Developmental regulation of heterochromatin-mediated gene silencing in

*Drosophila*

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Accepted 7 April; published on WWW 19 May 1998

SUMMARY

The roles of differentiation, mitotic activity and intrinsic promoter strength in the maintenance of heterochromatic silencing were investigated during development using an inducible lacZ gene as an in vivo probe. Heterochromatic silencing is initiated at the onset of gastrulation, approximately 1 hour after heterochromatin is first visible cytologically. A high degree of silencing is maintained in the mitotically active imaginal cells from mid-embryogenesis until early third instar larval stage, and extensive relaxation of silencing is tightly associated with the onset of differentiation. Relaxation of silencing can be triggered in vitro by ecdysone. In contrast, timing and extent of silencing at both the initiation and relaxation stages are insensitive to changes in cell cycle activity, and intrinsic promoter strength also does not influence the extent of silencing by heterochromatin. These data suggest that the silencing activity of heterochromatin is developmentally programmed.

Key words: Epigenetic silencing, Heterochromatin, Position effect, *Drosophila*

INTRODUCTION

Mitotically heritable gene silencing that occurs without changes in the underlying DNA sequence is termed ‘epigenetic silencing’. One form of epigenetic silencing is observed when genes are silenced by being positioned in specific chromosomal regions (position effects). Well-described examples of epigenetic silencing associated with specific chromosomal regions include X chromosome inactivation in mammalian females (Lyon, 1961), telomeric and homothallic mating-type silencing in budding yeast (Pillus and Grunstein, 1995), centromeric position effects in fission yeast (Allshire et al., 1994) and heterochromatic position-effect variegation (PEV) in *Drosophila* (Spofford, 1976).

Position-effect silencing in budding yeast is presently the best-characterized example of epigenetic silencing. Genes placed in either of the two silent mating-type cassettes HML or HMR, or within a few kilobase pairs of a telomere, become silenced by this juxtaposition. This silencing is mediated by a small number of structural proteins (the SIR proteins and core histones) as well as enzymes that modify their interactions with one another (e.g. histone acetyltransferases and histone deacetylases; see Pillus and Grunstein, 1995 and Grunstein et al., 1995 for recent reviews). Silencing appears to be the result of the formation of a distinctive chromatin structure at the silenced locus (Nasmyth, 1982; Gottschling, 1992).

Epigenetic silencing in metazoans is less well understood, despite having been studied for much longer. Here, the best-characterized example is heterochromatic PEV in *Drosophila* (for reviews see Spofford, 1976; Grigliatti, 1991; Weiler and Wakimoto, 1995). Again, silencing is believed to be the result of a distinct chromatin complex assembled at silenced loci (Wallrath and Elgin, 1995), but the composition and regulation of such complexes are only now being elucidated.

Heterochromatin-mediated silencing is developmentally regulated in mammals and *Drosophila*. X chromosome inactivation in mammalian females occurs at different times in different tissues (Tan et al., 1993). In germ cells, inactivation and reactivation of the X chromosome are triggered by local signals at different stages of development (Tam et al., 1994). Several studies in *Drosophila* (Noujadin, 1936; Baker, 1967; Spofford, 1969) have suggested a developmentally early establishment of heterochromatic silencing, probably during embryogenesis (reviewed in Spofford, 1976). Recently, we showed that in *Drosophila*, heterochromatic PEV of a heat shock-driven lacZ transgene in the adult eye is preceded by nearly complete silencing of the same marker in the undifferentiated larval imaginal disc cells containing precursors for the eye (Lu et al., 1996). This finding argues that variegation patterns in differentiated adult tissue result from a loss of silencing of the reporter gene in a subset of the cells some time during differentiation. However, the exact developmental stages at which silencing is initiated and subsequently relaxed was not determined.

The finding that heterochromatic silencing is dynamic
during development suggests that silencing is regulated. The establishment and/or maintenance of silencing has been shown in some forms of epigenetic silencing to be sensitive to the stage in the cell cycle (Miller and Nasmyth, 1984) or the nature of the silenced promoter (Lee and Gross, 1993; Walters et al., 1996). We previously reported an apparent correlation between terminal differentiation and a relaxation of heterochromatic silencing in Drosophila (Lu et al., 1996). Since the steroid hormone ecdysone regulates differentiation in Drosophila, and since differentiation is accompanied by exit from the cell cycle, ecdysone and/or cessation of mitotic activity are candidates for regulators of heterochromatic silencing.

In this report, we exploit variegating HS-lacZ transgenes as ubiquitously inducible markers for PEV to answer the following questions concerning the regulation of heterochromatic silencing: (1) When is heterochromatic silencing established in development? (2) How faithfully is silencing propagated in mitotically active tissue? (3) Can ecdysone promote the relaxation of heterochromatic silencing? (4) Does the time since the last cell division influence the formation of silencing heterochromatin? (5) Does intrinsic promoter strength influence sensitivity to silencing?

We show that PEV silencing begins by gastrulation, and that silencing is nearly complete among undifferentiated imaginal precursor cells by late embryogenesis. Silencing is maintained in these imaginal tissues until late third instar, when a marked relaxation of silencing occurs coincidentally with the onset of terminal differentiation. A similar relaxation of silencing can be induced in vitro with ecdysone. The establishment and subsequent relaxation of heterochromatic silencing appear to be regulated independently of mitotic activity, as the developmental course or the degree of PEV was insensitive to either blocking or adding cell cycles during developmentally critical periods. We also show that two genes with very different intrinsic promoter strengths are equally sensitive to heterochromatic silencing.

MATERIALS AND METHODS

Fly stocks and crosses

The P[w+] HS-lacZ(6SE), Int(3L)BL1 and Tp(3;Y)BL2 chromosomes were described previously (Fig. 1; Lu et al., 1996). Unless stated otherwise, these stocks were maintained as (1) Df(1)w, yw; P[w+] HS-lacZ(6SE), Sb/TM2, Ubx, (2) Df(1)w, yw; Int(3L)BL1/TM3, Ser and (3) P[w+]Tp(3;Y)BL2/ Df(1)w, yw. string refers to the t(tg) 7669 allele (Edgar and O’Farrell, 1989). From this, the stock Df(1)w, yw /Tp(3;Y)BL2; string/TM3, P[hb-lacZ] was created. string was also recombined onto the Int(3L)BL1 chromosome to generate Df(1)w, yw; Int(3L)BL1, string /TM3, P[hb-lacZ]. string embryos were identified as those without hb-lacZ expression (uniform lacZ staining in the anterior portion of the embryo).

Hsp70-DmcycE is a homozygous viable insertion on the 3rd chromosome. Df(1)w, yw; Hsp70-DmcycE females were crossed to Df(1)w, yw; Int(3L)BL1/TM6, Tb and Tp(3;Y)BL2/ Df(1)w, yw males to generate Df(1)w, yw; Int(3L)BL1/Hsp70-DmcycE and male Tp(3;Y)BL2/ Df(1)w, yw; Hsp70-DmcycE/+ larvae. Ectopic cycE expression was induced by a 30-minute heat shock at 37°C followed by a 60-minute recovery at room temperature, as described (Richardson et al., 1995).

glass-p21 contains a glass-driven p21 on the third chromosome (de Nooij and Hariharan, 1995). Df(1)w, y; glass-p21 females were crossed to Df(1)w, yw; Int(3L)BL1/TM6, Tb and Tp(3;Y)BL2/ Df(1)w, yw males to generate Df(1)w, yw; Int(3L)BL1/glass-p21 and male Tp(3;Y)BL2/Df(1)w, yw; glass-p21/+ larvae.

Enzymatic assay for lacZ expression

Tissues were incubated for at least 5 hours in X-gal staining solution (3.1 mM potassium ferricyanide, 3.1 mM potassium ferrocyanide, 10 mM sodium phosphate, pH 6.8, 0.15 M NaCl, 1 mM MgCl2, 10% DMSO and 0.1% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside; Gold Biotechnology). X-gal was stored at −20°C as a 10% stock solution in dimethylformamide and added immediately prior to staining. The staining solution was modified from Simon et al. (1985) by adding 10% DMSO and changing the pH from 7.2 to 6.8. These modifications resulted in uniform β-galactosidase staining of larval imaginal tissues in P[w+] HS-lacZ(6SE) hemizygotes.

Adult eyes were stained after a 1-hour heat shock at 37°C and 30-minute recovery at room temperature. Brain tissue behind the eyes was dissected away in PBS (130 mM NaCl, 7 mM Na2HPO4, 3 mM NaH2PO4). Fixation was performed in 1.5% glutaraldehyde (Electron Microscopy Sciences)/50 mM sodium cacodylate (pH 7.0) for 10

![Fig. 1. Generation and the phenotypes of transgenic stocks exhibiting heterochromatic PEV.](image-url)

As described in Lu et al. (1996), P[w+] HS-lacZ(6SE) contains a lacZ gene driven by Hsp70 with all the regulatory sequences required for maximal induction (Topol et al., 1985). A mini-white gene in tandem is regulated by an eye-specific enhancer (enh), scs and scs, domain boundary elements that block euchromatic but not heterochromatic position effect (Kellum and Schedl, 1991). P-element transposon termini necessary for insertional transposition. P[w+] HS-lacZ(6SE) harbors an insertion of the construct in the euchromatin of the left arm of the third chromosome at 65E. These flies exhibit uniform lacZ and white expression. X-irradiation of P[w+] HS-lacZ(6SE) flies juxtaposed the reporter gene locus to heterochromatic areas, resulting in two variegating rearrangements, Int(3L)BL1 and Tp(3;Y)BL2. Variegation of HS-lacZ and white is salt-and-pepper in the adult eye of Int(3L)BL1 and large-patch in Tp(3;Y)BL2.
minutes on a rotating platform. After photographing eye pigmentation, eyes were placed in X-gal staining solution overnight, washed several times with PBS and mounted in 90% glycerol/PBS. The staining solution extracted red eye pigment, allowing only X-gal staining to be photographed.

For staining of larval and pupal tissues, intact larvae/pupae were heat-shocked for 40 minutes in a 37°C water bath and recovered for 40 minutes at room temperature. Tissues were dissected in PBS, fixed in 5% formaldehyde (Electron Microscopy Sciences)/PBS for 2 minutes, washed with PBS and placed in X-gal staining solution. Tissues were then fixed again in 5% formaldehyde/PBS, washed with PBS and mounted in 90% glycerol/PBS. Cultured discs were treated similarly, but with a 10-minute heat shock and 20-minute recovery, and the 2-minute fixation prior to staining was omitted.

**Immunostaining for lacZ and esg expression in embryos**

Embryos were heat shocked for 30 minutes at 37°C and dechorionated with bleach for 5 minutes. They were placed in fixative (4% formaldehyde, 50 mM EGTA in PBS) and an equal volume of heptane for 20 minutes on a rotating platform. The lower aqueous phase was then removed and replaced by an equal volume of 50 mM EGTA/methanol. The tubes were shaken vigorously by hand. Devitellinized embryos that had settled to the bottom were collected, washed with methanol and stored at −20°C.

For antibody staining, 20-50 ml of fixed embryos in 1.5 ml Eppendorf tubes were treated with 3% H2O2/methanol for 15 minutes, washed with PBT (0.1% Tween 20 in PBS), incubated in blocking solution (5% goat serum/PBT, 2 hours), incubated in mouse β-galactosidase antibody (Promega, 1:5000 in blocking solution, 1 hour), washed with PBT, incubated in biotinylated anti-mouse antibody (Vector, 1:500 in blocking solution, 1 hour), washed with PBT, incubated in strepavidin-peroxidase conjugates (ABC kit, Vector, 30 minutes), washed with PBT, and developed using a DAB kit (Vector, 5 minutes). For double labeling with the esg antibody, embryos were re-blocked and similarly treated (esg antibody at 1:600 and biotinylated anti-rat antibody at 1:500; Vector). Staining was performed with the SG kit (Vector). After the color reaction, embryos were dehydrated in a 40%, 60%, 80%, 95% and 100% ethanol series, settled in xylene and mounted in Permount (Sigma).

We previously reported that, when In(3L)BL1 and Tp(3; Y)BL2 embryos were allowed to recover for 1 hour at room temperature after heat shock, inducible lacZ activity was not detected in blastoderm embryos (Lu et al., 1996). This contrasts with the uniform lacZ expression described in this report. The discrepancy can be explained by the hour-long recovery, during which pre-blastoderm embryos that were heat shocked when transcriptionally incompetent progress into the blastoderm stage and show no lacZ activity upon lacZ staining. By omitting the recovery period in our immunostaining protocol, we eliminated the delay between heat shock and detection of lacZ activity.

**Determination of heat shock-induced developmental arrest in early embryos**

Flies were first transferred to 3-5 successive apple agar plates with fresh yeast at 30-minute intervals to encourage females to lay freely. 15-minute collections of embryos were then made and aged at room temperature until the beginning of gastrulation (stage 7, approximately 185 minutes after egg deposition). Some of these collections were heat shocked at 37°C for 30 minutes while others were aged further for intervals from 0 to 30 minutes. After aging or heat shock, embryos were fixed as for immunostaining (see above) and mounted in Permount. Individual embryos from each collection were staged according to Campos-Ortega and Hartenstein (1985). Individual embryos were assigned discrete values (7, 8, 9, etc.), which were then averaged to give a mean stage for each collection. Extrapolating the developmental stage of heat-shocked embryos back to the developmental profile of their non-heat-shocked siblings showed that heat shock causes developmental arrest within minutes (Fig. 2).

**In vitro culture of larval imaginal discs**

Our methods were based on Fristrom et al. (1973) and Natzle and Vesenka (1994). Third instar larvae were surface-sterilized in 70% ethanol for 2 minutes and dissected in Ringer’s solution (130 mM NaCl, 5 mM KCl, 1.5 mM CaCl2, 2 mM Na2HPO4 and 0.37 mM KH2PO4). Discs were cultured in Robb’s medium containing 1% BSA and, when necessary, 1 ml of 20-hydroxyecdysone (Sigma) in ethanol (final concentration: 0.2 µg/ml or 0.4 mM) for 16 hours at room temperature with gentle shaking.

**RESULTS**

Heterochromatin-mediated silencing is initiated at the beginning of gastrulation

Heterochromatin first appears at blastoderm (Mahowald and Hardy, 1985; Vlassova et al., 1991). The heterochromatin-associated protein HP1 first appears to concentrate in heterochromatin at around this same time (James et al., 1989). Furthermore, HP1 becomes hyperphosphorylated between 1.5 and 2 hours of embryogenesis, suggesting a role for HP1 phosphorylation in the assembly of heterochromatin at blastoderm (Eisenberg et al., 1994). We mapped the onset of gene silencing by heterochromatin to determine whether these signs of heterochromatin assembly signify the partitioning of the eukaryotic nucleus into transcriptionally competent euchromatin and epigenetically silenced heterochromatin.

To determine when in embryogenesis heterochromatin silencing is initiated, we looked for the first time that variegated lacZ expression could be detected in embryos of two lacZ-variegating lines, In(3L)BL1 and Tp(3; Y)BL2 (see Materials and methods). A 30-minute heat shock at 37°C is sufficient to obtain uniform HS-lacZ expression from a euchromatin transgene (P[w+ HS-lacZ]/65E; see Materials and methods) beginning in blastoderm embryos (Fig. 3A-C). Prior to blastoderm, cleavage stage embryos are transcriptionally active.
incompetent (Edgar and Schubiger, 1986) and express no β-galactosidase (β-gal) in any of our transgenic lines. lacZ expression is uniform in syncytial blastoderm embryos from the variegating lines In(3L)BL1 and Tp(3;Y)BL2 (Fig. 3D,G).

As these embryos cellularize and begin to gastrulate, variegated silencing of HS-lacZ first becomes detectable (Fig. 3E,F,H,I), and the degree of silencing, i.e. the proportion of cells failing to express β-gal, continues to increase for the remainder of embryogenesis (see Discussion). Thus, silencing activity of heterochromatin is first detectable approximately 1 hour after cytologically visible heterochromatin is first seen in embryogenesis. Note that the heat-shock conditions used here arrest development during the 30 minutes of heat shock (see Materials and methods), as reported previously (Dura, 1981; Foe and Alberts, 1985). Therefore, the stage at which lacZ expression is visualized closely approximates the stage at which heat shock is first administered.

**Maintenance of the silent state during development and differentiation**

We previously showed that heterochromatic silencing is fully established at the HS-lacZ locus in third instar larval imaginal discs (Lu et al., 1996). To determine when in development this extreme level of silencing is established among the mitotically active imaginal anlagen, we examined HS-lacZ expression in embryonic imaginal tissue. Nuclei of embryonic imaginal cells (Hayashi et al., 1993) were immunolocalized with an antibody to the escargot gene product. escargot encodes a transcription factor normally expressed in diploid cells, and is required for the maintenance of diploidy. After stage 14, escargot protein

![Fig. 3. Establishment of variegated HS-lacZ silencing in early embryogenesis.](image)

(A-C) P[w+ HS-lacZ]/65E embryos. (D-F) Int(3L)BL1 embryos. (G-I) Tp(3;Y)BL2 embryos. (A,D,G) Syncytial blastoderm embryos (early stage 5). (B,E,H) Cellularized embryos at the beginning of gastrulation (late stage 5, early stage 6). Insets in E and H highlight variegated staining in the cellularized cortex of the embryo. (C,F,I) Gastrulating embryos (stage 7), lateral surface view. Two reproducible indications of the onset of silencing may be seen: staining in variegating stage 6 (E,H) and 7 (F,I) embryos is markedly reduced, and the staining is irregular on a cell-by-cell basis, indicating mosaic silencing. Embryos of each genotype were immunostained en masse for lacZ activity (brown) and staged according to Campos-Ortega and Hartenstein (1985). Embryos are shown with the anterior to the left. Bar, 150 μm.

![Fig. 4. Extensive silencing of HS-lacZ in embryonic imaginal precursor cells by stage 15.](image)

(A,B) P[w+ HS-lacZ]/65E. A is stained for ESG expression alone (blue), while B is doubly stained for ESG and β-galactosidase (brown). (C,D) Int(3L)BL1. (E,F) Tp(3;Y)BL2. Arrowheads mark the location of esg-expressing eye-antennal (A,B,C,E) and the wing and haltere (D,F) imaginal cells. The eye imaginal cells acquire a compact V-shaped distribution at this time (Foe et al., 1993). Double immunostaining with a β-galactosidase antibody (brown; indicated by arrows) and an esg antibody (blue) shows that lacZ activity, although present in tissues such as salivary glands (arrows in D and F) and amnioserosa (arrows in C and E), is absent in imaginal cells of the variegating lines. Bar, 150 μm.
Regulation of heterochromatic silencing specifically marks the nuclei of imaginal cells. In both In(3L)BL1 and Tp(3;Y)BL2 embryos, we detected no lacZ expression in the escargot-staining imaginal precursor cells at or after stage 14 (Fig. 4). This indicates that widespread silencing of HS-lacZ is already established in the imaginal precursors by this time in embryogenesis.

To test the maintenance of heterochromatic silencing in these same lineages at later stages of development, we stained variegating larval imaginal discs for β-gal activity. The sensitivity of this assay was increased over previously published conditions (Simon et al., 1985; see Materials and methods). We found that the nearly complete silencing established in embryonic imaginal disc precursors is stably maintained in early third larval instar eye imaginal discs from In(3L)BL1 and Tp(3;Y)BL2 larvae (Fig. 5A). However, as terminal differentiation ensues behind the morphogenetic furrow in later third instar eye discs (Wolff and Ready, 1993), these cells exhibit variegated HS-lacZ expression in patterns anticipating those seen in the adult eye (salt-and-pepper for In(3L)BL1 and large-patched for Tp(3;Y)BL2; Fig. 5A). Thus, the variegated phenotype seen in the adult eye emerges at the onset of terminal differentiation when variegated relaxation of HS-lacZ silencing occurs.

If the abrupt relaxation of silencing behind

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**Fig. 5.** Variegated relaxation of HS-lacZ silencing at the onset of imaginal disc differentiation. All tissues were heat shocked, fixed briefly in formaldehyde, and stained for β-galactosidase activity using X-gal. (A) Eye-antennal imaginal discs from early third instar larvae of both variegating lines show nearly complete silencing. In late third instar discs, cells ahead of the morphogenetic furrow retain extensive silencing, while cells behind the furrow show extensive, but variegated, expression of β-galactosidase. The anterior portion of each disc is oriented toward the top. Brackets designate the entirely undifferentiated early third instar eye disc and the undifferentiated anterior portion of the late third instar eye disc. Arrows mark the position of the morphogenetic furrow. (B) Onset of differentiation coincides with relaxation of silencing in variegating wing and leg imaginal discs. Early third instar discs show extensive silencing. Folds in late third instar discs signal the onset of differentiation in some cells. In early pupae (12-15 hours after puparium formation), wing and leg discs have everted; the wing blade begins to flatten and elongate, while the evverting leg discs show characteristic segmentation. The distal ends of the everted wings and legs are oriented toward the right. Bar, 150 μm.
the morphogenetic furrow in the eye were related to the normal developmental activation of the white gene at the transgene locus, then silencing should remain extensive in the other imaginal discs, since white is not expressed in any other disc. On the other hand, if relaxation of heterochromatic silencing is generally concomitant with differentiation, the extent of silencing in wing and leg imaginal discs should diminish with the onset of differentiation in these tissues. Differentiation in leg and wing discs is not as concerted as that of the eye, and involves a series of regionally restricted morphological changes until evagination in the pupal stage (Bodenstein, 1965; Cohen, 1993; Fristrom and Fristrom, 1993). Similarly, the extent of HS-lacZ expression increases gradually in the leg and wing discs, in contrast to the abrupt derepression seen behind the morphogenetic furrow in the eye disc. Nevertheless, by the time of disc evagination in early pupae, extensive relaxation of HS-lacZ silencing in the wing and leg discs is obvious when compared to the widespread silencing in early third instar (Fig. 5B).

**Relaxation of heterochromatic silencing can be triggered in vitro by ecdysone**

The onset of terminal differentiation, which accompanies relaxation of silencing, is triggered by peaks of the steroid hormone ecdysone (Riddiford, 1993). To test whether ecdysone alone is sufficient to drive relaxation of heterochromatic silencing, we cultured larval imaginal discs from Tp(3;Y)BL2 larvae and induced terminal differentiation in vitro with ecdysone. After 16 hours of culture in ecdysone, we observed disc differentiation in vitro similar to that reported in Fristrom et al. (1973). Ecdysone-dependent differentiation of Tp(3;Y)BL2 discs was accompanied by increased lacZ expression (Fig. 6). In contrast, neither significant differentiation nor significant relaxation of lacZ silencing were observed in control discs cultured without hormone. Thus, ecdysone is sufficient to promote relaxation of heterochromatic silencing.

**Extent of heterochromatic silencing is not cell cycle-dependent**

Differentiation in imaginal discs is accompanied by exit from the cell cycle. We considered the possibility that loss of mitotic activity per se could precipitate the relaxation of heterochromatic silencing that coincides with disc differentiation. To test this possibility, we genetically manipulated the cell cycle, both during the period at which silencing is established and the time at which relaxation of silencing is observed, and visualized the effect of these manipulations on heterochromatic PEV.

After cellularization, post-blastoderm embryos undergo at least three additional cell divisions before hatching (the 14th, 15th and 16th cell cycles) requiring zygotic expression of the string gene, a cdc25 homologue in Drosophila (Edgar and O’Farrell, 1990). In zygotically string-embryos, cells are arrested in G2 of the 14th cell cycle (the cycle in which silencing is first detected). Nevertheless, normal embryonic development continues until hatching (Edgar and O’Farrell, 1989), albeit with fewer cells. To determine whether the establishment of heterochromatic silencing in post-blastoderm embryos depends on mitotic activity, lacZ expression in post-blastoderm string- embryos was assayed. In both Int(3L)BL1 and Tp(3;Y)BL2 embryos, similar extents of variegation were observed in string- and string+ embryos (Fig. 7). Thus, the gradual establishment of PEV in post-blastoderm embryos proceeds independently of mitotic activity.

To test the role of cell cycle in the maintenance of silencing, we used dominantly acting transgenes to add or subtract a cell division in cells undergoing relaxation and tested the effect of this manipulation on the timing and extent of relaxation in the eye imaginal disc. In eye discs of late third instar larvae, mitoses occur asynchronously among the undifferentiated cells ahead of the morphogenetic furrow. As the morphogenetic furrow passes, a single burst of mitosis occurs, followed by exit from the cell cycle and the overt differentiation of the incipient ommatidial units (Tomlinson, 1990; Wolff and Ready, 1993). To test whether cell-cycle activity influences heterochromatic silencing behind the furrow, we asked if (1) ectopic mitosis in the cells behind the furrow suppresses the relaxation of heterochromatic silencing, or if (2) blocking the final mitotic wave immediately behind the furrow enhances the differentiation-coupled relaxation of silencing. Ectopic mitoses were induced with Hsp70-DmCycE, a transgene consisting of a heat shock-inducible Drosophila homologue of cyclin E to promote S-phase entry and ectopic mitoses (Richardson et al., 1995). Cell-cycle arrest behind the furrow was induced with glass-p21, a transgene expressing a human homologue of p21 in the cells behind the morphogenetic furrow (under the control of the glass enhancer), imposing a G1/S block behind the furrow (de Noolj and Hariharan, 1995). Neither of these genetic manipulations affected heterochromatic silencing in the eye disc (Fig. 8); variegated relaxation of HS-lacZ silencing behind the furrow was indistinguishable from that seen in mitotically normal discs. Furthermore, widespread silencing persisted among the undifferentiated cells undergoing ectopic mitoses ahead of the morphogenetic furrow in Hsp70-DmCycE discs, indicating that mitotic activity per se does not destabilize silencing.

**Fig. 6. Relaxation of HS-lacZ silencing in cultured imaginal discs treated with ecdysone.** Tp(3;Y)BL2 imaginal discs were cultured in ecdysone (0.4 mM) for 16 hours (filled bars). Each disc was scored as staining (+) or not staining (−) for β-galactosidase activity. Bars indicate the percentage of similarly treated discs scored for each classification. Control discs (open bars) were treated with the same volume of ethanol (the solvent for ecdysone). The numbers of discs scored in the ecdysone experiment are 44 (eye), 31 (wing) and 29 (leg) with ecdysone and 51 (eye), 18 (wing) and 43 (leg) without ecdysone.
Promoters of differing intrinsic strength do not differ in their sensitivity to silencing by heterochromatin.

In some cases, strong promoters can escape silencing by repressive chromatin (Renaud et al., 1993; Lee and Gross, 1993; Walters et al., 1996). Although many genes in Drosophila have been shown to variegate under the influence of heterochromatin (including Hsp70), the possible influence of intrinsic promoter strength on silencing has never been tested, either because different heterochromatin regions were involved or because the genes lay quite far apart. To test the role of intrinsic promoter strength in PEV silencing, we evaluated the degree of concordance of variegated HS-lacZ and white expression in adult flies carrying the Tp(3;Y)BL2 chromosome. Since Tp(3;Y)BL2 flies exhibit large-patch PEV (see Materials and methods), the degree of concordance can be determined by inspection. The Hsp70 promoter driving HS-lacZ expression is capable of directing high levels of transcription after induction, and is one of the strongest promoters known in Drosophila (Velazquez et al., 1983; Hunt et al., 1992). The white gene, which lies in tandem with HS-lacZ, is transcribed at much lower levels than Hsp70 (Steller and Pirotta, 1985). In the variegating Tp(3;Y)BL2 rearrangement, the white and Hsp70 promoters lie 5 kb apart and are flanked by the same heterochromatin. If the Hsp70 promoter resists heterochromatic silencing better than the nearby white promoter, the proportion of eye cells expressing inducible β-galactosidase in Tp(3;Y)BL2 flies should be greater than the proportion of pigmented cells in the same eye. HS-lacZ and white variegation is coextensive at the light microscope level (Fig. 9), arguing that silencing by heterochromatin is insensitive to differences in intrinsic promoter strength and that Hsp70 induction does not antagonize heterochromatic silencing in Drosophila.

DISCUSSION

Heterochromatic silencing follows its structural maturation in early embryos.

In embryonic development, cytologically visible heterochromatin first appears in syncytial blastoderm embryos at approximately 1.5 hours of embryogenesis (Vlassova et al., 1991). We find that heterochromatin first acquires the ability to silence genes approximately an hour after heterochromatin is first visible in cleavage stage embryos. Establishment of variegated silencing of HS-lacZ at the end of the blastoderm stage coincides with G2 of the 14th cell cycle (Foe et al., 1993) and with previously reported physical changes in heterochromatin. During the interphase of the 14th cell cycle, heterochromatin undergoes a rapid compaction and remains consistently condensed thereafter (Foe et al., 1993; Hiraoka et al., 1993). Also beginning in the 14th cell cycle, heterochromatin is replicated progressively later during subsequent S phases (Edgar and O’Farrell, 1990; Foe et al., 1993). We therefore propose that the initiation of heterochromatic PEV, beginning at the end of blastoderm stage, reflects the functional maturation of heterochromatin. Earlier studies identified a temperature-sensitive period for heterochromatic silencing during the first 3-4 hours of embryogenesis (Hartmann-Goldstein, 1967; Spofford, 1976). This coincides with the period in embryogenesis when we find that silencing of HS-lacZ is being initiated. Thus, our results confirm earlier speculation that the embryonic temperature-sensitive period of PEV corresponds to the time of heterochromatinization (Hartmann-Goldstein, 1967). It is likely that temperature changes within the first few hours of embryogenesis influence the maturation of heterochromatin, higher temperatures reducing the extent of heterochromatic spreading and lower temperatures favoring greater spreading. Once heterochromatic silencing is fully established, sensitivity to subsequent temperature change diminishes. The extent of relaxation of heterochromatic silencing upon differentiation would be influenced by the extent of heterochromatin spreading established in embryogenesis.

We considered the possibility that the shared cytoplasm in syncytial blastoderm embryos permits diffusion of β-gal (the product of lacZ) and might obscure any variegated lacZ silencing before cellularization. However, previous studies employing lacZ promoter fusions driven by promoters of early zygotic genes show that β-gal diffusion is limited in syncytial blastoderm embryos. Expression of β-gal approximates the mRNA distribution of the early zygotic gene in bands as narrow as 3-4 nuclei across (Howard and Struhl, 1990; Pankratz et al., 1990). Furthermore, if silencing were established sometime in the pre-cellularized embryo, the overall intensity of the lacZ staining should be reduced compared to control P[w+ HS-lacZ]/(65E) embryos, but this was not observed.

Although silencing is initiated in some embryonic cells by the beginning of gastrulation, the extent of silencing increases as embryonic development progresses. This can be seen by comparing the extent of β-galactosidase staining in embryos shown in Fig. 3 (early gastrulation; end of stage 6, about 3 hours of development) to that of embryos in Fig. 7 (germband extension; stage 11, between 5.5 and 7.5 hours of development) and Fig. 4 (dorsal closure; stage 15, between 11.5 and 13 hours of development). We did not determine whether this progressive silencing is uniform or cell type-specific in early stage embryos, but by dorsal closure, tissue-specific differences in silencing reminiscent of those reported (Lu et al., 1996) for third instar larvae are established: the undifferentiated and mitotically active imaginal precursors show nearly complete silencing, while silencing appears more relaxed in the differentiated postmitotic salivary glands. Silencing was previously shown to be maintained even during extended culture in proliferating serially transplanted discs (Hadorn et al., 1970). Although the necessity to subdivide discs and the consequent effects of sampling and differential proliferation of certain cell types suggests caution in interpreting the results of transplantation experiments, they fit well with our direct observation of stable silencing in the proliferating cells of late embryo and larval imaginal tissue.

A model for the developmental regulation of heterochromatic PEV

The progression of heterochromatic silencing in the mitotically active precursors of the adult throughout development is shown in Fig. 10. There are two periods during which silencing is stably maintained: one during the mitotic proliferation from mid-embryogenesis through early third instar, and the other during the largely postmitotic period from the time silencing...
is relaxed at the onset of differentiation through the eclosion of the adult fly. Extensive silencing of HS-lacZ in imaginal cells from late embryogenesis (Fig. 4) through third larval instar (Fig. 5) indicates that silencing is propagated faithfully in mitotically active tissues. After the relaxation of HS-lacZ silencing at the onset of differentiation, the degree of PEV also appears to be preserved for the remainder of pupation, as judged by the concordant pigment mosaicism and lacZ expression in Tp(3;Y)BL2 eyes. Eye pigment mosaicism reflects the variegated chromatin state at the transgene locus in the first 2 days of the pupal stage, when white expression is required for pigmentation (Summers et al., 1982; Steller and Pirrotta, 1985), while variegated lacZ expression reflects the chromatin state of the transgene locus at the time of heat shock. Co-variegation of white and lacZ variegation in the adult eye (Fig. 9), therefore, demonstrates that the chromatin state at the transgene locus is stable after the initial relaxation of silencing at the beginning of eye differentiation.

The late larval/early pupal stage is thus a critical period in the establishment of adult patterns of PEV. Ecdysone-driven relaxation of silencing in cultured Tp(3;Y)BL2 discs suggests that this hormone may represent the developmental signal that triggers heterochromatin remodeling. However, we were unable to elicit a similar ecdysone-dependent relaxation in In(3L)BL1 tissue (data not shown). It appears that the particular heterochromatic context in In(3L)BL1 demands more stringent conditions for lacZ reactivation, conditions not satisfied by our tissue culture protocol. Silencing of HS-lacZ in In(3L)BL1, like that in Tp(3;Y)BL2, does respond to other modifiers of PEV, as does variegation of the linked mini-white gene in the construct (Lu et al., 1996; B. Y. Lu, D. Zhu, G. Reuter and J. C. Eissenberg, unpublished data). There is good evidence that the composition of heterochromatin differs in different chromosomal domains (Sinclair et al., 1983; Bishop, 1992); such differences could underlie the difference in responsiveness of our two lacZ-variegating lines to ecdysone in culture.

Locke et al. (1988) proposed a mass action model of PEV in which the extent of heterochromatin assembly depends on the concentrations of various heterochromatin subunits. Although this model explains why PEV is sensitive to changes in the dosage of a particular subunit, it postulates a highly unstable heterochromatin structure that is extremely sensitive to small developmental changes in nuclear volume. Such changes would exert dramatic effects on the extent of PEV if silencing depended continuously on mass action-driven assembly throughout development, and this is inconsistent with the mitotic stability of an epigenetic silencing mechanism (Henikoff, 1996). We propose that heterochromatin is sensitive to mass action-driven assembly only during the period when silencing is relaxing. This hypothesis is supported by preliminary data showing that several genetic PEV modifiers have little or no effect on HS-lacZ silencing in undifferentiated eye imaginal cells but exert dramatic effects in the differentiating cells behind the morphogenetic furrow (B. Y. Lu, G. Reuter and J. C. Eissenberg, unpublished data).

Remarkably, the distinct patterns of variegation in the In(3L)BL1 and Tp(3;Y)BL2 lines both emerge in their adult form immediately behind the morphogenetic furrow (B. Y. Lu, G. Reuter and J. C. Eissenberg, unpublished data). A cartoon model for how each pattern could arise in the eye disc is shown in Fig. 11. In In(3L)BL1, it is proposed that heterochromatic spreading in undifferentiated cells (e.g. ahead of the furrow) extends past the transgene in nearly every cell,

Fig. 7. *string* mediated mitotic block has no effect on heterochromatic PEV in embryos. (A) In(3L)BL1, (B) Tp(3;Y)BL2, (C) In(3L)BL1 stg/stg, (D) Tp(3;Y)BL2; stg/stg. Germ band-extended embryos (stage 11) are shown. In *string* embryos at this stage, a majority of cells have completed the 16th cell cycle (Foe et al., 1993), whereas *string* embryos at the same stage are arrested in G2 of the 14th cell cycle (Edgar and O’Farrell, 1990). Embryos are oriented with anterior to the left and dorsal toward the top. Bar, 150 μm.

Fig. 8. Ectopic p21 and cyclin E have no effect on heterochromatic PEV in eye imaginal discs. (A,D) P[w+] HS-lacZ [65E]. (B,E) In(3L)BL1. (C,F) Tp(3;Y)BL2. (A-C) Eye imaginal discs expressing glass-p21. (D-F) Eye imaginal discs expressing Hsp70-DmcycE. Arrows mark the position of the morphogenetic furrow. The discs are oriented with anterior at the top. Bar, 150 μm.
Regulation of heterochromatic silencing

resulting in widespread silencing of the lacZ transgene. When heterochromatin silencing is relaxed during differentiation, the boundary of heterochromatin spreading retreats to a position close to the site of the transgene. Thus, behind the furrow, lacZ may escape silencing in a subset of cells depending on relatively small differences in the extent of spreading. The sectors in the Tp(3;Y)BL2 line appear clonal, suggesting that the adult variegation pattern in this line is influenced by a distinct process. In Tp(3;Y)BL2, it is proposed that heterochromatic spreading in undifferentiated cells extends past the transgene in every cell. Furthermore, in a subset of cells, a continuous domain of heterochromatin is established between the blocks of Y-chromosome material that flank both sides of the Tp(3;Y)BL2 insertion. We propose that the continuous domain state is established during embryogenesis in some cells and clonally inherited throughout development, and that this state is relatively refractory to the relaxation of heterochromatic silencing that occurs upon differentiation. The establishment of the continuous domain state corresponds to the developmental timing of sectored inactivation proposed by Baker (1967) and Becker (1957; 1961). Behind the furrow, the boundary of heterochromatin spreading retreats to a position beyond the transgene in those cells in which a continuous domain was not established. To summarize, we suggest that silencing is unstable at the boundary between euchromatin and heterochromatin, and that in cases such as Tp(3;Y)BL2, spreading blocks of heterochromatin may converge and form a continuous domain with no boundary, making such blocks stable to developmentally programmed relaxation.

By this model, the clonal sectors of silencing in Tp(3;Y)BL2 are the result of stable transmission of an epigenetic state. This inference is supported by Southern blot analysis comparing transgene copy number in nearly white-eyed Tp(3;Y)BL2 adult heads to nearly red-eyed sibs (T. Zhao and J. C. Eissenberg, unpublished observations), which showed no evidence for somatic DNA loss (Karpen and Spradling, 1990) or underrepresentation due to DNA modification (Glaser and Spradling, 1994).

Developmental course of heterochromatic silencing is cell-cycle insensitive

Heterochromatic silencing is sensitive to mutations in several factors known to regulate DNA replication (Henderson et al., 1994; Seum et al., 1996; Pak et al., 1997), and it has been proposed that the time since last cell division influences the formation of heterochromatin (Csink and Henikoff, 1996). However, in no case has it been shown that genotypes causing modification of heterochromatic silencing are associated with changes in the cell cycle. Since terminal differentiation is accompanied by cessation of mitotic activity, we considered the possibility that the temporal coupling of cell-cycle cessation and relaxation of silencing could be mechanistically related. If so, an unscheduled or precocious cell-cycle block should promote the relaxation of silencing (that is, suppress PEV in the affected cells) and an unscheduled or supernumerary cell cycle should inhibit the relaxation of silencing (that is, enhance PEV in the affected cells). We found that the progressive onset of heterochromatic silencing during embryogenesis is unaffected by a block in G2 of nuclear cycle 14 imposed by zygotic string mutation. Thus, neither establishment nor stability of silencing in embryonic cells is influenced by the time since the last cell division; rather, the developmental state determines the degree of heterochromatic PEV in embryogenesis. In larval eye imaginal discs, blocking the final mitosis by glass-p21 (effectively initiating the final interphase in the last division ahead of the furrow) has no effect on the extent of HS-lacZ reactivation. Similarly, forcing cells both ahead of and behind the furrow through an additional unscheduled cell cycle has no effect on the extent of silencing.

We considered the possibility that cell cycle manipulations in the eye disc could perturb disc differentiation and thereby confound our interpretation. However, de Nooij and Harhiran (1995) found that after blocking the final mitotic wave in the eye disc by ectopic p21 expression, specification of most cell types occurred normally and the arrested cells were not obliged

![Fig. 9. Concordant expression of lacZ and white in Tp(3;Y)BL2 eyes. (A,C) Pigmentation in the adult eye. (B,D) lacZ expression in the adult eye. A and B are pictures of the same eye; C and D are pictures of the same eye, but from another individual. Bar, 150 μm.](image)

![Fig. 10. The developmental course of heterochromatic PEV in imaginal cells. The degree of heterochromatic PEV in imaginal cells is shown (dark line). Horizontal bars mark the stages of development and periods of mitosis and terminal differentiation. The vertical axis shows the extent of PEV. The graph depicts the gradual acquisition of silencing during early embryogenesis and precipitate relaxation of silencing in the imaginal discs at the onset of differentiation.](image)
and efficiently overcome HMRE silencing of basal heterochromatic silencing. In yeast, however, Lee and Gross demonstrating that heat-shock promoters are not immune to salivary glands under the influence of heterochromatin, heat-shock puff containing three copies of 1994; Walters et al., 1996). Henikoff (1981) reported that a and mammalian cells (Lee and Gross, 1993; Renauld et al., 1994) have been shown to counteract epigenetic silencing in yeast tissues. Promoter activity and induction of assay the extent of silencing by heterochromatin in different In these studies, we employ an ommatidial clustering behind the furrow as a result of either of defects in morphogenetic furrow migration or the timing of developmental signals that initiate relaxation of heterochromatin boundary.

Intrinsic promoter strength does not influence the extent of heterochromatic PEV

In these studies, we employ an Hsp70-driven lacZ reporter to assay the extent of silencing by heterochromatin in different tissues. Promoter activity and induction of cis-acting enhancers have been shown to counteract epigenetic silencing in yeast and mammalian cells (Lee and Gross, 1993; Renaud et al., 1994; Walters et al., 1996). Henikoff (1981) reported that a heat-shock puff containing three copies of Hsp70 variegates in salivary glands under the influence of heterochromatin, demonstrating that heat-shock promoters are not immune to heterochromatic silencing. In yeast, however, Lee and Gross (1993) found that heat-shock induction of Hsp82 can rapidly and efficiently overcome HMRE silencing of basal transcription, leading these authors to conclude that silencing is inversely correlated with the target gene’s promoter strength.

Our data show that promoters of different intrinsic strength succumb equally to heterochromatic silencing: although the Hsp70 promoter in HS-lacZ represents one of the strongest promoters known in Drosophila (generating several thousand transcripts per cell; Velazquez et al., 1983), HS-lacZ expression in adult Tp(3;Y)BL2 eyes is concordant with expression of the adjacent white gene, which is expressed under a much weaker promoter (producing no more than 50 transcripts per white-expressing cell; Fjose et al., 1984). Furthermore, daily heat shock of In(3L)BL1 or Tp(3;Y)BL2 flies throughout development failed to antagonize the silencing of the adjacent mini-white gene (data not shown), suggesting that even repeated Hsp70 induction does not result in significant conversion of heterochromatic domains into euchromatin.

In summary, we have defined two developmental stages for the regulation of heterochromatic silencing: (1) the initiation of variegated silencing in cellularized, gastrulating embryos and (2) the relaxation of silencing at the onset of terminal differentiation in imaginal tissues. Furthermore, developmental regulation of heterochromatic silencing can be uncoupled from the cell cycle at both stages. Finally, we show that heterochromatin in Drosophila silences with equal efficiency promoters of widely different intrinsic strengths. Taken together, these results suggest that heterochromatic silencing is regulated by developmental signals, such as edcsyne, that initiate various determinative decisions during development. The recent observation linking developmental repression to heterochromatic associations in lymphocyte development (Brown et al., 1997) suggests that differentiation-dependent remodeling of heterochromatin may have wider implications in the maintenance of developmental silencing. The mechanism by which developmental signals like edcsyne regulate the extent of silencing is unknown, but two lines of evidence implicate changes in histone acetylation: (1) the histone deacetylase inhibitor trichostain A (TSA) can effect a similar suppression of heterochromatic silencing to edcsyne, and (2) both edcsyne and TSA cause similar changes in core histone acetylation patterns (J. Ma, B. Y. Lu and J. C. Eissnenberg, unpublished observations). It is likely that histone acetylation plays a general role in regulating various forms of epigenetic silencing in metazoan development, in addition to Drosophila PEV. This is supported by a recent finding demonstrating that TSA can antagonize silencing of the paternally derived H19 gene in mouse (Svensson et al., 1998).

We thank I. Duncan for TM3, P[hs-lacZ], J. de Nooij for glass-p21, S. Hayashi for the escargot antibody, K. Matthews for TM3, Ser, J. Natzle for advice on imaginal disc culture, C. Nusslein-Volhard for string7869, H. Richardson for Hsp70-DmcycE, E. Ward and J. Caspermeyer for advice on antibody staining of embryos, and D. E. Coulter, H. P. Zassenhaus, A. Waheed and S. I. Tsubota for critical reading of the manuscript. This work was supported by National Science Foundation grant IBN 9506103. B. Y. Lu was supported in part by NIH training grant T32GM08109.

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