to the *white* eye-specific enhancer element. 'HS-lacZ' refers to the minimal *D.melanogaster Hsp70* promoter, 5′ UTR and first seven codons of *Hsp70*, fused to the *E.coli lacZ* gene coding sequence and *D.melanogaster Hsp70* 3′ UTR. 'scs' and 'scs′' refer to the domain boundary elements flanking the 87A7 heat shock locus. Scs elements block euchromatic but not heterochromatic position effects (Kellum and Schedl, 1991). 'P' refers to the P-element transposon terminal elements which support insertional transposition (Spradling, 1986). Arrows over the map indicate the relative direction of transcription of the *lacZ* and mini-*white* sequences.

Using P-element-mediated germ line transformation of white-eyed flies (Spradling, 1986), we generated a homozygous-viable, fertile transgenic line, *P[w$^+$ HS-lacZ] (65E)*, with a single-copy transgene insertion in the euchromatin of the third chromosome at cytological position 65E (Figure 2A). These transgenic flies have uniform red eyes (Figure 3A) and, when induced for β-galactosidase, exhibit similarly uniform staining in various structures such as the adult eyes (Figure 3B), larval polytene salivary glands (Figure 3C), larval diploid imaginal discs (Figure 3D) and embryos (Figure 3E).

To verify that the uniform induction of *Hsp70-lacZ* seen in whole tissues also occurs at the cellular level, the duration of heat shock was minimized to lessen the intensity of staining so that individual cells exhibiting no heat-dependent response may be discerned. Such cell by cell resolution can be obtained easily in the salivary glands because of the enormous size of the polytene cells. After establishing that the *Hsp70* promoter, at the euchromatic locus in *P[w$^+$ HS-lacZ] (65E)*, exhibits a low but detectable basal activity (Figure 4A), a brief heat induction resulted in intermediate levels of increased β-galactosidase in every cell (Figure 4B) and, with successively longer heat shocks, the intense uniform staining was restored (Figure 3C).

Generation of transgenic lines exhibiting heterochromatic position-effect variegation of the reporter gene

Heterochromatic PEV of the transgene locus was generated by treating *P[w$^+$ HS-lacZ] (65E)* males with X-rays (3200 R) to generate chromosomal rearrangements that would juxtapose the transgene to heterochromatin. Progeny of these irradiated fathers and white-eyed mothers were then screened for *white* variegation. Two variegating lines established from this screen are subjects of this report.

One line, *In(3L)BL1*, displays 'salt and pepper' variegation of *white* (Figure 3F) while the other, *Tp(3;Y)BL2*, shows Y-linked sectored or 'large patch' mosaicism (Figure 3K), and, after heat shocking the adults of both lines, inducible *Hsp70-lacZ* variegation becomes apparent in various structures such as the eye, where a striking resemblance to the variegated pattern of *white* suggests a similar extent of heterochromatic silencing on both transgenes (Figure 3G and L).

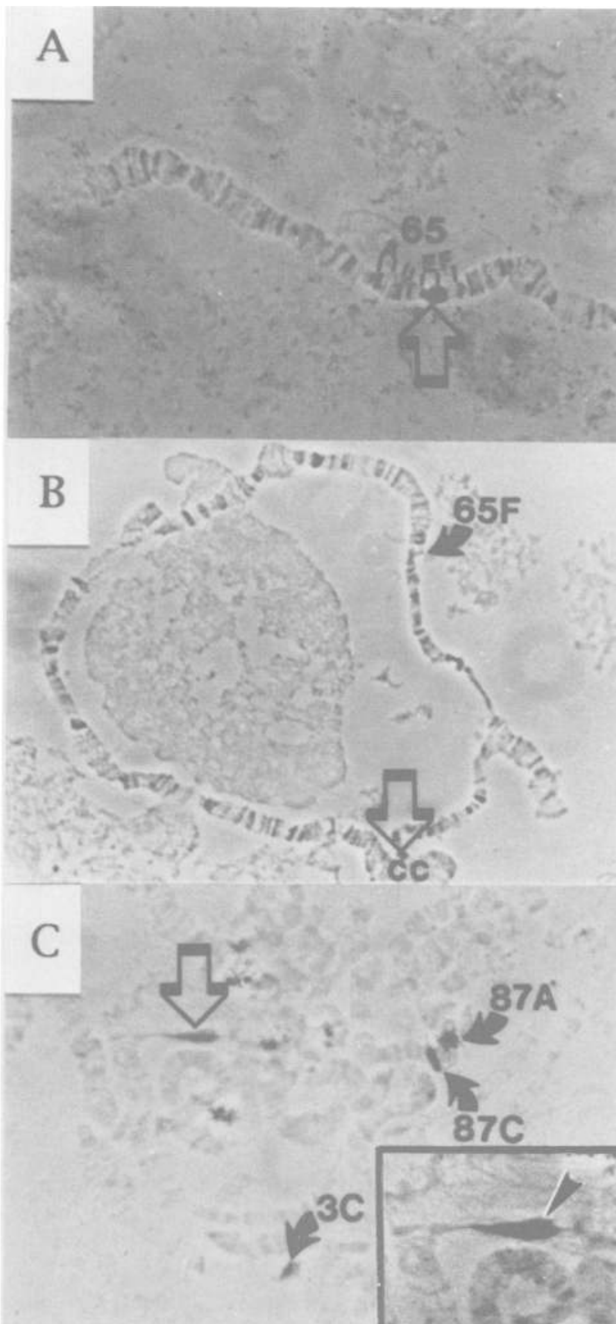

Fig. 2. Cytological analysis of transgenic lines. (**A**) Localization of the transgene insertion site in *P[w⁺ HS-lacZ] (65E)* to cytological position 65E by *in situ* hybridization (arrow). (**B**) Inversion loop in the left arm of chromosome 3 in an *In(3L)BL1/+* larva. A breakpoint is seen at 65F in the inversion homolog (arrow) with the distal portion of the rearranged arm emerging from the heterochromatic chromocenter (cc). Thus, *In(3L)BL1* carries a simple inversion in chromosome 3, with one breakpoint just proximal to the transgene and the other in pericentric heterochromatin. (**C**) *In situ* hybridization to *Tp(3;Y)BL2* male polytene chromosomes using the transgene probe. Expected hybridization occurs to *Hsp70* sequences at 87A and 87C and to *white* sequences at 3C (arrows). The open arrow points to an additional site of hybridization in a stretched region of attenuated chromosomal material associated with the chromocenter. This is consistent with the observed Y-linkage of the transgene in this line, as the Y is heterochromatic in somatic tissue. Inset: magnification of the heterochromatic hybridization in the chromocenter (arrowhead). Hybridization is associated with several attenuated strands stretched by pressure applied during squashing. This is a diagnostic feature of the polytene chromocenter (Lefevre, 1976).

Variegation in both of these lines has been verified to represent examples of classical PEV because they also demonstrate known structural and genetic properties of heterochromatic silencing. Heterochromatic PEV is believed to result from changes in chromosome structure because (i) regions containing the variegating locus acquire the condensed and attenuated morphology of heterochromatin in at least a subset of nuclei and (ii) remobilization of the variegating gene away from heterochromatin restores its wild-type activity (Prokofjeva-Belgovskaya, 1947; Hartmann-Goldstein, 1967; Henikoff, 1981; Reuter *et al.*, 1982; Zhimulev *et al.*, 1988; Belyaeva and Zhimulev, 1991). In our variegating lines, *In(3L)BL1* contains an inversion of the left arm of chromosome 3 with one breakpoint at 65F, just proximal to the transgene insertion site, and the other in pericentric heterochromatin (Figure 2B). In *Tp(3;Y)BL2*, a transposition inserting a small piece of chromosome 3, including the transgene, onto the heterochromatic Y chromosome has taken place (Figure 2C). Both of these arrangements have resulted in the juxtaposition of the transgene to heterochromatin. In addition, after confirming the integrity of the transgene construct with a Southern blot analysis, we have successfully remobilized the reporter gene cassette out of its heterochromatic context using the Δ2-3 chromosome (Robertson *et al.*, 1988) and restored uniform *white* and inducible *lacZ* expression (unpublished data).

Besides meeting the structural criteria for heterochromatic silencing, variegation in both *In(3L)BL1* and *Tp(3; Y)BL2* also responds to genetic modifiers of classical PEV. Heterochromatin-mediated variegation is known to be sensitive to the dosage of several loci thought to encode structural components of heterochromatin or their modifiers (Locke *et al.*, 1988; Wustmann *et al.*, 1989). The level of silencing can either be increased with a PEV enhancer such as *Dp(2;2)P90* (Wustmann *et al.*, 1989) or reduced with a suppressor such as *Su(var)2-1*^{01} (Dorn *et al.*, 1986). In our variegating stocks, flies carrying either *Dp(2;2)P90* or *Su(var)2-1*^{01} also exhibit the anticipated enhancement or suppression of both *white* variegation in the eye and *Hsp70-lacZ* variegation in the entire fly (Figure 5). In addition, the effect of increased Y chromosome dosage, which suppresses PEV (Gowen and Gay, 1934), is also evident (Figure 5).

Heterochromatic position-effect variegation begins early in embryogenesis and persists in germline tissues

In both *In(3L)BL1* and *Tp(3;Y)BL2* embryos, variegated silencing can already be perceived. Silencing is most extensive at cellular blastoderm, after which islands of inducible *lacZ* expression begin to emerge (Figure 3J and O). Although it has been known that adult variegation can be influenced by environmental and genetic modifiers of PEV during embryogenesis (Hartmann-Goldstein, 1967; Eissenberg and Hartnett, 1992), this is the first direct visualization of heterochromatic silencing in embryonic tissues.

Studies demonstrating parental source effects on variegation have implicated chromosome imprinting in the germline (Grigliatti, 1991; Reuter and Spierer, 1992; Bishop and Jackson, 1995). Since DNA methylation is absent in *Drosophila* (Urieli-Shoval *et al.*, 1982), it is

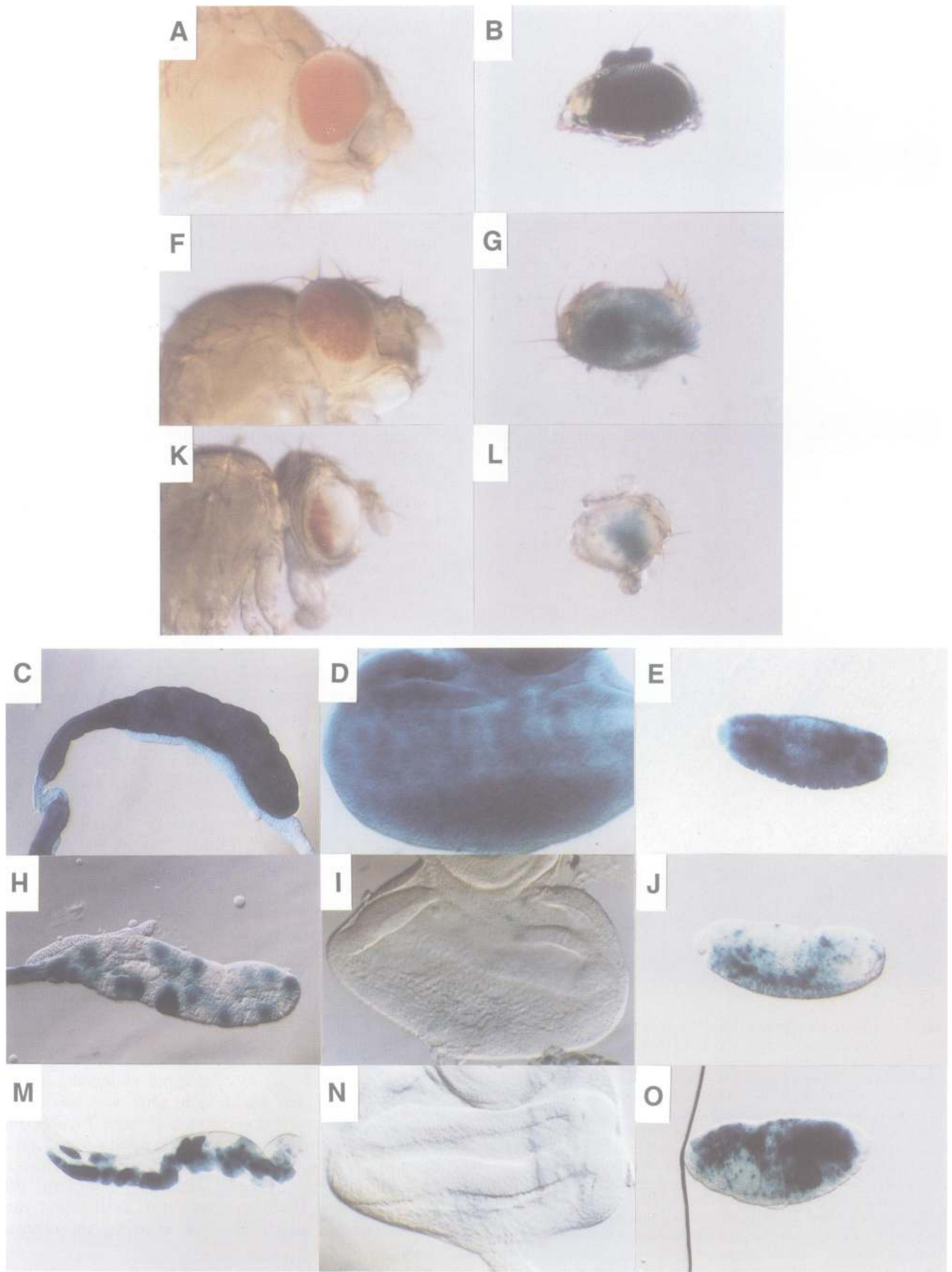

Fig. 3. Expression patterns of *white* and *lacZ* in founder and variegating transgenic lines. (A–E) Founder *P[w⁺ HS-lacZ] (65E)* line. (F–J) *In(3L)BL1* variegating line. (K–O) *Tp(3;Y)BL2* variegating line. (**A**, **F** and **K**) Unstained adult heads showing *white* expression. (**B**, **G** and **L**) Adult heads stained with X-gal [not the same heads as in (A, F and K)]. (**C**, **H** and **M**) Third instar larval salivary glands stained with X-gal. (**D**, **I** and **N**) Eye portion of eye-antennal imaginal discs from third instar larvae. While the number of staining cells varied somewhat between individuals, in no instance was β-galactosidase expression in discs as extensive as in adult eyes. (**E**, **J** and **O**) Post-gastrulation embryos stained with X-gal.

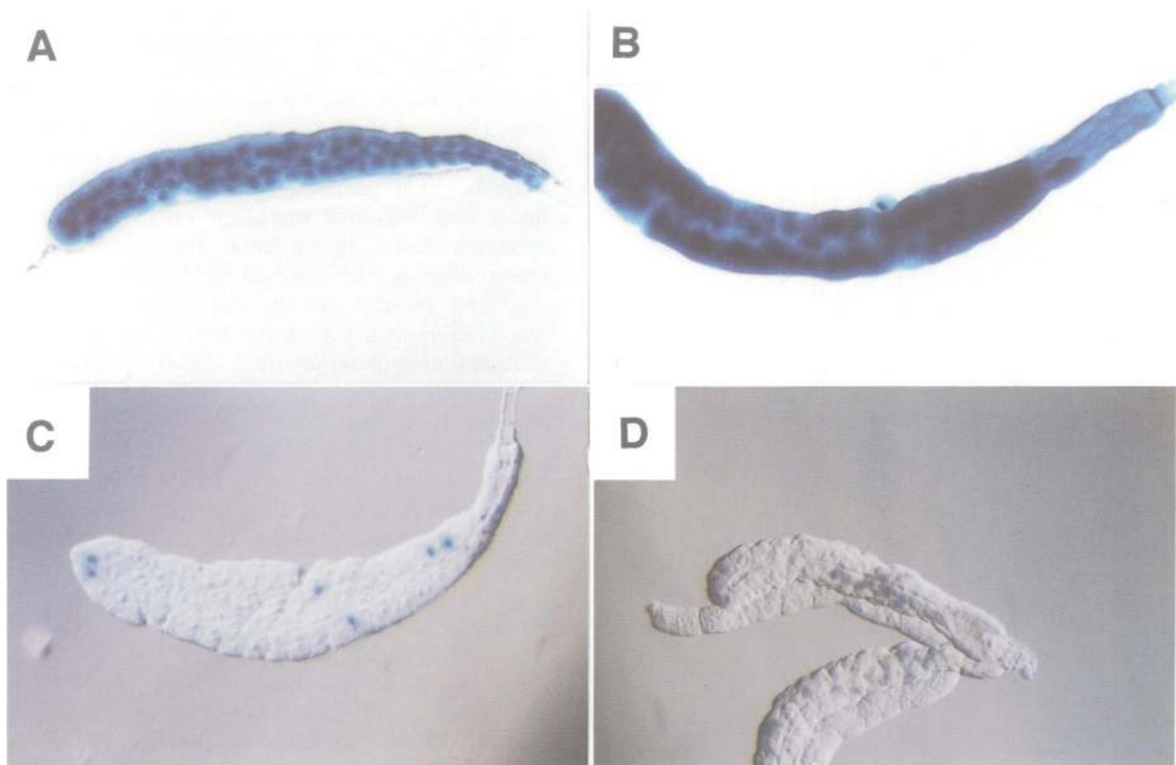

Fig. 4. Basal activity and induction of the *Hsp70* promoter at various transgenic loci. Because of the enormous size of polytene cells in salivary glands, the degree of *lacZ* expression in each cell can be easily discerned. Each panel shows staining for β-galactosidase activity in third instar salivary glands. (**A**) Non-heat shocked *P[w$^+$ HS-lacZ] (65E)*. (**B**) *P[w$^+$ HS-lacZ] (65E)* heat shocked for only 5 min. (**C**) Non-heat shocked *In(3L)BL1*. (**D**) Non-heat shocked *Tp(3;Y)BL2*.

possible that chromosomal imprinting of a variegating locus may be mediated directly by heterochromatin. An examination of ovaries and testes reveals that germline silencing is already apparent in the stem cells (Figure 6). This finding provides direct evidence for heterochromatic silencing in both male and female *Drosophila* germlines.

Tissue-specific differences in the extent of PEV suggest that adult variegation patterns arise following a relaxation in heterochromatic silencing

In the third instar larvae of each variegating line, mosaic patterns resembling those in the adult eyes can be seen in the salivary glands. *In(3L)BL1* shows a 'salt and pepper' variegation (Figure 3H), whereas groups of contiguous *lacZ*-expressing cells often yield a sectored variegation in *Tp(3;Y)BL2* (Figure 3M).

In contrast to variegated expression in salivary glands, widespread silencing prevails in the diploid third instar imaginal discs, which contain the precursor cells for various adult structures. For example, unlike the dramatically different variegated patterns seen in the adult eyes, an almost uniform silencing is seen in the imaginal discs for the eye (Figure 3I and N). Such extensive silencing in the eye imaginal disc suggests that derepression of the heterochromatin-silenced loci must have occurred in a portion of the cells so that variegated expression can be seen in the adult eye.

Finally, because the *Hsp70* promoter in *P[w$^+$ HS-lacZ] (65E)* exhibits a uniform basal activity (Figure 4A), we examined if such background also occurs in the variegating lines and, if so, contributes to inducible β-galactosidase activity. However, in all non-heat-shocked *In(3L)BL1* and *Tp(3;Y)BL2* tissues, widespread silencing rather than uniform expression of the *Hsp70* promoter is observed (Figure 4C and D). It appears that the juxtaposition of *Hsp70* to heterochromatin has rendered both its basal activity and induction susceptible to heterochromatic silencing. Since the uniform basal activity seen in *P[w$^+$ HS-lacZ] (65E)* is no longer detectable in the *In(3L)BL1* and *Tp(3;Y)BL2* lines variegated β-galactosidase expression must result from the activity of the *Hsp70-lacZ* reporter in cells carrying a non-silenced transgenic locus, and, therefore, accurately represents the extent of heterochromatic silencing.

Discussion

Our present investigation signifies a systematic effort to visualize directly the developmental timing and stability of heterochromatic silencing in *Drosophila*, without the spatial and temporal restrictions inherent in other PEV studies. Sequestered in a domain independent of euchromatic position-effect and inducible in almost all cell types, our novel PEV reporter gene offers a versatile assay for heterochromatic silencing in different tissues throughout development. We have generated variegating rearrangements that place the reporter gene construct adjacent to heterochromatin and demonstrated that this variegation responds appropriately to known PEV modifiers. In particular, its sensitivity to dosage of HP1, a heterochromatin-associated non-histone chromosomal

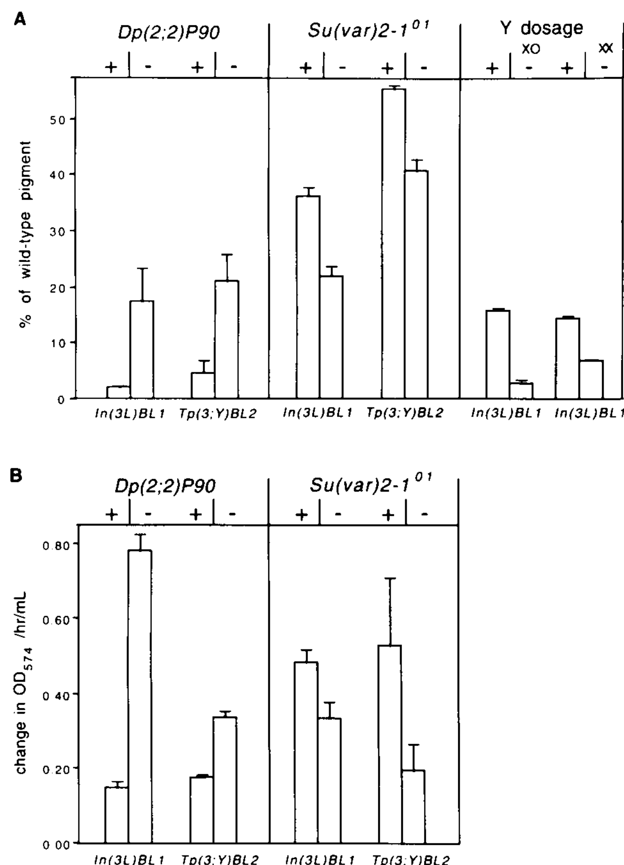

Fig. 5. Effects of genetic modifiers of variegation. (**A**) Modification of *white* variegation in the eye. (**B**) Modification of *Hsp70-lacZ* variegation in the entire fly. Modifier-carrying females were mated to males from each variegating line, and male progeny were collected for eye pigmentation assay or β-galactosidase assay. For Y dosage, the effect of a maternally or paternally derived compound XY chromosome on *white* variegation was assayed in both sexes. '+' denotes progeny bearing the modifier while '–' denotes those without. *Dp(2;2)P90* carries a duplication of the *Su(var)205* locus (encoding heterochromatin protein 1) and enhances classical PEV (James *et al.*, 1989; Wustmann *et al.*, 1989; Eissenberg *et al.*, 1992). In both variegating lines, *Dp(2;2)P90* reduced *white* and *Hsp70-lacZ* expression compared with non-duplication-bearing sibs. *Su(var)2-1^{01}* suppresses PEV and is associated with impaired histone deacetylation (Dorn *et al.*, 1986; Reuter and Spierer, 1992). Progeny carrying *Su(var)2-1^{01}* exhibited significantly higher *white* expression than those without. The classically observed suppressing effect of increased Y chromosome dosage (Gowen and Gay, 1934) was only verified in *In(3L)BL1*; since the transgene is Y-linked in *Tp(3;Y)BL2*, a similar comparison was not done.

protein (James *et al.*, 1989), and the *Su(var)2-1^{01}* mutation, which interferes with histone deacetylation (Dorn *et al.*, 1986), argues that variegation is associated with perturbation of chromosomal structure and establishes further that our variegating rearrangements represent examples of classical PEV.

Even though our reporter gene construct contains two discrete transcriptional units, interaction between them appears to be minimal. A previous study has demonstrated that when the *Hsp70* and *white* promoters are placed ~1 kb apart, daily induction of *Hsp70* produced no apparent changes in *white* expression (Steller and Pirrotta, 1985). This is also confirmed by a quantitative pigment assay indicating that a heat shock on each day of development has not increased *white* expression in our variegating lines while a similar treatment on flies carrying a simple classical *white* variegating allele has only caused a slight increase in pigmentation (unpublished data). Conversely, despite proximity to the eye-specific *white* enhancer, which activates *white* transcription in third instar eye imaginal discs (Fjose *et al.*, 1984), *Hsp70* appears to be independent of such programed *white* activation. The promoter remains inducible in all heat-shocked tissues before and after third instar and becomes repressed only when silenced by heterochromatin. In particular, the observed *Hsp70-lacZ* reactivation in the variegating lines is unlikely to be mediated by the eye-specific *white* enhancer because reactivation occurs after the third instar and also in other differentiating imaginal discs, which do not express *white*.

The introduction of *Hsp70-lacZ* as a PEV marker has allowed us to show that heterochromatic silencing begins early in embryogenesis shortly after the appearance of heterochromatin in late syncytial blastoderm (James *et al.*, 1989). This finding is consistent with studies showing that silencing of a variegating locus can be modified either by altering the rearing temperature or changing the dosage of a PEV modifier during embryogenesis (Hartmann-Goldstein, 1967; Eissenberg and Hartnett, 1993).

Heterochromatic silencing may also be initiated early in the germline, which attains its identity ~2.5 h after fertilization, and then propagated in various germline-specific larval and adult organs. Genetic evidence of chromosomal imprinting of a variegating locus has been well documented (Grigliatti, 1991; Reuter and Spierer, 1992, Bishop and Jackson, 1995), and such an imprint would presumably be protein mediated since *Drosophila* lacks detectable DNA methylation (Urieli-Shoval *et al.*, 1982). One candidate could be a heterochromatin complex maintained through meiosis and transmitted directly to the zygote at fertilization. Even in the Y chromosome, where the expression of several genes is essential for spermatogenesis (Giatti and Pimpinelli, 1983), heterochromatin-mediated silencing should also operate, as indicated by the repression of the transgene in *Tp(3;Y)BL2* testis. Finally, since the entire parental genetic content must be preserved and equally distributed during meiosis, silencing, at least in the germline, is unlikely to be caused by DNA elimination or underrepresentation.

It is likely that the transition from widespread silencing in third instar imaginal discs to variegated expression in adult tissues results from a derepression of heterochromatin-silenced loci in a subset of the cells. In the developing eye, such variegated reactivation must occur between late third instar and the 50th hour of pupation when eye pigments appear (Summers *et al.*, 1982). This very same period has already been shown to be pivotal in the transcription of a variegating *white* gene so that its mosaic phenotype can be seen in the adult eye (Steller and Pirrotta, 1984). Because mitosis in the eye imaginal disc persists until the end of third instar when terminal differentiation begins (Wolff and Ready, 1993), it may be that silencing is mitotically stable in undifferentiated cells and that terminal differentiation ushers in reactivation. Such coupling between differentiation and derepression is likely to account for variegated *lacZ* expression in other adult structures subsequent to the widespread silencing in their precursor cells (unpublished data), and it may also explain variegated expression in the terminally differentiated larval salivary glands.

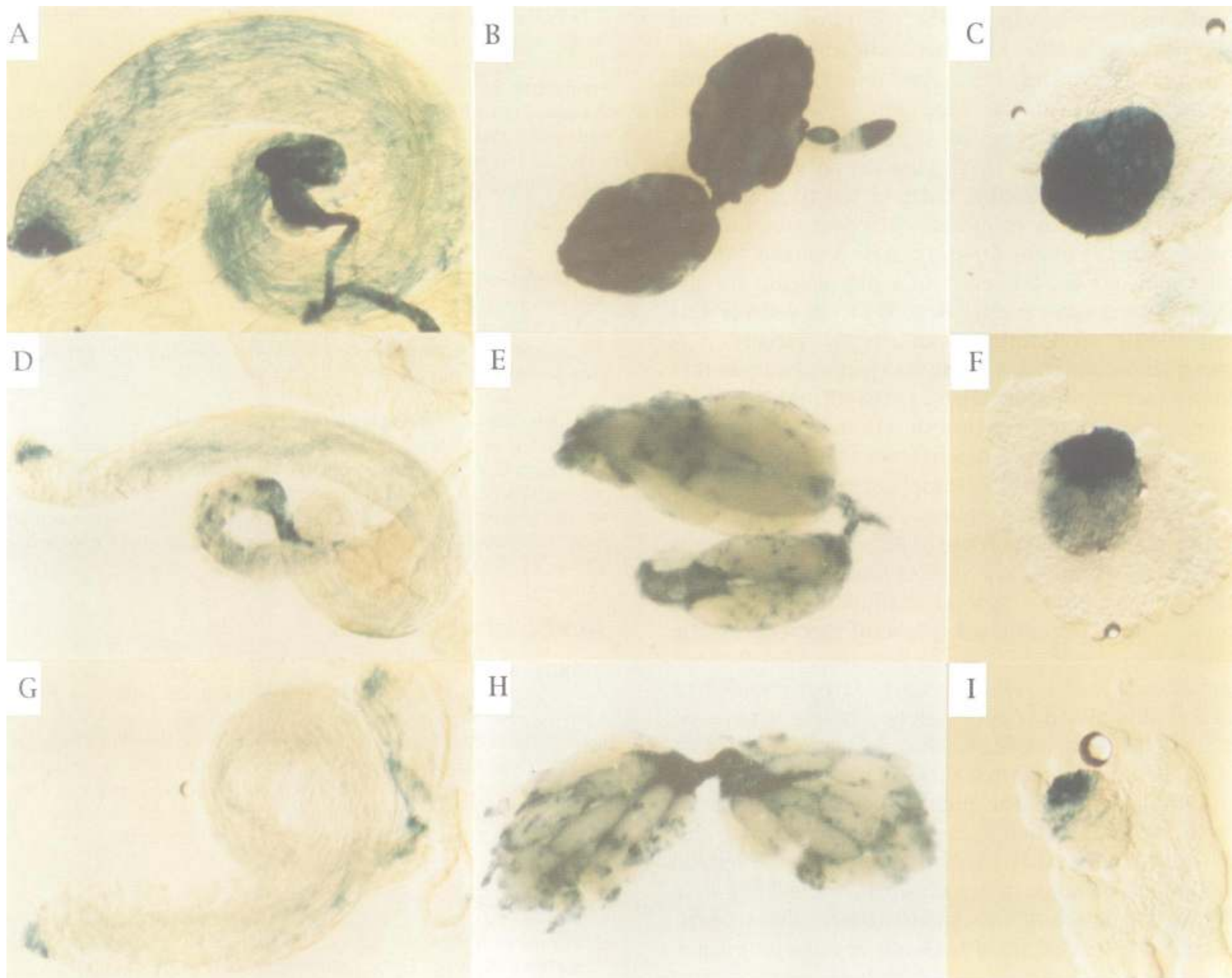

Fig. 6. Heterochromatic position-effect silencing operates in germline cells. (A–C) Founder *P[w$^+$ HS-lacZ] (65E)* line. (D–F) *In(3L)BL1* variegating line. (G–I) *Tp(3;Y)BL2* variegating line. β-Galactosidase activity throughout adult testes (**A**, **D** and **G**), adult ovaries (**B**, **E** and **H**) and larval testes (**C**, **F** and **I**) is much reduced in the variegating lines compared with that in *P[w$^+$ HS-lacZ] (65E)*. In (A, D and G), the interceding region between the apical stem cells on the left and the testicular duct at the distal end contains sperm bundles that exhibit little heat shock response (Bonner *et al.*, 1984) and, thus, shows incomplete uniform staining in *P[w$^+$ HS-lacZ]*. In (C, F and I), the prominently stained apical region in the larval testes is composed of both somatic and germline cells.

Prevailing models on *Drosophila* PEV assume that variegated expression stems from a determinative gene suppression occurring in a portion of the cells. Therefore, mosaic patterns containing larger clonal patches would suggest an earlier onset of determination while the 'salt and peppered' would indicate a later onset (Baker, 1968; Spofford, 1976; Tartof *et al.*, 1984; Pirrotta and Rastelli, 1994; Singh, 1994). Accordingly, the proportion of β-galactosidase-expressing cells in *In(3L)BL1* and *Tp(3; Y)BL2* eye discs should equal or exceed that in the adult eye, and, because the 'large patch' phenotype in *Tp(3; Y)BL2* implies an early silencing, an already sectored *lacZ* variegation is anticipated in the eye discs. On the contrary, our data demonstrate an almost complete silencing in the eyes discs followed by a developmentally late reactivation in both variegating lines. Thus, timing and stability of epigenetic silencing cannot be inferred from the adult variegated pattern. Alternatively, although our data asserts that the variegated transcriptional state of a gene is not established until differentiation, it is possible that a determinative event may still have taken place earlier at different times for different mosaic phenotypes. For example, in lines exhibiting 'large patch' mosaicism, the phenotype may already have been conceived by the first larval instar (Baker, 1967), but it is not realized until differentiation triggers a concerted relaxation of silencing in a clonal fashion.

Dynamic mechanisms analogous to a stage- and tissue-specific control of heterochromatic silencing and reactivation are numerous. In *Drosophila*, derepression of a *Polycomb*-silenced transgene also coincides with differentiation (Chan *et al.*, 1994); *Polycomb* is an essential constituent of homeotic silencing that not only shares molecular genetic and cytological properties with heterochromatic silencing (Paro, 1990; Grigliatti, 1991; Paro and Hogness, 1991; Pirrotta and Rastelli, 1994) but also can cause variegated expression of nearby genes (Fauvarque and Dura, 1993; Chan *et al.*, 1994). In mammals, variegated reactivation is seen in X-linked genes (Wareham *et al.*, 1987) and in autosomal loci translocated to the X chromosome (Cattanach, 1974). Also, timing of X chromosome inactivation is lineage specific (Tan *et al.*, 1993) and, in the germline, reactivation of the X chromosome appears to depend on inductive interactions (Tam *et al.*, 1994).

Overall, our results support the model of PEV as an example of a metastable, epigenetic silencing (Grigliatti, 1991; Reuter and Spierer, 1992), and this is demonstrated by the reactivation of the *lacZ* reporter gene during pupation. Therefore, variegated silencing in our study is unlikely to be caused by DNA elimination (Karpen and Spradling, 1990). In addition, because reactivation takes place after the cessation of mitosis, it appears that heterochromatic silencing in *Drosophila* can be reversed without the cell cycle, which, however, is a prerequisite for the metastability of epigenetically silenced loci in yeast (Pillus and Rine, 1989; Gottschling *et al.*, 1990). Finally, it is interesting that reactivation of the transgene occurs within a putative temperature-sensitive period (Spofford, 1976) for *white* variegation. However, while these temperature studies may have tested the same mechanism that we have uncovered, they also perturb other temperature-dependent processes such as stress response and the length of development, which are known to modify PEV (Michailidis *et al.*, 1988). In contrast, our system has minimized these variables and sought to detect developmental switches within the naturally programed course of heterochromatic silencing.

In conclusion, our novel reporter gene has enabled us to visualize directly the developmental timing and tissue specificity of heterochromatin-mediated silencing. Developmental studies on factors that dictate the dynamic nature of PEV during development, such as those associated with cell cycle and differentiation, are now possible with this system. Furthermore, it is likely that many of the known PEV modifiers regulate heterochromatic silencing in a spatially and temporally restricted manner; these possibilities can also be examined with our reporter construct.

Materials and methods

Construction of the white–lacZ bipartite reporter

The mini-*white* and eye enhancer sequences, flanked by scs and scs′ elements and P-element termini, were contained in the plasmid pw5′scs (gift from M.Muller and P.Schedl, Princeton University). An *XhoI–BglII* fragment containing the *Hsp70* promoter, 5′ untranslated region (UTR), first seven codons of *Hsp70, Escherichia coli lacZ* and *Hsp70* 3′ UTR was excised from the plasmid HS190.70ZT (gift of J.T.Lis, Cornell University), and the ends polished with Klenow polymerase. The pw5′scs plasmid was digested with *XhoI* (which has a unique cleavage site between the eye-enhancer and scs), polished with Klenow polymerase and the *Hsp70-lacZ* fragment inserted by ligation.

Germline transformation and screen for variegating rearrangements

Host flies for germline transformation were *Df(1)w, y w*. The bipartite reporter gene construct was co-injected with pπ25.7wc helper (Karess and Rubin, 1984) essentially as described (Spradling, 1986). Transformants were identified by red eye color. To generate variegating rearrangements, 360 2–3-day-old adult *P[w$^+$ HS-lacZ] (65E)* males were irradiated (3200 R) and mated to *Df(1)w* females. Seven *white*-variegating individuals were recovered from a screen of 26 436 progeny.

In situ hybridization

Chromosome squashes were performed on glands fixed in 45% acetic acid. Transgene plasmid DNA was labeled by nick-translation (Rigby *et al.*, 1977) in the presence of biotin-11-dUTP. *In situ* hybridization and detection were by the method of Engels *et al.* (1986).

Histochemical localization of β-galactosidase

Heat shock was done by incubation of adult flies, larvae or embryos for 45 min at 37°C, followed by a 1 h recovery at room temperature. Staining was by immersing tissues in a 0.2% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) assay buffer (Simon *et al.*, 1985). Adult heads were prepared and stained as described by Qian *et al.* (1992). Embryos were prepared by the method of Ashburner (1989), except that formaldehyde was substituted for glutaraldehyde and that fixation lasted 20 min. They were dechorionated in bleach and fixed in formaldehyde-saturated heptane. After washing in PBS (10 mM sodium phosphate, pH 7.2, 150 mM NaCl), embryos were devitellinized manually and stained for β-galactosidase activity. Larval tissues were dissected in PBS, fixed in 5% formaldehyde for 15 min, washed with PBS, incubated in the X-gal assay buffer overnight and mounted in 90% glycerol/PBS.

Quantitation of eye pigments

Red eye pigment was extracted from groups of 10 adult males, aged 2–5 days after eclosion, by the method of Ephrussi and Herold (1944). Values are reported as percent of wild-type (Canton S) red eye pigment with standard deviation.

Quantitation of transgenic β-galactosidase

The method of Ashburner (1989) was followed with minor modifications. Flies aged 2–6 days after eclosion were heat shocked for 1 h and recovered for 2 h. Each measurement was derived from a 50 μl extract of five homogenized flies. After endogenous β-galactosidase activities were subtracted, transgenic β-galactosidase activity was expressed as change in OD_{574}/h/ml of extract.

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References

Ashburner,M. (1989) *Drosophila: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Baker,W.K. (1967) A clonal system of differential gene activity in *Drosophila. Devel. Biol.*, **16**, 1–17.

Baker,W.K. (1968) Position-effect variegation. *Adv. Genet.*, **14**, 133–169.

Belyaeva,E.S. and Zhimulev,I.F. (1991) Cytogenetic and molecular aspects of position effect variegation in *Drosophila*. III. Continuous and discontinuous compaction of chromosomal material as a result of position effect variegation. *Chromosoma*, **100**, 453–466.

Bishop,C.P. and Jackson,C.M. (1996) Genomic imprinting of chromatin in *Drosophila melanogaster. Genetica*, in press.

Bonner,J.J., Parks,C., Parker-Thornbury,J., Martin,M.A. and Pelham,H.R.B. (1984) The use of promoter fusions in *Drosophila* genetics: isolation of mutations affecting the heat shock response. *Cell*, **37**, 979–991.

Cattanach,B.M. (1974) Position effect variegation in the mouse. *Genet. Res.*, **23**, 291–306.

Chan,C.-S., Rastelli,L. and Pirrotta,V. (1994) A *Polycomb* response element in the *Ubx* gene that determines an epigenetically inherited state of repression. *EMBO J.*, **13**, 2553–2564.

Chen,S.Y. (1948) Action de la temperature sur trois mutants a panachure *de Drosophila melanogaster*: $w^{258\text{-}18}$, w^{m5} et *z*. *Bull. Biol. France Belg.*, **82**, 114–129.

Dorer,D.R. and Henikoff,S. (1994) Expansions of transgene repeats cause heterochromatin formation and gene silencing in *Drosophila. Cell*, **77**, 993–1002.

Dorn,R., Heymann,S., Lindigkeit,R. and Reuter,G. (1986) Suppressor mutation of position-effect variegation in *Drosophila melanogaster* affecting chromatin properties. *Chromosoma*, **93**, 398–403.

Dura,J.-M. (1981) Stage dependent synthesis of heat shock induced proteins in early embryos of *Drosophila melanogaster. Mol. Gen. Genet.*, **184**, 381–385.

Eissenberg,J.C. and Hartnett,T. (1993) A heat shock-activated cDNA rescues the recessive lethality of mutations in the heterochromatin-associated protein HP1 of *Drosophila melanogaster. Mol. Gen. Genet.*, **240**, 333–338.

Eissenberg,J.C., Morris,G.D., Reuter,G. and Hartnett,T. (1992) The heterochromatin-associated protein HP-1 is an essential protein in

Drosophila with dosage-dependent effects on position-effect variegation. *Genetics*, **131**, 345–352.

Engels,W.R., Preston,C.R., Thompson,P. and Eggleston,W.B. (1986) *In situ* hybridization to *Drosophila* salivary chromosomes with biotinylated DNA probes and alkaline phosphatase. *Focus*, **8**, 6–8.

Ephrussi,B. and Herold,J.L. (1944) Studies of eye pigments of *Drosophila*: I. Methods of extraction and quantitative estimation of the pigment components. *Genetics*, **29**, 148–175.

Fauvarque,M.-O. and Dura,J.-M. (1993) Polyhomeotic regulatory sequences induce developmental regulator-dependent variegation and targeted P-element insertions in *Drosophila*. *Genes Dev.*, **7**, 1508–1520.

Fjose,A., Polito,L.C., Weber,U. and Gehring,W.J. (1984) Developmental expression of the *white* locus of *Drosophila melanogaster*. *EMBO J.*, **9**, 2087–2094.

Gatti,M. and Pimpinelli,S. (1983) Cytological and genetic analysis of the Y chromosome of *Drosophila melanogaster*. *Chromosoma*, **88**, 349–373.

Gottschling,D.E., Aparicio,O.M., Billington,B.L. and Zakian,V.A. (1990) Position effect at *S.cerevisiae* telomeres: reversible repression of PolII transcription. *Cell*, **63**, 751–762.

Gowen,J.W. and Gay,E.H. (1934) Chromosome constitution and behavior in ever-sporting and mottling in *Drosophila melanogaster*. *Genetics*, **19**, 189–208.

Grigliatti,T.A. (1991) Position-effect variegation—an assay for nonhistone chromosomal proteins and chromatin assembly and modifying factors. *Methods Cell Biol.*, **35**, 587–627.

Hartmann-Goldstein,I.J. (1967) On the relationship between heterochromatinization and variegation in *Drosophila* with special reference to temperature sensitive periods. *Genet. Res.*, **10**, 143–159.

Henikoff,S. (1981) Position-effect variegation and chromosome structure of a heat shock puff in *Drosophila*. *Chromosoma*, **83**, 381–393.

James,T.C., Eissenberg,J.C., Craig,C., Dietrich,V., Hobson,A. and Elgin,S.C.R. (1989) Distribution patterns of HP1, a heterochromatin associated nonhistone chromosomal protein of *Drosophila*. *J. Cell Biol.*, **50**, 170–180.

Karess,R.E. and Rubin,G.M. (1984) Analysis of P transposable element functions in *Drosophila*. *Cell*, **38**, 135–146.

Karpen,G.H. (1994) Position-effect variegation and the new biology of heterochromatin. *Curr. Opin. Genet. Dev.*, **4**, 281–291.

Karpen,G.H. and Spradling,A.C. (1990) Reduced DNA polytenization of a minichromosome region undergoing position-effect variegation in *Drosophila*. *Cell*, **63**, 97–107.

Kellum,R. and Schedl,P. (1991) A position-effect assay for boundaries of higher order chromatin conformation. *Cell*, **64**, 941–950.

Kornher,J.S. and Kauffman,S.A. (1986) Variegated expression of the Sgs-4 locus in *Drosophila melanogaster*. *Chromosoma*, **94**, 205–216.

Laird,C.D. (1987) Proposed mechanism of inheritance and expression of the human fragile-X syndrome of mental retardation. *Genetics*, **117**, 587–599.

Laurenson,P. and Rine,J. (1992) Silencers, silencing, and heritable transcriptional states. *Microbiol. Rev.*, **56**, 543–560.

Lefevre,G.Jr (1976) A photographic representation and interpretation of the polytene chromosomes of *Drosophila melanogaster* salivary glands. In Ashburner,M. and Novitski,E. (eds), *The Genetics and Biology of Drosophila*, Vol. 1a. Academic Press, New York, pp. 31–66.

Locke,J., Kotarski,M.A. and Tartof,K.D. (1988) Dosage dependent modifiers of position effect variegation in *Drosophila* and a mass action model that explains their effect. *Genetics*, **120**, 181–198.

Lorentz,A., Ostermann,K., Fleck,O. and Schmidt,H. (1994) Switching gene *swi6*, involved in repression of silent mating-type loci in fission yeast, encodes a homologue of chromatin-associated proteins from *Drosophila* and mammals. *Gene*, **143**, 139.

Lyon,M.F. (1992) Some milestones in the history of X-chromosome inactivation. *Annu. Rev. Genet.*, **26**, 16–28.

Michailidis,J., Murray,N.D. and Marshall Graves,J.A. (1988) A correlation between development time and variegated position effect in *Drosophila melanogaster*. *Genet. Res.*, **52**, 119–123.

Nasmyth,K. and Shore,D. (1987) Transcriptional regulation in the yeast life cycle. *Science*, **237**, 1162–1170.

Paro,R. (1990) Imprinting a determined state into the chromatin of *Drosophila*. *Trends Genet.*, **6**, 416–421.

Paro,R. and Hogness,D.S. (1991) The *Polycomb* protein shares a homologous domain with a heterochromatin-associated protein of *Drosophila*. *Proc. Natl Acad. Sci. USA*, **88**, 263–267.

Pillus,L. and Rine,J. (1989) Epigenetic inheritance of transcriptional states in *S.cerevisiae*. *Cell*, **59**, 637–647.

Pimpinelli,S. and Goday,C. (1989) Unusual kinetochores and chromatin diminution in Parascaris. *Trends Genet.*, **5**, 310–315.

Pirrotta,V. (1988) Vectors for P-mediated transformation in *Drosophila*. In Rodriguez,R.L. and Denhardt,D.T. (eds), *Vectors: A Survey of Molecular Cloning Vectors and their Uses*. Butterworths, Boston, pp. 437–456.

Pirrotta,V. and Rastelli,L. (1994) White gene expression, repressive chromatin domains and homeotic gene regulation in *Drosophila*. *BioEssays*, **16**, 549–555.

Prokovjeva-Belgovskaya,A.A. (1947) Heterochromatinization as a change of chromosome cycle. *J. Genet.*, **48**, 80–98.

Qian,S., Varjavand,B. and Pirrotta,V. (1992) Molecular analysis of the *zeste–white* interaction reveals a promoter-proximal element essential for distant enhancer–promoter communication. *Genetics*, **131**, 79–90.

Reuter,G. and Spierer,P. (1992) Position effect variegation and chromatin proteins. *BioEssays*, **14**, 605–612.

Reuter,G., Werner,W. and Hoffmann,H.J. (1982) Mutants affecting position-effect heterochromatinization in *Drosophila melanogaster*. *Chromosoma*, **85**, 539–551.

Rigby,P.W.J., Dieckmann,M., Rhodes,C. and Berg,P. (1977) Labeling deoxyribonucleic acids to high specific activity *in vitro* by nick translation with DNA polymerase I. *J. Mol. Biol.*, **113**, 237–251.

Robertson,H.M, Preston,C.R., Phillis,R.W, Johnson-Schlitz,D., Benz,W.K. and Engels,W.R. (1988) A stable genomic source of P element transposase in *Drosophila melanogaster*. *Genetics*, **118**, 461–470.

Saunders,W.S. *et al.* (1993) Molecular cloning of a human homologue of *Drosophila* heterochromatin protein HP1 using anti-centromere autoantibodies with anti-chromo specificity. *J. Cell Sci.*, **104**, 573–582.

Simon,J.A., Sutton,C.A., Lobell,R.B., Glaser,R.L. and Lis,J.T. (1985) Determinants of heat shock-induced chromosome puffing. *Cell*, **40**, 805–817.

Singh,P.B. (1994) Molecular mechanisms of cellular determination: their relation to chromatin structure and parental imprinting. *J. Cell Sci.*, **107**, 2653–2668.

Singh,P.B., Miller,J.R., Pearce,J., Kothary,R., Burton,R.D., Paro,R., James,T.C. and Gaunt,S.J. (1991) A sequence motif found in a *Drosophila* heterochromatin protein is conserved in animals and plants. *Nucleic Acids Res.*, **19**, 789–794.

Spofford,J.B. (1976) Position-effect variegation in *Drosophila*. In Ashburner,M. and Novitski,E. (eds), *The Genetics and Biology of Drosophila*, Vol. 1c. Academic Press, New York, pp. 955–1018.

Spradling,A.C. (1986) P element-mediated transformation. In Roberts,D.B. (ed.), *Drosophila—A Practical Approach*. IRL Press, Oxford, pp. 175–198.

Spradling,A.C. (1993) Position effect variegation and genomic instability. *Cold Spring Harbor Symp. Quant. Biol.*, **58**, 585–596.

Steller,H. and Pirrotta,V. (1985) Expression of the *Drosophila white* gene under the control of the *hsp70* heat shock promoter. *EMBO J.*, **4**, 3765–3772.

Summers,K.M., Howells,A.J. and Pyoliotis,N.A. (1982) Biology of eye pigmentation in insects. *Adv. Insect Physiol.*, **16**, 119–166.

Tam,P.P.L., Zhou,S.X. and Tan,S.-S. (1994) X-chromosome activity of the mouse primordial germ cells revealed by the expression of an X-linked *lacZ* transgene. *Development*, **120**, 2925–2932.

Tan,S.-S., Williams,E.A. and Tam,P.P.L. (1993) X-chromosome inactivation occurs at different times in different tissues of the postimplantation mouse embryos. *Nature Genetics*, **3**, 170–174.

Tartof,K.D. and Bremer,M. (1990) Mechanisms for the construction and developmental control of heterochromatin formation and imprinted chromosome domains. *Development*, Suppl., 35–45.

Tartof,K.D., Hobbs,C. and Jones,M. (1984) A structural basis for variegating position effects. *Cell*, **37**, 869–878.

Thompson,J.S., Hecht,A. and Grunstein,M. (1993) Histones and the regulation of heterochromatin in yeast. *Cold Spring Harbor Symp. Quant. Biol.*, **58**, 247–256.

Urieli-Shoval,S., Gruenbaum,Y., Sedat,J. and Razin,A. (1982) The absence of detectable methylated bases in *Drosophila melanogaster* DNA. *FEBS Lett.*, **146**, 148–152.

Wallrath,L.L. and Elgin,S.C.R. (1995) Position effect variegation in *Drosophila* is associated with an altered chromatin structure. *Genes Dev.*, **9**, 1263–1277.

Wareham,K.A., Lyon,M.F., Glenister,P.H. and Williams,E.D. (1987) Age related reactivation of an X-linked gene. *Nature*, **327**, 725–727.

Wolff,T. and Ready,D.F. (1993) Pattern formation in the *Drosophila* retina. In Bate,M. and Arias,A.M. (eds), *The Development of*

Drosophila melanogaster. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 1277–1325.

Wustmann,G., Szidonya,J., Taubert,H. and Reuter,G. (1989) The genetics of position-effect variegation modifying loci in *Drosophila melanogaster*. *Mol. Gen. Genet.*, **217**, 520–527.

Zhimulev,I.F., Belyaeva,E.S., Bgatov,A.V., Baricheva,E.M. and Vlassova,I.E. (1988) Cytogenetic and molecular aspects of position effect variegation in *Drosophila melanogaster*. II. Peculiarities of morphology and genetic activity of the 2B region in the *T(1;2)dor*^{var7} chromosome in males. *Chromosoma*, **96**, 255–261.

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