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Time out: developmental regulation of heterochromatic silencing in *Drosophila*

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Abstract. Transcriptional silencing by heterochromatin represents a model for developmental gene silencing. Current models of heterochromatin envision DNA-protein complexes that prevent access by euchromatic transcription factors. Here, we summarize the evidence that heterochromatin acts at the chromatin level to silence genes and the status of current models of heterochromatin silencing, and we highlight some recent progress in understanding the composition and regulation of heterochromatin in *Drosophila*.

Key words. Heterochromatin; position-effect variegation; *Drosophila*; epigenetic; silencing; development.

Von Koenigswald went to a basin of water, meaning to wash his hands. 'When I turned to look at him,' he told me, his hands poised over the water, 'he was dead – as hard as a statue, just as you see him. I brushed my fingers over his lips. They looked so peculiar.' He put his hands into the water. 'What chemical could possibly . . .' The question trailed off.
Von Koenigswald raised his hands, and the water in the basin came with them. It was no longer water, but a hemisphere of *ice-nine*.
Von Koenigswald touched the tip of his tongue to the blue-white mystery.
Frost bloomed on his lips. He froze solid, tottered, and crashed.
. . . At last I had seen *ice-nine*!

Kurt Vonnegut
"Cat's Cradle"

Introduction

Gene expression in eukaryotes relies upon the accessibility of the DNA template to RNA polymerase and a variety of regulatory proteins. This accessibility can be controlled by the chromatin structure at a given locus. Thus, a gene can remain silent in a given cell independent of the availability of all of the requisite DNA binding proteins. Importantly, this silenced state can be propagated faithfully through mitosis and meiosis. As the underlying DNA sequences being transmitted in each instance are the same regardless of the activity state of the gene, the mechanisms by which cell memory and genomic imprinting occur are 'epigenetic' and are believed to involve the assembly of special chromatin structures [1].

The chromatin structure of genomic DNA correlates with its transcriptional potential. Differential chromatin packaging in eukaryotic cells was first recognized by Heitz [2], who coined the term 'heterochromatin' to designate the fraction of the nuclear material that remained condensed after mitosis. In general, heterochromatin is rich in repetitive DNA [3], poor in classical genes [4], late replicating in the cell cycle [5], and rich in hypoacetylated core histone [6, 7]. Genetic evidence suggests that heterochromatin interferes with expression of normally euchromatic genes: for example, all but one X chromosome in mammalian cells appear to be transcriptionally inactive and heterochromatic [8], and chro-

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mosome rearrangements placing normally euchromatic genes adjacent to a heterochromatic breakpoint usually result in the inactivation of the euchromatic loci ('heterochromatic position effect') [9]. The molecular basis for the cis-inactivation by heterochromatic position effect is unclear. Possibly, the distinctive structure of heterochromatin is sufficient to occlude sites of transcription factor binding necessary for gene activation.

Heterochromatic position-effect variegation (PEV) silencing in *Drosophila* has been reviewed numerous times in the past decade [10–19]. This recent enthusiasm for a phenomenon first described over 65 years ago [20] is owing to two factors: the emergence of new tools and materials to test long-standing hypotheses about the mechanism of heterochromatic silencing, and the renewed interest in epigenetic phenomena in fungi [21, 22], plants [23] and mammals [24]. Our goals in this review are to summarize the evidence that heterochromatin acts at the chromatin level to silence genes and the status of current models of heterochromatin silencing, and to highlight some recent progress in understanding the composition and regulation of silencing by heterochromatin in *Drosophila*.

What is the evidence that heterochromatic silencing is mediated by chromatin?

Gene silencing by heterochromatin occurs at the transcriptional level [25]. The most direct evidence that transcriptional inactivation by heterochromatin is accompanied by a modification of chromatin structure is cytological. In flies exhibiting phenotypic variegation in gene expression, the cytological locus containing the gene (in polytene chromosomes) is seen to take on the distinctive densely staining, attenuated appearance of heterochromatin in a subset of cells. Genetic and environmental factors that increase or decrease the proportion of cells experiencing phenotypic silencing also modify the frequency with which the chromosomal region containing the locus shows the cytological appearance of heterochromatin [26, 27]. The conclusion that such cytological variegation reflects a change in the composition and conformation of the chromatin at the silenced locus is buttressed by genetic, histological, pharmacological, and biochemical evidence:

- The proportion of cells in which a gene is silenced depends upon histone gene dosage as well as the dosage of several loci that encode nonhistone chromosomal proteins or chromatin protein modifiers [28].
- The normally heterochromatin-associated protein HP1 is found at the silenced locus [29].
- *Drosophila* food containing the histone deacetylase inhibitor sodium butyrate relieves heterochromatic silencing [30].
- The DNA at a variegating locus is more resistant to nuclease attack than the same sequence at a euchromatic locus [31].

Taken together, these observations suggest that proximity to heterochromatin modifies the chromatin structure of otherwise euchromatic genes in a way that interferes with normal transcription.

Current models

In 1988, Tartof and his colleagues proposed a mass action model to explain the genetic behaviour of variegating heterochromatic position effect [32]. Figure 1A shows a cartoon representation of this model. Briefly, heterochromatin is considered to be a complex of DNA and a set of chromosomal proteins that establish an equilibrium between a disassembled (or at least nonchromosomal) state and an assembled state on the chromatin fibre. The assembly of a functional subunit of heterochromatin depends upon the proper stoichiometry of several distinct proteins (cartooned as circles and triangles in figure 1), and is initiated at one or several initiation points within chromosomal regions that normally form heterochromatin. Once initiated, a heterochromatin complex may spread cooperatively in *cis* down the chromatin fibre as a continuous array until some boundary, termination or 'stop' signal is reached. In chromosomal rearrangements (such as the inversion in figure 1), the 'stop' signal is removed to a distal position, and placed in the path of the spreading heterochromatin is a euchromatic reporter gene (symbolized by the light bulb in figure 1). In some cells, it is imagined that the concentration of heterochromatin proteins is sufficiently high that the assembled complex invades the neighbouring euchromatin across the breakpoint, resulting in heterochromatic silencing of the reporter gene (bottom of figure 1). In other cells of the same tissue, slightly lower concentrations of one or several heterochromatin proteins result in less heterochromatin assembly/spreading, and the reporter gene remains euchromatic. The chief features of the mass-action model are that it explains (i) the fact that mutations in several genes can each exert dosage-dependent effects on silencing and (ii) the extreme sensitivity of silencing to relatively modest changes in gene dosage.

Recently, Henikoff [33] pointed out that the mass action model predicts a silencing complex that is extremely unstable and unrealistically sensitive to changes in cell/nuclear volume. Such instability would be incompatible with the developmental stability of

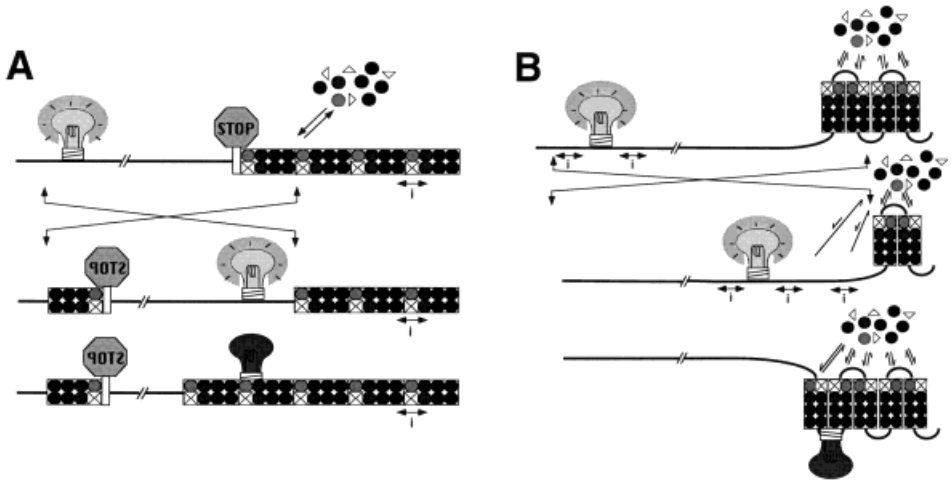

Figure 1. Two models for heterochromatic position effect. (*A*). The mass action spreading model. Heterochromatin-associated proteins (represented by the circles and triangles) equilibrate between a dissociated state and an assembled state on the chromosome. In a normal chromosome (top chromosome) heterochromatic protein complexes initiate assembly at one or several initiation points ('i') and complexes spread cooperatively in *cis* until a termination ('STOP') signal is reached. Euchromatic genes normally located beyond the farthest reaches of heterochromatic spreading will be activated normally (illuminated light bulb). A chromosomal inversion removes the termination signal, and places the normally euchromatic genes near the proximal inversion breakpoint, in the path of spreading heterochromatin. In cells carrying the variegating rearrangement and expressing somewhat lower amounts of heterochromatin protein (middle chromosome), silencing complex spreading falls short of euchromatic genes, which are activated normally. In cells expressing higher levels of heterochromatin protein (bottom chromosome), silencing complexes invade neighbouring euchromatic sequences, forming heterochromatin over euchromatic genes, thus inactivating them (darkened bulb). The proportions of cells experiencing the greater or lesser degree of spreading are reflected in the degree of variegation or mosaicism for expression of the euchromatic gene. (*B*) Pairing-dependent silencing model. In a normal chromosome (top chromosome), heterochromatin proteins assemble at mutiple target sites, which pair with one another to form a compartment in the nucleus. Concentration of heterochromatin proteins is highest in this compartment. In a chromosome inversion, a normally euchromatic gene is brought into proximity with the heterochromatin compartment. In some cells carrying the variegating rearrangement, cryptic heterochromatin binding sites ('i') fail to nucleate heterochromatin complexes, and euchromatic genes are activated normally (middle chromosome). This failure of heterochromatin assembly could be because these cryptic binding sites have lower affinity or because they are present at a lower density per unit chromosome length. In other cells, heterochromatin protein assembly at heterochromatin-binding sites flanking the euchromatic gene results in this region being drawn into the heterochromatic compartment, and consequently silencing of the euchromatic gene.

silencing. Henikoff suggested that a multiple binding site model, proposed by Pirrotta and Rastelli [34] to account for dosage-dependent effects on Polycomb-dependent silencing, would bypass the problematic aspects of the mass-action model and still account for dosage-dependent effects. Instead of a single heterochromatic complex, the multiple binding site model invokes multiple complexes which are in turn capable of looping in *cis* to form silencing aggregates. While the multiple binding site model of heterochromatin assembly is compatible with existing data, our recent finding that variegation levels in adult tissues are a consequence of a developmentally late relaxation of silencing (see below) [35] means that the conditions of extreme instability necessitated by the Tartof model only need apply to a brief developmental period; for the imaginal precursors, this period appears to be in mid-third instar (B. Y. Lu, J. Ma and J. C. Eissenberg, unpublished data). Thus, the effects of developmental changes in nuclear or cellular volume would be minimized.

A pairing-driven compartmentalization model of heterochromatin assembly was proposed by Wakimoto and Hearn [36], whereby exchange of heterochromatic proteins in *trans* can occur as a result of the tendency of regions of heterochromatin to aggregate or pair [37]. Higher local concentration of silencing proteins in heterochromatin-rich nuclear compartments could promote silencing of euchromatic genes dragged to such compartments because of their abnormal placement next to a heterochromatic breakpoint. A cartoon depicting this model is shown in figure 1B. The important distinction between this model and the *cis*-spreading model is that the pairing-driven compartmentalization

does not require the silencing of all genes lying between the silenced reporter and other blocks of heterochromatin. Rather, naturally occurring binding sites for heterochromatin complexes near most euchromatic genes (which are not used when the gene is in its normal chromosomal position) nucleate heterochromatin complexes in some cells when the gene is positioned abnormally close to heterochromatin in a rearrangement.

Structural proteins of heterochromatin in *Drosophila*

A fruitful genetic strategy to identify candidates for structural proteins of heterochromatin has been the use of mutagenesis screens for dominant suppressors of PEV [38, 39]. In cases where such suppressors have been cloned and characterized, several have proven to encode heterochromatin-associated proteins. Among the best characterized of these are:

– Heterochromatin protein 1 (HP1). The HP1 protein was cloned using monoclonal antibodies generated to a fraction of tightly bound nonhistone chromosomal proteins [40, 41]. Subsequently, mutations were found in the HP1 transcription unit in each of five independent allelic suppressors of PEV [42–44] (J. C. Eissenberg, unpublished data) and a heat shock-driven HP1 complementary DNA (cDNA) was shown to complement both dominant suppression of silencing and recessive lethality associated with these alleles [43, 45]. HP1-like proteins have been identified in organisms as diverse as yeast [46] and human [47–49]. This level of evolutionary conservation suggests a fundamental role in nuclear organization. All HP1-like proteins have in common two somewhat diverged copies of a motif termed the 'chromo domain' [50, 51]. In the *Drosophila* HP1, each chromo domain has been shown to have heterochromatin targeting activity [52, 53], and mutations in the N-terminal chromo domain abolish silencing activity [44]. The chromo domain motif has also been found in a variety of otherwise unrelated chromosomal proteins [50, 51]. So far, the data suggest that the chromosome-binding activity associated with this motif is mediated by protein-protein interactions, rather than DNA-protein interactions [44, 53].

– Suppressor of variegation 3-7 [SU(VAR)3-7]: Like HP1, SU(VAR)3-7 is a dosage-dependent modifier of PEV [54]. The protein contains seven widely spaced zinc-finger motifs, making it an attractive candidate for a DNA-binding component of heterochromatin complexes. Interestingly, SU(VAR)3-7 colocalizes with HP1 on interphase chromosomes, and the two proteins coimmunoprecipitate from nuclear extracts [55]. Although such results cannot distinguish between direct and indirect interactions, they support the view that HP1 and SU(VAR)3-7 cohabit a common complex in heterochromatin.

– Suppressor of variegation 3-9 [SU(VAR)3-9]: Initially identified as a haplo-insufficient suppressor of PEV, the SU(VAR)3-9 protein also contains the chromo domain [56]. The SU(VAR)3-9 protein is heterochromatin-associated, and shows a strong interaction with HP1 by the yeast two-hybrid protein assay (G. Reuter, personal communication), making it another strong candidate for a component of heterochromatin complexes.

In addition to these nonhistone chromosomal proteins, the histone H4 isoform acetylated at lysine 12 is enriched in heterochromatin [57]. Indeed, post-translational modification of the nonhistone structural proteins of heterochromatin may also modulate heterochromatic silencing. Indirect evidence for this is the finding that mutation of the *Su(var)3-6* locus, a type I protein phosphatase and dominant suppressor of PEV [58], is epistatic to the enhancement of PEV caused by an extra copy of the *Su(var)3-7* gene [15]. Additionally, the correlation of HP1 hyperphosphorylation with heterochromatin assembly suggests a role for phosphorylation of this structural protein in silencing [59].

DNA structure and the assembly of heterochromatin

While heterochromatin is a nearly universal feature of interphase eukaryotic nuclei, no single conserved sequence has yet been identified that is common to the heterochromatic regions of all eukaryotes. Instead, the common DNA property shared by constitutive heterochromatin is enrichment for repeat sequence DNA. The possible significance of repeated sequences to the initiation of heterochromatin was dramatically highlighted by Dorer and Henikoff, who showed that short arrays of a mini-*white* gene were sufficient to initiate ectopic heterochromatin at euchromatic chromosome sites [60]. Perhaps, then, local homologous pairing can drive heterochromatin formation, and since pericentric DNA is highly enriched for repetitious DNA, these regions are naturally heterochromatic. Clearly, not all repeat arrays are equally efficient in forming silencing heterochromatin: in their natural locations, the rDNA (ribosomal DNA) and histone gene cluster consist of extended arrays of highly homologous repeats, and artificially created arrays of the *brown* gene [61] and 5S genes [62] appear to lack the ability to form silencing heterochromatin at several euchromatic sites. In all likelihood, the proteins that bind to different sequences will influence the efficiency or stability of pairing.

Regulation of heterochromatic silencing during development

One classical example of heterochromatic PEV in *Drosophila* is the mosaic red-and-white patches of an adult eye that occurs when the normally euchromatic

white gene, essential for the production of red eye pigments, is juxtaposed to heterochromatin. As a result, *white* becomes silenced by the adjacent heterochromatin in some cells but remains active in others, leading to variegated patterns ranging from a fine-grained 'salt-and-pepper' mosaicism to a sectored 'large-patch' mosaicism. Such an array of variegated patterns raises questions as to how the mosaic state of *white* expression is determined during development. Can variegated silencing be established at any of several developmental times, or is it initiated at one particular developmental stage? Also, is the variegated state acquired by inactivating *white* in a subset of the cells or by relieving silencing of *white* in a subset of cells containing an initially silenced *white* gene? Answers to these questions will provide insight into the developmental regulation of gene silencing by heterochromatin.

To track the developmental course of heterochromatic PEV, the direct approach is to monitor the activity of the variegating reporter gene through time and detect changes in the degree of silencing. However, the expression of most PEV reporter genes, such as *white*, is spatially and temporally confined. For example, in eye development, *white* is not transcribed until the pupal stage so that the chromosomal state at the *white* locus prior to the pupal stage cannot be determined by a direct assay for *white* activity. Given such a limitation, two indirect strategies have been employed. One involves a clonal analysis of the variegated patches through somatic recombination, and the other focuses on deriving temperature-sensitive periods (TSP) of PEV.

Findings from somatic recombination analysis and TSPs

In eye development, eye precursor cells remain mitotically active until the beginning of terminal differentiation shortly before the pupal stage. It is, therefore, often assumed that the determinative event for large-patch variegation must occur earlier than that for salt-and-pepper. To estimate the timing for large-patch variegation, somatic recombination of eye marker genes was induced at various developmental times to generate distinguishable recombinant patches on the adult eye. Technically, this involves irradiating flies heterozygous for wild-type and null alleles of an eye marker to induce recombination between the marker and the centromere. Daughter cells in the mitosis following irradiation are genetically marked in this way, and cells inheriting two copies of the null allele are phenotypically distinguishable. Since all descendants of such genetically marked cells are similarly marked, the number of similarly marked cells indicates the number of mitoses (deducting for any cell death) between the time of the first mitosis following irradiation and the time at which the marked cells are scored. When the sizes of genetically marked recombinant patches were compared with those resulting from large-patch variegation, it was found that the patch sizes were the most similar when recombination was induced in first instar larvae, suggesting that the decision for the variegated state is made as early as this time [63].

Another effort in deciphering the developmental regulation of PEV involves TSPs, which represent developmental windows within which shifts in the rearing temperature can alter the degree of silencing. These windows presumably represent stages when the extent of heterochromatic PEV is determined. TSPs have been identified for various PEV reporter genes [9, 26, 64]. A comparison of major TSPs shows that they usually occur (i) within the first hours (0–4 h) of embryogenesis in blastoderm embryos and (ii) around the onset of terminal differentiation of tissues that will eventually exhibit the mosaic phenotype. In some cases, the entire period from embryogenesis to terminal differentiation shows temperature sensitivity. Generally, it appears that, as long as the terminally differentiated state has not been reached, the degree of silencing is malleable by temperature fluctuations.

Although these findings offer clues to when PEV may be developmentally regulated, they do have a few shortcomings. For instance, in somatic recombination analysis, a pigmented patch can be seen to partially overlap a recombinant patch, indicating that the decision for variegated expression occurs independently of clonal origin. If so, it is possible that the variegated large-patch state is not established by the first larval instar. Also, prolonged temperature manipulations in TSP experiments may perturb not only the regulation of heterochromatic PEV but also other temperature-sensitive processes, which can subsequently affect the degree of silencing.

***Hsp70* as an in vivo probe of heterochromatic silencing**

To generate a PEV reporter gene that can be directly assayed at any time in development, we devised a *lacZ* reporter gene driven by the *Drosophila Hsp70* promoter (*HS-lacZ*) and generated variegating chromosomal rearrangements of this gene (fig. 2) [35]. One rearrangement, *In(3L)BL1*, exhibits salt-and-pepper variegation, whereas another, *Tp(3;Y)BL2*, displays large-patch mosaicism in the adult eye. *HS-lacZ* represents an ideal reporter gene because it can be induced in virtually all cells throughout development with a transient heat shock, and the *lacZ* product is stable and easily assayed in situ by enzymatic or immunological assay. Further experiments also showed that the induction of *HS-lacZ* does not antagonize heterochromatic silencing, validating *Hsp70* as a specific in vivo probe of heterochromatic PEV (see below).

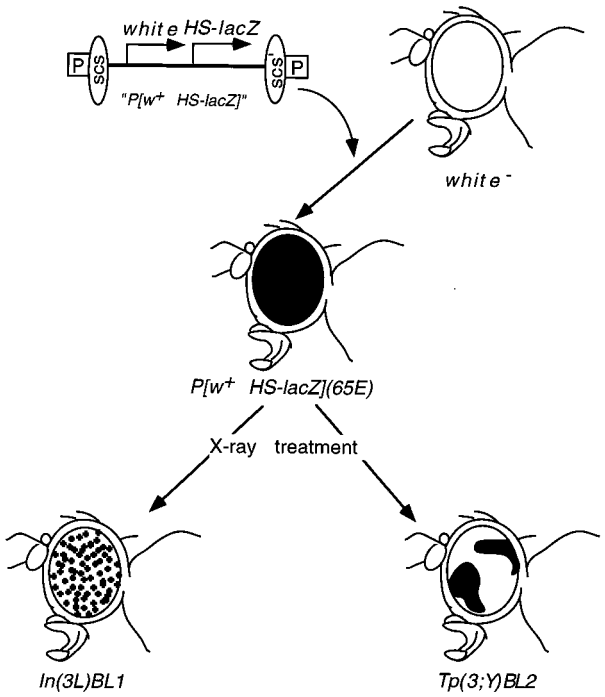

Figure 2. Generation and the phenotypes of fly stocks exhibiting heterochromatic PEV. A P-element reporter gene construct, *P[w⁺HS-lacZ]*, containing P element termini ('P'), scs and scs′ elements (boundary elements against euchromatic position-effect), a *white* gene driven by an eye-specific enhancer ('*white*'), and a *Hsp70*-driven *lacZ* ('*HS-lacZ*'), was transformed into white-eyed flies. A euchromatic insertion, *P[w⁺HS-lacZ](65E)*, shows uniform reporter gene expression. Two fly stocks, *In(3L)BL1* and *Tp(3;Y)BL2*, that display heterochromatic PEV of the reporter gene construct were generated by X ray-induced chromosomal rearrangements. Mosaic patterns are salt-and-pepper in *In(3L)BL1* and large-patch in *Tp(3;Y)BL2*.

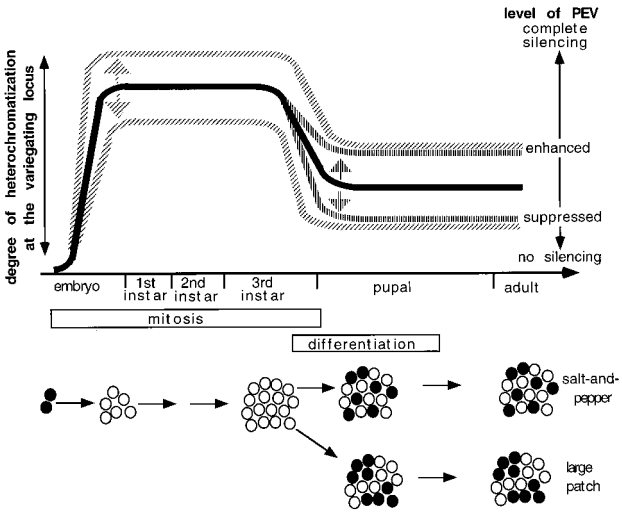

Figure 3. A model on the developmental regulation of heterochromatic PEV. The course of heterochromatic PEV in the development of adult structures is shown here. Top: In the *Drosophila* life cycle (horizontal axis in the center), the theoretical degree of heterochromatization at the variegating locus (left vertical axis) and the corresponding level in variegation (right vertical axis) is shown (dark line). The hatched arrows indicate times in which PEV modifiers modulate the degree of silencing (diagonal hatches: during the maturation of heterochromatin; vertical hatches: during terminal differentiation). The results of these modifications are displayed as hatched lines. Bottom: Mitosis and differentiation of cells in the eye-specific lineage are shown. Filled circles represent cells with a nonsilenced variegating reporter gene locus, while open circles represent those with a silenced locus. After extensive silencing is established in embryogenesis, cells undergo terminal differentiation and variegated reporter gene derepression toward the end of the third larval instar, establishing various types of mosaic patterns.

By assaying *lacZ* expression at various times of eye development in *In(3L)BL1* and *Tp(3;Y)BL2* (B. Y. Lu, J. Ma and J. C. Eissenberg, unpublished data), we found that *HS-lacZ* in the eye precursor cells is silenced as early as mid-embryogenesis. Extensive silencing is maintained until the onset of terminal differentiation in late third larval instar, when variegated relaxation of silencing occurs and gives rise to the mosaic phenotypes seen in adults. These findings argue that the mosaic state in heterochromatic PEV becomes manifest only after the beginning of terminal differentiation. To test if this hypothesis also holds in the development of other tissues, we followed the development of wings and legs and obtained similar results.

How early is heterochromatic PEV established? This question was also answered with our variegating *HS-lacZ*. We showed that in early syncytial blastoderm embryos, *HS-lacZ* is uniformly active in both *In(3)BL1* and *Tp(3;Y)BL2*. However, as the embryo cellularizes and begins to gastrulate, variegated silencing becomes detectable, signifying the functional maturation of heterochromatin.

A model for the developmental course of heterochromatic PEV

The use of *HS-lacZ* as an in vivo probe of heterochromatic silencing has uncovered two developmental stages in which silencing by heterochromatin is modified: (i) the establishment phase in post-blastoderm embryos and (ii) the relaxation phase in terminal differentiation (fig. 3). The establishment of variegated silencing in gastrulating embryos coincides with the maturation of the chromosome architecture: processes such as homologous chromosome pairing, condensation of heterochromatic regions and the delayed replication of heterochromatic sequences are not completed until this time [65–67]. On the other hand, TSPs spanning the blastoderm stage suggests that the process of heterochromatin maturation is temperature-sensitive, allow-

ing temperature shifts to perturb the extent of heterochromatinization.

Similar to models proposed for the developmental regulation of epigenetic silencing in mammalian systems [68], our results showed that the level of heterochromatic PEV in *Drosophila* remains stable throughout development except for brief periods within which the extent of silencing is modified according to the availability of factors that either promote or suppress silencing, such as the genetic PEV modifiers (see below). In the development of adult structures such as the eye, the critical period corresponds to the onset of terminal differentiation, when silencing becomes reversible. This also accounts for those TSPs that coincide with the terminal differentiation of tissues expressing the PEV reporter gene. The exact mechanism for such developmentally programmed modulation of silencing remains unknown, but our preliminary data suggest that ecdysone, the major growth and molting hormone in *Drosophila*, may function upstream of this pathway (B. Y. Lu, J. Ma and J. C. Eissenberg, unpublished data).

Interestingly, our findings also indicate that the variegated phenotypes for both large-patch and salt-and-pepper variegation arise at the same time, contrary to the assumption that large-patch variegation necessitates a much earlier determinative event. Further studies are necessary to conclude whether large-patch variegation stems from a regional determinative event at the onset of differentiation or reflects an earlier mitotically maintained cryptic decision that only becomes manifest with terminal differentiation. One approach may involve a revisit to the somatic analysis, but the emphasis should be on whether the recombinant and variegated patches consistently lie within one another rather than a comparison of their absolute sizes.

Possible role of genetic PEV modifiers

When in development do genetic PEV modifiers affect the level of heterochromatic PEV? Do they participate in (i) the functional and physical maturation of heterochromatin in blastoderm embryos, (ii) the maintenance of silencing or (iii) switches in silencing during terminal differentiation? Information on this has been scarce, because until now PEV reporter genes are not continuously inducible throughout development. However, it appears that some PEV modifiers function in determining the degree of variegated expression during terminal differentiation (fig. 3). By examining the effects of some PEV enhancers on *y* (*yellow*) variegation in the adult wing, Tartof and Bremer [64] suggested that the enhancers decrease *y* expression in a nonclonal fashion, indicating that they function very late in development, at a time when mitosis ceases and terminal differentiation begins. They also noted that, although some enhancers examined are known to be expressed early in development, they apparently do not perturb the stability of silencing until differentiation. Similarly, the well-characterized HP1 protein also appears to modulate PEV at terminal differentiation. Eissenberg and Hartnett [45] reported that ectopic HP1 must be induced before the end of the third instar (the time for terminal eye differentiation) to exert a significant enhancement of *white* variegation in the eye. We also have a preliminary finding that, in third instar larval eye imaginal discs, PEV suppressors, including those allelic to HP1, dramatically increase the degree of derepression of *HS-lacZ* in differentiating cells but have little or no effect on silencing in undifferentiated cells (B. Y. Lu, G. Reuter and J. C. Eissenberg, unpublished data).

It is likely that at least some genetic PEV modifiers regulate heterochromatin not only at the onset of terminal differentiation but also during its physical and functional maturation in blastoderm embryos (fig. 3). Possible candidates that play a role in early embryogenesis are those that have maternal effects on PEV. In this case, the maternally expressed modifier proteins participate in the assembly of heterochromatin and thereby affect the degree of heterochromatization around the variegating locus. Further experiments are needed to determine if heterochromatic PEV in postblastoderm embryos are subject to the effects of these modifiers.

Drosophila heterochromatic PEV is not sensitive to the cell cycle or promoter strength

As mentioned previously, epigenetic silencing in yeast and mammalian cells has been extensively studied. In telomeric PEV and repression at the silent mating-type loci in yeast, switches in the silent state respond to or depend on passage through the S phase [69–71]. Also, promoter activity has been shown to relieve silencing and suppress PEV [72, 73]. Heterochromatic silencing in *Drosophila*, on the other hand, appears to be insensitive to these factors. By exploiting the *HS-lacZ* reporter gene, we have tested the role of mitosis in the establishment and maintenance of silencing in two ways:

1) Silencing in variegating embryos mitotically blocked (by zygotic *string* mutation) at the point we have determined that silencing begins was compared to normally mitosing embryos. By the end of germ band extension, several hours after the mitotic block, mutant and mitotically normal embryos showed levels of silencing that were phenotypically indistinguishable (B. Y. Lu, J. Ma and J. C. Eissenberg, unpublished data). Similarly, blocking the final mitosis before the onset of terminal differentiation in the compound eye (by expressing human p21 under a *glass* enhancer) resulted in levels of variegated silencing indistinguishable from that seen in cells in which the final mitosis proceeded normally (B. Y. Lu, J. Ma and J. C. Eissenberg, unpublished data). These results suggest that mitotic activities are dispens-

able in both the embryonic establishment of heterochromatic PEV and the variegated relaxation of silencing in terminal eye differentiation.

2) A supernumerary mitosis was induced before the onset of terminal differentiation in eye precursor cells (by inducing ectopic cyclin E expression); again, silencing was neither stabilized nor antagonized by an additional mitotic cycle (B. Y. Lu, J. Ma and J. C. Eissenberg, unpublished data). Thus, ectopic S-phase entry and mitoses are insufficient to counteract silencing in nondifferentiated eye precursor cells.

In addition, we also showed that, even though *Hsp70* in our *HS-lacZ* reporter gene represents one of the strongest promoters known in *Drosophila*, it is unable to suppress silencing, as illustrated by the concordant expression of *HS-lacZ* and *white* (driven by a much weaker promoter), when the two genes are positioned in tandem at a variegating locus (fig. 4).

Future problems

Both the cis-assembly and pairing-driven compartment models provide ways of thinking about how different chromosomal positions cause the same DNA sequence to acquire different protein compositions and different transcriptional potential, but neither model answers important mechanistic questions: What are the structural constraints of heterochromatin that impose silencing on virtually any gene that comes under its influence? By what mechanism does heterochromatin act to block gene transcription?

Future progress in understanding the mechanism of heterochromatin silencing will depend on the development of an assay for heterochromatin formation. Assays for euchromatic nucleosome assembly (proper spacing, protein:DNA stoichiometry) and DNA-binding protein specificity for euchromatic transcription factors have been essential for the current progress in euchromatic regulatory mechanisms, and corresponding assays are still absent for heterochromatin.

The binding sites for heterochromatin-associated proteins at silenced loci are unknown. Are heterochromatin protein complexes bound uniformly throughout silent domains? Or are discreet binding sites used which, in turn, aggregate so as to involve the DNA in regions between binding sites? Cross-linking studies analogous to those used to map binding domains of Polycomb silencing complexes in the *Bithorax* complex [74, 75] might be used to test whether heterochromatin complex formation is continuous or discontinuous. Synthetic constructs with binding sites for T7 RNA polymerase, for example, can be tested in variegating rearrangements for in vivo accessibility; analogous experiments with a T7 promoter in the *Bithorax* complex found the promoter active in segments where Polycomb-dependent silencing blocks the activity of eukaryotic promoters [76].

Finally, it will be necessary to identify the protein-protein and protein-DNA contacts among structural heterochromatin proteins that give rise to silencing complexes. Such interactions include those involving core and/or linker histones.

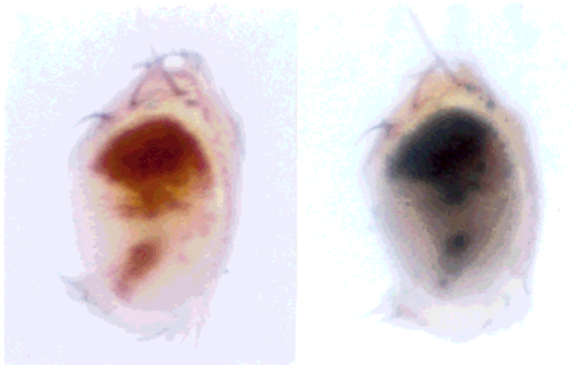

Figure 4. Concordant expression of *white* and *HS-lacZ*. *white* expression (red eye pigmentation, left panel) and inducible *HS-lacZ* activity (enzymatic X-gal staining, right panel) of the same *Tp(3;Y)BL2* eye are shown. This correspondence in the expression of two genes at the same locus shows that heterochromatic silencing is insensitive to promoter differences.

Summary

Heterochromatic PEV is a paradigm for mechanisms of mitotically heritable gene silencing. In several cases, genes that act to modify heterochromatic silencing have been shown to encode chromosomal proteins or their modifiers. In *Drosophila*, heterochromatic silencing is initiated by the onset of gastrulation and is maintained in the mitotically active, undifferentiated adult precursors until the onset of differentiation, when silencing is relaxed in a subset of cells. Thus, the variegated state seen for such terminal differentiation markers such as *white* is acquired as a result of the extent of heterochromatic relaxation occurring as a concomitant of differentiation late in development. The establishment and relaxation phases of silencing appear to be subject to developmental regulation, but the mechanism of this regulation is not influenced by cell cycle or the strength of the silenced promoter. A profitable focus of future studies would be the composition of heterochromatin, the nature of the protein-DNA and protein-protein interactions governing silencing, and the regulation of these interactions.

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