Chromosome Rearrangements Induce Both Variegated and Reduced, Uniform Expression of Heterochromatic Genes in a Development-Specific Manner

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ABSTRACT
In Drosophila melanogaster, chromosome rearrangements that juxtapose euchromatin and heterochromatin can result in position effect variegation (PEV), the variable expression of heterochromatic and euchromatic genes in the vicinity of the novel breakpoint. We examined PEV of the heterochromatic light (lt) and concertina (cta) genes in order to investigate potential tissue or developmental differences in chromosome structure that might be informative for comparing the mechanisms of PEV of heterochromatic and euchromatic genes. We employed tissue pigmentation and in situ hybridization to RNA to assess expression of lt in individual cells of multiple tissues during development. Variegation of lt was induced in the adult eye, larval salivary glands and larval Malpighian tubules for each of three different chromosome rearrangements. The relative severity of the effect in these tissues was not tissue-specific but rather was characteristic of each rearrangement. Surprisingly, larval imaginal discs did not exhibit variegated lt expression. Instead, a uniform reduction of the lt transcript was observed, which correlated in magnitude with the degree of variegation. The same results were obtained for cta expression. These two distinct effects of rearrangements on heterochromatic gene expression correlated with the developmental stage of the tissue. These results have implications for models of heterochromatin formation and the nuclear organization of chromosomes during development and differentiation.

The parameters that govern normal gene expression extend beyond a gene to its chromosomal and nuclear contexts. The discovery by Muller (1930) of position effect variegation (PEV) in Drosophila melanogaster first illustrated that chromosome rearrangements can have a profound effect on the expression of genes distant from the physical breakpoint. Further examples of PEV, as well as the discovery of position effects exerted upon transgenes in numerous experimental systems, support the conclusion that gene expression is sensitive to the chromosomal environment. The study of position effects provides an avenue to increase our understanding of the role of chromosome structure in the regulation of gene expression.

The underlying causes and resulting phenotypes of position effects are quite varied. Position effects in multicellular eukaryotes typically fall into two broad categories. The examples most simple to explain are those in which the regulatory elements of a resident gene interfere, in either a negative or a positive fashion, with those of a translocated gene or a transgene, resulting in temporal and/or tissue-specific changes in its expression pattern (reviewed by Wilson et al. 1990). Less well defined are examples of altered gene expression due to a change in chromatin environment. For example, in mammalian systems transgenes frequently insert into what is referred to as repressive chromatin, because of the resulting decreased or variegated transgene expression (reviewed by Martin and Whitelaw 1996). Repressive chromatin is present in both the heterochromatin and the euchromatin of mammalian chromosomes, although the nature, distribution, and variety of regions of repressive chromatin are still unclear. In Drosophila, however, chromosomal regions that induce variegated expression of transgenes correspond to only a few localized domains, most notably the pericentric heterochromatin and telomeres (reviewed by Weiler and Wakimoto 1995).

The study of position effects induced by chromosome rearrangements in Drosophila led to the establishment of PEV as a model system for studying how gene expression is influenced by higher order chromatin structure (reviewed by Grigliatti 1991; Reuter and Spierer 1992; Weiler and Wakimoto 1995). While stable effects on gene expression are typically associated with rearrangements having two euchromatic breakpoints, variegated gene expression (PEV) has been the hallmark of rearrangements that juxtapose the continually condensed pericentric heterochromatin with euchromatin, which decondenses during interphase of the cell cycle (Lewis 1950). Interestingly, the latter rearrangements can induce the variegated expression of genes that are euchromatic as well as genes that normally reside in...
the heterochromatin. Although first suggested by cytological observations, the idea that a change in chromatin structure underlies PEV is strengthened by more recent genetic and biochemical studies (reviewed by Weiler and Wakimoto 1995). PEV has been shown to be sensitive to histone dosage (More et al. 1983) and mutations and compounds that affect histone acetylation (Mottus et al. 1980; Reuter et al. 1982; Dorn et al. 1986; Fantl et al. 1994). In some cases, PEV correlates with altered nucleosome sensitivity (Wallrath and Elgin 1995).

Analyses of heterochromatic genes have suggested that PEV is, in addition, a model system for the study of the role of nuclear organization in gene expression. The study of light (lt)-variegating rearrangements (Wakimoto and Hearn 1990) revealed that heterochromatic genes require a heterochromatic environment for their normal expression, and implicated chromosomal interactions in the formation of heterochromatic compartments. Wakimoto and Hearn (1990) proposed that the association between displaced heterochromatin and pericentric heterochromatin within a compartment allowed for the exchange of heterochromatic proteins and/or the induction of the appropriate chromatin state necessary for normal heterochromatic gene expression. Support for this model has come from genetic analyses of position effects on the heterochromatic rolled gene (Eberl et al. 1993) and cytological studies showing the predicted variable association of a heterochromatic insertion and the pericentric heterochromatin (Csink and Henikoff 1996; Dernburg et al. 1996). Thus, chromosome rearrangements might also indirectly influence higher order chromatin structure, and consequently gene expression, through changes in chromosome organization within the nucleus.

Much has been learned about chromatin-induced position effects upon gene expression through work on silencing in yeast (reviewed by Pillus and Grunstein 1995). However, studies with D. melanogaster provide the opportunity to address the influences of tissue specificity and development on position effects. Specifically, the timing of the onset of variegated gene expression, as well as its plasticity, provide a window into the dynamics of higher order chromatin structure during development. In addition, determining if variegation of a gene shows tissue-specific differences should be enlightening as to the etiology of PEV. For practical reasons, the vast majority of studies of PEV have concentrated on chromosome rearrangements that cause mosaicism of pigmentation, most typically in the eye. Consequently, relatively little is known about variegated expression of genes in diverse tissue types or when, during development, the rearrangements first have their effect. Early cell lineage studies using two white (w)-variegating alleles, w\textsuperscript{wm256} and w\textsuperscript{wm264}, suggested that in rare cases the eventual expression state of the w gene in ommatidial cells may be predetermined very early in development and clonally inherited (Becker 1961; Baker 1963; Janning 1970). This conclusion was by necessity indirect, as w gene expression cannot be monitored until late in eye development. Potentially more informative are analyses utilizing variegating alleles of genes that are ubiquitously expressed. Examination of whole larvae and adult flies bearing a variegating allele of the widely-expressed purple gene suggested that variegation may not begin until pupal development (Tobler et al. 1979; but see also Kim et al. 1996). A second study analyzing variegating alleles of the Pgd gene revealed a decrease in 6-PGD protein level in only a subset of larval tissues, suggesting tissue specificity (Slobodyanyuk and Serov 1987). However, in neither study was expression monitored at the cellular level. This caveat was recently addressed by the developmental studies of Lu et al. (1996) that employed a hsp70-lacZ reporter transgene, which was inducible in a wide variety of tissues and readily assayable for expression in individual cells. Two chromosome rearrangements were isolated which placed the hsp70-lacZ reporter near heterochromatin, and both caused variegation of heat shock-induced lacZ expression in embryonic, larval, and adult tissues. Interestingly, repression was more extensive in the larval antennal imaginal disc than would have been predicted by the subsequent adult eye expression patterns, suggesting that a relaxation of repression (reactivation) occurred during eye differentiation.

Studies of lt variegation provide several advantages for investigating the dynamics of heterochromatin formation during development and in different tissues. First, the activation of lt transcription by heterochromatin reflects a normal function of heterochromatin, as opposed to its ability to repress euchromatic genes. Second, variegated expression of lt appears to reflect chromosome organization, thus yielding insight into nuclear architecture. Third, the lt gene is widely expressed throughout development (Devlin et al. 1990), allowing us to compare the effects of chromosome rearrangements on gene expression for multiple tissues and developmental stages.

This article describes an analysis of the effects of three chromosome rearrangements on lt expression in multiple tissues. We show that the relative strengths of the effects of the rearrangements on lt expression are consistent for a given lt-variegating allele in all tissues examined. Remarkably, we find that a rearrangement can either induce variegated expression or cause reduced nonvariegated expression. We have confirmed this result for a second heterochromatic gene, concertina (da), suggesting that this may be a general property of PEV of heterochromatic genes. We attribute the differing effects of the chromosome rearrangements to the developmental stage of the tissue, and suggest that variegation of heterochromatic genes is restricted to differentiated cell types.
MATERIALS AND METHODS

Drosophila stocks and culture conditions: Stocks were maintained at 25°C on standard cornmeal-molasses-agar medium. All of the mutations described in this study are listed within Flybase (http://flybase.indiana.bio.edu/).

Larvae for the Malpighian tube assays and RNA in situ analyses were cultured at 25°C under identical conditions of low crowding for each experiment. In order to eliminate potential sex differences only female larvae were used for the assays. For the Lt and cta expression studies, larvae were derived from Df(2L)lt16 Bc, LtF1 females, to effectively eliminate any maternal contribution to Malpighian tubule pigmentation. The paternal parent was Canton S, for the positive control, or carried the TSTL14, Tb balancer and either a Lt-variegating allele or the Lt deficiency, Df(2L)lt120. The Lt/Df(2L)lt116 larval progeny were identified as Bc and Tb individuals.

Assays of Malpighian tubule pigmentation: Wandering third instar female larvae of the appropriate genotype were collected as described above. The Malpighian tubules were dissected in 0.7% NaCl, and stained in 0.1 μg/ml DAPI, 0.7% NaCl on a multiwell slide. For each larva, thirty contiguous cells from each posterior arm of the tubules were scored for the presence of pigment granules using UV illumination and ×100 magnification. Data for each genotype were accumulated from two to three experiments. Statistical analysis was performed using Statview 4.5 (Abacus Concepts, Inc., Berkeley, CA).

In situ hybridization to whole mount third instar larval tissues: For a typical experiment, at least 15 wandering third instar female larvae of each genotype were processed. The anterior halves of the larvae were isolated and inverted in cold phosphate-buffered saline + 0.1% Tween-20 (PBT), in <30 min per sample. Each sample was immediately fixed in fresh 4% formaldehyde (EM grade; Electron Microscopy Sciences, Fort Washington, PA), 0.1% deoxycholate in PBT, for 20 min at room temperature, and then washed three times for 5 min each in PBT. The remaining pretreatments and hybridization procedure were a modification of the protocol of Tautz and Pfeifer (1989). The tissue samples were digested with 10 μg/ml proteinase K for 9 min, rinsed twice for 2 min each with 2 mg/ml glycine in PBT, rinsed twice for 1 min each in PBT, refixed for 20 min with 4% formaldehyde in PBT, washed 5 min in 2 mg/ml glycine in PBT, and finally washed twice for 5 min each in PBT. The tissues were then equilibrated in a 1:1 dilution of PBT and hybridization solution (50% formamide, 5× SSC, 50 μg/ml heparin, 0.1% Tween-20) for 5 min followed by 5 min in hybridization solution. Prehybridization was performed for at least 1 hr at 55°C in hybridization solution supplemented with 100 μg/ml denatured salmon sperm DNA. Digoxigenin-labeled RNA probes were synthesized using digoxigenin-UTP (Boehringer Mannheim, Indianapolis), T3 or T7 polymerase, and 1 μg template [cDNA clones of the light (Devlin et al. 1990), concertina (Parks and Wieschaus 1991) and string (Edgar and O’Farrell 1989) genes] under standard reaction conditions, and reduced in length by limited alkaline hydrolysis. The tissue samples were hybridized in 500 μl hybridization solution supplemented with 100 μg/ml denatured salmon sperm DNA and denatured RNA probe (1/15 of the transcription reaction) overnight at 55°C. Washes consisted of five 30-min incubations in hybridization solution at 55°C, 20 min each in 80, 60, 40, and 20% hybridization solution in PBT at room temperature, followed by five 5-min washes in PBT.

Color detection of hybridized probe was performed by incubating the samples with alkaline phosphate-conjugated anti-digoxigenin antibody (Boehringer Mannheim), which had been preabsorbed against unhybridized tissues, at 1:2000 dilution in PBT overnight at 4°C. Following four 20-min washes in PBT, the tissues were rinsed twice for 5 min in freshly prepared blocking buffer (100 mm NaCl, 50 mm MgCl2, 100 mm Tris pH 9.5, 0.1% Tween-20, 0.1% levamisole) and then incubated in blocking buffer supplemented with 4.5 μl/ml NBT and 3.5 μl/ml BCIP (Boehringer Mannheim) until the desired level of staining was achieved. The tissues were then stained for 10 min in 0.1 μg/ml DAPI, and mounted in 50% glycerol. The patterns of staining were visualized using a Nikon (Garden City, NY) Microphot microscope equipped with DIC optics.

Fluorescence detection of hybridized probe was performed as above except that the anti-digoxigenin antibody (1:500; Boehringer Mannheim) was unconjugated, the samples were washed in PBT four times for 30 min, incubated 4 hr in fluorescein-conjugated anti-sheep antibody (1:100; Jackson ImmunoResearch Labs., Inc., West Grove, PA) at room temperature, and washed again in PBT four times for 30 min. The tissues were mounted in 80% glycerol and examined using a ×60 (1.4 NA) objective on a Bio-Rad (Richmond, CA) MRC-600 confocal imaging system.

Assays of transcription in salivary glands: Salivary gland nuclei were visualized using DAPI staining and UV illumination. Each nucleus in the distal three-fourths of each gland was scored for the presence or absence of a focus of probe hybridization, using DIC optics. An average of 74 nuclei were scored per gland. The data for each Lt/ Df genotype derive from two experiments, and for Lt/Df from six, with 10 to 20 glands assayed per experiment. Statistical analysis was performed using Statview 4.5 (Abacus Concepts).

RESULTS

The effects of light gene variegation on tissue pigmentation: The Lt gene is essential for viability and required for the normal pigmentation of several tissues including the adult eyes and larval Malpighian tubules. More than 50 chromosome rearrangements that cause variegated expression of the Lt gene in ommatidia have been isolated (Hessler 1958; Wako moto and Hearn 1990). Three of these, Lt116, Lt120, and Lt115, were selected for analyses of Lt expression in additional tissues because they represented examples of severe, moderate, and weak Lt variegation, respectively. The effects of these rearrangements on viability and eye pigmentation are included in Table 1, and the eye phenotype of flies heterozygous for an Lt-variegating allele and Lt is illustrated in Figure 1. The Lt allele bears a hypomorphic mutation that results in a uniform, low level of pigmentation but does not reduce viability. We reasoned that a comparison of the extent of Lt variegation in multiple tissues derived from individuals carrying each of these variegating alleles might allow us to detect potential tissue-specific differences in variegation behavior among the rearrangements. Additionally, commonalities in the variegation behavior of the three Lt-variegating alleles, such as tissue or developmental specificity, would likely be representative of Lt variegation-inducing chromosome rearrangements, in general.

To compare the effects of the three Lt-variegating rearrangements (collectively denoted Ltvar) on viability...
and eye pigmentation to their effects on lt expression in the larval Malpighian tubules, we utilized the presence of pigment granules in individual tubule cells as an indicator of gene activity. The Malpighian tubules of female third instar larvae bearing a deficiency of the lt gene and an lt-variegating rearrangement were dissected and assayed as described in materials and methods. In lt/Df(2L)ltG10 (abbreviated lt/Df) larvae bearing the lt+ gene situated on an unrearranged chromosome, 100% of the cells were pigmented. Individuals bearing two lt deficiency chromosomes, Df(2L)ltG10/Df survive until pupariation, making it possible to assay pigmentation in larvae deleted for the lt gene. The Malpighian tubules of these larvae were completely unpigmented. In contrast, the Malpighian tubules of larvae having a single lt-variegating allele were a mosaic of pigmented and unpigmented cells. A tabulation of the frequencies of pigmented cells in ltG10/Df, ltG12/Df and ltG13/Df larvae is shown in Figure 2. The frequency of pigmented cells varied between individuals of the same genotype, as is characteristic of PEV. Malpighian tubules from ltG10/Df larvae had very few or no pigmented cells per larva, indicating very severe variegation. The ltG12/Df Malpighian tubules showed much greater pigmentation and a broad range in pigmentation frequency. The ltG13/Df Malpighian tubules exhibited the weakest variegation, and showed less variability between larvae than the ltG12/Df tubules. These results paralleled the effects of each rearrangement on viability (Table 1) and eye pigmentation (Figure 1): ltG10 greatly reduces viability and eye pigmentation; ltG12 causes a moderate reduction in viability and moderate eye variegation that is quite variable between individuals; and ltG13 does not reduce viability and has a weak effect on eye pigmentation.

**Expression of the light gene in wild-type third instar larval tissues, as detected by RNA in situ hybridization.**

In order to assess the expression of the lt-variegating alleles in additional, unpigmented tissues, RNA in situ hybridization assays were undertaken. We first determined the extent and pattern of lt expression in tissues of wild-type third instar larvae. Single-stranded sense and anti-sense RNA probes were synthesized from the lt cDNA clone and hybridized to whole mount tissues. In agreement with the results of previous Northern analyses (Devlin et al. 1990), expression of the lt gene was detected using the anti-sense probe in all tissues examined (e.g., larval brain, digestive system, circulatory system, imaginal discs, Malpighian tubules, fat body, gonads). In imaginal discs, cytoplasmic RNA appeared to be present in all cells, at similar levels (Figure 3A). The uniformity of staining was apparent during microscopic examination as the focal plane was adjusted through the full depth of the tissue (as well as by confocal microscopy, see below). However, since out-of-focus staining contributed to the intensity as observed in photographs, the staining appeared darker in areas of greater tissue thickness and where the tissue had folded over. Under the experimental conditions used in this study, the degree of staining appeared quantitative. Comparisons of staining levels were made only for tissues treated identi-
Figure 2.—Pigmentation of Malpighian tubule cells in larvae bearing \(lt\)-variegating chromosome rearrangements. The percentage of pigmented cells in tubules of individual larvae was assayed as described in materials and methods (60 cells per larva), and plotted as a histogram. Twenty third instar larvae were scored per genotype, from two or three experiments. Malpighian tubules from \(lt^{+}/Df\) larvae were fully pigmented, and those from \(Df(2L)ltX120/Df\) larvae were unpigmented. Data obtained for \(lt/G10/Df\), \(lt X2/Df\), and \(lt X13/Df\) differ significantly from \(lt^{+}/Df\), using a Mann-Whitney U-test of median values \((P < 0.0001)\). The \(lt^{+}/Df\) and \(lt^{11}/Df\) data are also significantly different \((P < 0.0068)\).

Figure 3.—Expression of the \(lt\) gene in tissues of late third instar larvae, detected by in situ hybridization to \(lt\) RNA. Imaginal discs from \(lt^{+}/lt^{+}\) larvae (A) exhibited a more intense cytoplasmic staining pattern than did those from \(lt^{+}/Df\) larvae (B). Nuclear staining was observed in polytene tissues such as the salivary gland from a \(lt^{+}/Df\) larva (C) and the foregut and gastric caeca from a \(lt^{+}/lt^{+}\) larva (D). Many nuclei of the gut and gastric caeca are not in the plane of focus and therefore do not show a nuclear dot.

cally within a single experiment. The sensitivity of this technique to detect different levels of \(lt\) transcript is illustrated by comparing the staining of tissues having two copies of the \(lt^{+}\) gene (\(lt^{+}/lt^{+}\) in Figure 3A) to those having a single copy (\(lt^{+}/Df\) in Figure 3B). Furthermore, as described below, certain \(lt\) genotypes yielding staining levels lower than one wild-type copy were easily distinguishable. In polytene tissues such as the gut, fat body, and salivary glands, a focus of nuclear RNA representing the site of transcription (O’Farrell et al. 1989; Boyd
et al. 1991) was detected (Figure 3, C and D). The intensity of nascent transcript staining in salivary gland nuclei exhibited slight cell-to-cell variability, possibly reflecting differences in cell physiology and polyteny. Staining was rarely observed in the cytoplasm of the salivary gland cells. These results were in contrast to those obtained using an α-sense strand probe as a control, which showed no hybridization in the imaginal discs or the salivary glands (data not shown). As an additional control, larvae deleted for both copies of the It gene were included in each experiment to reveal any nonspecific staining of imaginal discs. Nuclear foci were never observed for Df(2L)ltX13/Df salivary glands.

Salivary glands exhibit a mosaic light expression pattern, as a result of lt var rearrangements: Having established the wild-type expression pattern of the lt gene, we then determined the effects of the lt var rearrangements on lt expression in multiple unpigmented cell types. For these lt var assays, the genotype of the larvae assayed was lt +/Df so that only expression from the gene on the rearranged chromosome was monitored. A single focus of lt probe hybridization was observed within all nuclei of most lt +/Df salivary glands (see Figures 3C and 4A; data in Figure 5). In contrast, the glands derived from larvae bearing a lt-variegating allele were a mosaic of cells having the nuclear staining and cells devoid of the nuclear staining (e.g., lt G10/Df in Figure 4B). We assessed the severity of variegation in this tissue for each lt-variegating allele by quantitating the fraction of cells expressing the lt gene. The percentage of expressing nuclei in individual salivary glands from lt +/Df, lt G10/Df, lt X2/Df and lt G10/Df larvae is illustrated in Figure 5. The effect of each rearrangement on lt expression in the salivary glands correlated in magnitude with its effect on lt expression in other tissues (compare with Figures 1 and 2). Only a few salivary gland cells from lt G10/Df larvae expressed the lt gene. Salivary glands from lt X2/Df and lt G10/Df larvae showed a much higher frequency of staining nuclei than lt G10/Df larvae, but were not statistically different from each other. As in the eye and Malpighian tubules, there was a large degree of variability between lt G10/Df individuals in the frequencies of salivary gland cells showing lt expression.

Imaginal discs exhibit reduced, nonvariegated expression of the light gene, as a result of lt var rearrangements: As stated above, in situ hybridization to lt mRNA yielded a uniform staining pattern for lt +/Df imaginal discs. When imaginal discs from larvae bearing any one of the three lt var rearrangements were assayed, the staining pattern was less intense but still uniform. Tissues derived from at least four experiments were examined for each lt +/Df genotype, and for three experiments all three lt var/Df genotypes (and controls) were processed simultaneously and identically. Typical results from a single experiment in which individuals of all three lt var/Df genotypes were processed identically are illustrated in Figure 6 for leg imaginal discs and

![Image](309x397 to 548x743)

Figure 4.—Variegated lt expression in lt G10/Df salivary glands. In situ hybridization to the polytene salivary glands revealed the nascent lt transcript in each nucleus of lt +/Df glands (A). Only a subset of the cells appeared to be actively transcribing the lt gene within lt G10/Df glands (B).

![Figure 7](link)

Figure 7 for eye-antennal imaginal discs. The staining obtained for lt X2/Df imaginal discs (Figures 6B and 7B) was strong but generally less intense than lt +/Df imaginal discs (Figures 6A and 7A). The level of imaginal disc staining of lt X2/Df larvae (Figures 6C and 7C) was intermediate between that of lt +/Df larvae and Df(2L)lt X120/Df larvae (Figures 6E and 7E). The imaginal discs of lt X2/Df larvae (Figures 6D and 7D) showed an extremely low level of staining, which was generally equivalent to or only slightly darker than that of Df(2L)lt X120/Df imaginal discs. Therefore, the chromosome rearrangements affected lt expression by reducing it to a level characteristic of each allele, but did not induce mosaicism of expression in these imaginal tissues. Uniform staining was observed upon examination of tissues at up to 400-fold magnification. We confirmed that cell-to-cell differences in gene expression could be detected using our in situ hybridization protocol by assaying imaginal discs for the string transcript, which is present in single cells scattered throughout the discs because of differences in stage of the cell cycle (data not shown;
In addition, the accessibility of the imaginal discs to Lt probe and antibody in these studies was supported by the observation that hemocytes associated with the interior pockets formed by folds of the eye-antennal disc epithelia showed a variegated Lt RNA staining pattern (Figure 7F). It should be noted that within an experiment small differences in the intensity of staining among Lt+/Df imaginal discs were observed. A similar variability in staining between discs was observed for LtG10/Df and LtX13/Df larvae, whereas LtX2/Df imaginal discs exhibited a broader range of staining. The greater variability among LtX2/Df imaginal discs is likely a manifestation of the position effect.

It remained a formal possibility that the imaginal discs were composed of Lt-expressing and Lt-nonexpressing cells, but that the distinctions between cells were masked by convolutions of the tissue and/or associated hemocytes (which include the melanotic crystal cells; see Figure 7). Therefore, we performed in situ hybridization to Lt mRNA in imaginal discs using fluorescence detection and confocal microscopy. We focused our analysis upon the portion of the eye-antennal disc that gives rise to the ommatidia, since Lt variegation occurs in the adult eye. We examined 0.1-μm optical sections spanning the full thickness of the tissue for nine Lt+/Df eye-antennal discs and three LtX2/Df eye-antennal discs, but variegation was never observed. Rather, the staining was cytoplasmic and uniform across the disc, and was not observed within the nuclei. However, a comparison of the intensity of staining of Lt+/Df and Df(2L)LtX12/Df tissues processed simultaneously showed that Lt+/Df eye-antennal discs were more brightly stained (data not shown). As the LtX2/Lt1 adult eye phenotype frequently shows large patches of dark pigmentation (Figure 1D), a comparable pattern of variegation at the RNA level should have been readily apparent but was not detected. A representative section from an LtX13/Df eye-antennal disc is shown in Figure 8. These results confirmed the results obtained using colorimetric detection of Lt mRNA, in showing that Lt RNA levels were uniform in the eye-antennal imaginal discs of Lt-variegating strains.

The absence of mosaic expression in imaginal discs is observed for another variegating heterochromatic gene: To determine if the reduced nonvariegated mRNA staining pattern was unique to variegating alleles of the Lt gene or was characteristic of other variegating heterochromatic genes, we assayed expression of the ctA gene. The ctA gene encodes a subunit of a G protein complex, and was identified as a maternal effect gene because it is required in female Drosophila for the normal development of their embryos (Parks and Wieschaus 1991). Using in situ hybridization to ctA RNA, we determined that the wild-type ctA gene was widely expressed in third instar larval tissues and showed uniform expression in the imaginal discs. The nascent ctA transcript was also detected as a single focus of staining in salivary gland nuclei. Variegation of the ctA gene in ovaries was initially detected as a variable reduction in progeny yield from individual females carrying a single wild-type copy of the ctA gene on an Lt variegation-inducing chromosome rearrangement (Wakimoto and Hearn 1990). We looked for variegation of ctA in larval tissues using LtX13/Df larvae, which have a single variegating copy of the ctA gene (Wakimoto and Hearn 1990). We determined that ctA was expressed in a variable fraction of the cells of the salivary gland (Figure 9, A and B). In...
Figure 6.—Expression of $\text{lt}$ in leg imaginal discs. In situ hybridization to $\text{lt}$ mRNA of leg discs from larvae bearing a $\text{lt}$-variegating rearrangement showed a reduced, nonvariegated staining pattern. Typical staining levels are shown for leg discs from larvae bearing a $\text{lt}$ deficiency chromosome heterozygous with the indicated $\text{lt}$ allele, from a single experiment.

contrast, the $\text{cta}$ gene appeared to be uniformly expressed in the cells of the imaginal discs. Figure 9 also illustrates the relative $\text{cta}$ mRNA staining levels of $\text{cta}^{+/}\text{Df}(C \text{ and } F)$, $\text{lt}^{G10}/\text{Df}(D \text{ and } G)$, and $\text{Df}(2L)\text{lt}^{102}/\text{Df}(E \text{ and } H)$ leg imaginal discs (C-E) and eye-antennal imaginal discs (F-H). Analogous to results for $\text{lt}$ expression, a reduced level of $\text{cta}$ mRNA staining was observed in $\text{lt}^{G10}/\text{Df}$ imaginal discs, as compared to wild-type discs.

**DISCUSSION**

This study explores tissue-specific and developmental changes in chromosome structure using PEV of the heterochromatic $\text{lt}$ and $\text{cta}$ genes as tools. The aims of this study were to determine if heterochromatic gene expression is variegated in all larval tissues and if rearrangements may show tissue-specific differences in the ability to induce variegation. Our analyses revealed that each of three chromosome rearrangements reduced $\text{lt}$ expression in all tissues examined. Interestingly, the relative severity of variegation in the adult eye, the larval Malpighian tubules, and the larval salivary glands was consistent in these tissues for the three $\text{lt}$-variegating alleles and reflected their effects on viability. These results suggest that variegation-inducing rearrangements do not have tissue-specific effects on gene expression. However, we did discover a developmental specificity to $\text{lt}$ variegation. For example, the imaginal eye-antennal disc showed a uniform, reduced level of $\text{lt}$ mRNA at the third larval instar although the adult eye exhibited variegated, sometimes patchy, $\text{lt}$ expression. This non-variegated mRNA staining pattern was observed in all imaginal discs for all three $\text{lt}$-variegating alleles. Moreover, the level of $\text{lt}$ mRNA detected in these discs was consistent with the severity of $\text{lt}$ variegation observed in variegated tissues. A second heterochromatic gene exhibiting PEV, the $\text{cta}$ gene, was similarly found to have a variegated pattern of expression in the salivary glands but reduced, nonvariegated mRNA levels in the imaginal discs.

Our observation that rearrangements can cause both a uniform reduction and a variegated pattern of expression for a single gene was unexpected. Previous phenotypic observations have been consistent with the classification of chromosome rearrangements into two categories by Lewis (1950): those involving only euchromatic breakpoints can exert stable effects on gene expression and others involving both heterochromatic
Strong staining for \textit{l}t RNA was observed in a subset of the hemocytes and was most apparent against a background of weak staining, as was obtained for discs from \textit{l}t\textsuperscript{X}2/Df and \textit{l}t\textsuperscript{G10}/Df larvae. A high magnification view of hemocytes associated with a \textit{l}t\textsuperscript{G10}/Df disc is shown in F. The \textit{l}t staining pattern was typically cytoplasmic, but a nuclear dot was also observed for some cells (arrow).

and euchromatic breakpoints can exert variegated effects on gene expression. However, the present analyses of the effects of rearrangements with heterochromatic and euchromatic breakpoints on \textit{l}t and cta expression illustrate that rearrangements can no longer be simply characterized as inducing either stable or variegated effects. For consistency, we retain the designation of "\textit{l}t-variegating" to describe these rearrangements, even though the current study illustrates that the effect of this class of rearrangements was decreased nonvariegated expression in the imaginal discs. A greater understanding of the relationship of the two elicited phenotypes should be enlightening as to the mechanisms of chromatin-induced position effects.

**Analyses of the effects of chromosome rearrangements on gene expression using in situ hybridization to RNA:** We have used RNA in situ hybridization to examine \textit{l}t expression in nonpigmented tissues. This approach monitors expression at the cellular level and has the added advantage of more accurately reflecting the transcriptional state of a gene compared to methods measuring its protein product or ultimate phenotype. Several factors can influence the concentration of specific cellular mRNAs, including the frequency of transcription initiation, the efficiency of RNA processing steps, and mRNA stability. While certain mutations and conditions can modulate these steps, the effects of variegation-inducing chromosome rearrangements are believed to be mediated through altered chromatin structure. We therefore think it most likely that we have assayed changes in transcription initiation or transcript elongation. For simplicity, the models presented below refer to transcription initiation, although they apply as well to synthesis of a full-length transcript.

We conclude from the nonvariegated imaginal disc staining pattern observed for \textit{l}t\textsuperscript{X}2/Df larvae that all imaginal cells transcribed the \textit{l}t gene, but at a lower level than that of cells bearing the nonrearranged allele. An alternate possibility, which we do not favor, is that variegated expression of \textit{l}t occurred in imaginal cells, but the \textit{l}t mRNA was sufficiently stable to mask the variegation pattern. If so, every imaginal cell was either currently expressing \textit{l}t or had inherited \textit{l}t mRNA due to expression in a previous generation. The uniformity of staining makes this possibility unlikely because it would require that the combined amount of \textit{l}t message was similar regardless of when and how long \textit{l}t transcription was "on" versus "off" in each cell's lineage. At the least, we would expect to have observed the effect of the exponential dilution of \textit{l}t RNA resulting from cell divi-
Figure 8.—Fluorescent detection of m RNA in eye-antennal imaginal discs from /t+/Df larvae. Uniform staining of eye-antennal discs was observed using in situ hybridization to m RNA and confocal microscopy. A representative 0.1-μm optical section from the lower eye portion of an eye-antennal imaginal disc is shown. The nuclei appear as black holes.

Dramatic remodeling of chromatin components during mitosis (Aparicio and Gottschling 1994; Martinez-Balbas et al. 1995). Furthermore, heterochromatic portions of eukaryotic genomes are generally later replicating than euchromatic genes, and chromosome rearrangements with breakpoints within these two domains could conceivably alter replication patterns in diploid cells. Indeed, rearrangements which cause It variegation do decrease the degree of DNA representation of the It gene in polytene tissues (M. Hearn and B. Wakimoto, unpublished data; M. Howe and B. Wakimoto, unpublished data).

A second model is based on the idea of a decreased rate of transcription, to explain the reduction of It and cta transcripts in imaginal tissues. It predicts that initiation or elongation of transcription is impeded as a result of disruption of normal heterochromatin formation in the vicinity of these heterochromatic genes. The consequence of chromosome rearrangements with breakpoints in the heterochromatin proximal to the It gene and in the distal euchromatin, is the isolation of a subregion of heterochromatin including It and other heterochromatic genes. This isolation reduces the variety and quantity of heterochromatin in the vicinity of the It gene, and in this way might restrict its ability to associate with particular heterochromatic proteins. For example, the affinity of a heterochromatic region for particular proteins and/or its propensity to assume specific chromatin conformations may be proportional to the quantity of certain repetitive sequence elements. Subdividing the total amount of any one repetitive element could have a dramatic effect on the efficacy of that region to attract a protein and/or take on a particular chromatin structure. If this rearrangement-induced heterochromatin protein deficiency includes one or more heterochromatic proteins that are required indirectly as local chromatin morphogens or directly as transcription factors for It transcription, then expression would be expected to be reduced. We favor this second model because it shares much in common, mechanistically, with the compartment model proposed to explain the variegated expression of heterochromatic genes (see below).

What determines whether a chromosome rearrangement causes variegated or reduced, uniform heterochromatic gene expression? We attribute the disparate effects of the It variegation-inducing chromosome rearrangements, i.e., mosaic expression in the adult eye, and larval Malpighian tubules and salivary glands, but nonvariegated reduced expression in imaginal tissues, to the differing developmental states of these cells. A diagram illustrating this model is shown in Figure 10. The imaginal disc cells are undifferentiated and are cycling or newly postmitotic. We propose that the heterochromatic factors required for It transcription are abundant within these undifferentiated nuclei. However, the impaired ability of the displaced heterochromatic region to attract the appropriate quantity and variety of heterochro-
matic proteins (as proposed above) results in reduced Lt transcription. In the differentiated nucleus, the displaced subregion of heterochromatin is likewise compromised in its ability to attract heterochromatic proteins. However, we suggest that a restriction in the abundance of these factors (perhaps related to the cessation of mitotic chromosome condensation) and the establishment of chromosomal interactions accompany differentiation and result in cell-to-cell differences in expression state. In the nuclear context of limiting concentrations of heterochromatin proteins, the relative ability of the isolated heterochromatic region to compete with pericentric heterochromatin for these components might often be insufficient to support any transcription. However, in a subset of the cell population physical interactions between the displaced heterochromatic region and pericentric heterochromatin could ameliorate this situation and allow for full expression (the compartment model; Wakimoto and Hearn 1990). We propose that the transition of the imaginal cells into a postmitotic, differentiated state allows for the formation of associations between different heterochromatic domains that are absent or transient in dividing cells. These associations are postulated to be important for the exchange of heterochromatin proteins and/or induction of a heterochromatic state necessary for heterochromatric gene expression. The correlation between the reduction of Lt transcription in the imaginal discs and the severity of variegation in the eye, salivary gland, and Malpighian tubules for the Lt\textsuperscript{G10}, Lt\textsuperscript{X2}, and Lt\textsuperscript{X13} alleles suggests a common factor influencing both phenotypes. According to our model, this correlation reflects the importance of the quantity of the displaced subregion of heterochromatin to heterochromatic gene expression throughout development. Cytological analyses of the Lt-variegating rearrangements indicated that the Lt gene-containing heterochromatic block was smaller for the Lt\textsuperscript{G10} chromosome than...
Figure 10.—A model to explain developmental differences in the effect of \textit{lt} var rearrangements on \textit{lt} expression. Expression of the heterochromatic \textit{lt} gene in both undifferentiated and differentiated cells is sensitive to the association of heterochromatin proteins with surrounding repetitive DNAs. Within the undifferentiated nucleus, heterochromatin proteins are abundant. When the \textit{lt} gene is present on a nonrearranged chromosome, it has a full complement of heterochromatin proteins and is expressed normally. However, the isolation of a subregion of heterochromatin due to chromosome rearrangement, as shown in A, leads to a reduction in the association of heterochromatin protein(s) and reduced \textit{lt} expression. Following differentiation (B), heterochromatic gene expression may be further compromised as a result of a decrease in the nuclear concentration of heterochromatin proteins. However, one or a few areas of high local concentration (compartments) of heterochromatin are formed by associations between regions of heterochromatin. Cells bearing an \textit{lt}-variegating chromosome rearrangement either express the \textit{lt} gene or do not express the \textit{lt} gene, depending on whether the displaced heterochromatic region is located within a heterochromatin compartment.

for the \textit{lt}^{12} and \textit{lt}^{13} chromosomes (that were not distinguishable from each other cytologically), consistent with the more severe effect of \textit{lt}^{10} on gene expression (Wakimoto and Hearn 1990). We propose that in imaginal cells, the expression level of the translocated \textit{lt} gene is reduced due to the decrease in quantity of associated heterochromatic factors, which in turn reflects the amount of surrounding heterochromatic DNA. When these cells differentiate, the magnitude of the displaced heterochromatin is also likely to influence its frequency of interaction with other heterochromatic compartments, and thus the severity of variegation.

The model we propose to explain the developmental change in the effects of chromosome rearrangements on heterochromatic gene expression bears similarity to the transvection effects model of Golic and Golic (1996). They proposed that the pairing ability of homologous chromosomes in mitotic cells was governed by cell cycle length such that homolog asynapsis induced by structural heterozygosity might be overcome given a long enough interphase. Our model that the interaction between displaced heterochromatin and pericentric regions is transient or absent in imaginal disc cells, but forms in a subset of differentiated cells, follows a similar rationale. However, we have yet to demonstrate these inferred heterochromatin associations and to show that they correlate with \textit{lt} expression. Although a few studies on variegation of the euchromatic brown (\textit{bw}) gene have examined the association of the \textit{bw} heterochromatic insertion with pericentric heterochromatin, the cells examined do not express the \textit{bw} gene (Talbert et al. 1994; Csík and Henikoff 1996; Dernburg et al. 1996). In the experiment most relevant to our studies of \textit{lt} variegation, the \textit{bw}^3-pericentric heterochromatin association was measured using squashed cell preparations derived from the eye-antennal imaginal disc (Dernburg et al. 1996). Only a low frequency of association was observed, perhaps reflecting the transient nature of the association in imaginal cells, or a mixed population of cell types in the preparation. Interestingly, the \textit{bw}^3-pericentric heterochromatin association was not observed in embryonic cells (Dernburg et al. 1996), possibly attributable to their short cell cycle length.

The relationship between position effect variegation of euchromatic and heterochromatic genes: It should be informative to study the properties of variegating alleles of other genes in order to gain further insight into chromatin behavior during development. Expression analyses of variegating genes conducted at the cellular level in multiple tissues at different stages of development have been performed for only one euchromatic gene (Lu et al. 1996) and two heterochromatic genes (this study), and may not reveal the full range of phenotypic effects of rearrangements. The characterization of variegating alleles of additional euchromatic genes is especially important as housekeeping genes or genes that are constitutively expressed may not behave similarly to the inducible \textit{hsp70-lacZ} gene. Preliminary analyses of a variegating allele of the euchromatic glycerol-3-phosphate dehydrogenase (\textit{gpdh}) gene by in situ hybridization to RNA suggest that differences may exist. Larvae bearing the \textit{T(Y:2)D222} rearrangement that in-
duces gpdh variegation (R. MacIntyre, personal communication) showed reduced, uniform imaginal disc expression (K. S. Weller, unpublished data). However, we cannot firmly conclude that gpdh variegation shows developmental specificity because of the absence of homozygous gpdh RNA null larvae to serve as a negative control for nonspecific staining. It may also be relevant that two adjacent euchromatic reporter genes present on a variegation-inducing rearrangement were observed to differ in the severity and pattern of variegation (Wines et al. 1996). However, these phenotypic differences could result from differences in the cell types compared and/or the transcriptional regulation of the two reporter genes.

The analyses of PEV of hsp70-lacZ, Lt, and cta suggest that heterochromatin formation is more dynamic during development than would have been predicted based upon early studies. Our conclusion that variegation of the Lt and cta genes is established during differentiation is consistent with the results of Lu et al. (1996) indicating that hsp70-lacZ variegation changes during eye differentiation. We postulate that a decrease in abundance of some heterochromatin proteins upon differentiation is in part responsible for the transition in Lt expression from a uniform to a variegated pattern. This idea would similarly explain the relaxation in repression observed for hsp70-lacZ induction during the same time period.

Previous studies have shown that heterochromatic genes require a heterochromatine environment for their normal expression (Wakimoto and Hearn 1990; Eberl et al. 1993), and we propose that a failure to achieve an optimally heterochromatine state is responsible for the observed decreased expression of the Lt gene in imaginal discs. However, this suboptimal heterochromatine state may be quite sufficient to have an antagonistic effect on euchromatic gene expression. Hence, the model we have proposed to explain the nonvariegated effect of chromosome rearrangements on heterochromatine gene expression in imaginal discs can accommodate the results of Lu et al. (1996) showing variegation of inducible hsp70-lacZ expression in these tissues. The mosaicism of hsp70-lacZ expression could reflect cell-to-cell differences in the association of one or more heterochromatine proteins at the site of the transposon insertion (i.e., spreading), whereas heterochromatine gene expression could reflect the status of the entire heterochromatine domain. In support of this model, we have previously shown that the severity of variegation of the euchromatic w gene was not indicative of the quantity of adjacent heterochromatin (Howe et al. 1995). Rather, w variegation was sensitive to the nature of the juxtaposed repetitive DNA. Taken together, these studies reflect the diverse biological activities of heterochromatin and the fact that position effects exerted by rearrangements on euchromatic vs. heterochromatine genes are not strictly reciprocal phenomena.

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