Analysis of Properties of Heterochromatin Relative to Meiotic Recombination and Heterochromatic Gene Expression

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Abstract

To better understand the genetic properties of heterochromatin, I have pursued two avenues: meiotic recombination around the border of euchromatin and heterochromatin, and position effects at the heterochromatic *light* locus. Heterochromatin lacks recombination, and using meiotic recombination frequencies, I show that the recombination inhibition border concurs with the previously defined molecular border based on changes in histone proteins, specifically histone 3 methylation characteristic of heterochromatin. I also show that the heterochromatic gene *light* behaves in a similar fashion to a previously studied heterochromatic gene, in that its function is impaired when moved out of the heterochromatic environment, but can be restored when brought near to large blocks of heterochromatin. These findings support the idea that gene function and recombination can be tightly controlled by the molecular environment of heterochromatin.

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CHAPTER 1

General Introduction

1.1 Early history of heterochromatin

The study of heterochromatin, i.e., regions of the genome which remain compacted throughout most of the cell cycle, originates mainly with Emil Heitz. Although largely unrecognized in his own time, his cytological explorations are fundamental to the work being done to explore the nature of heterochromatin today. In his landmark paper on cytological properties of liverwort mosses (genus: *Pellia*), Heitz coined the term “heterochromatin”, derived from the earlier term “heterochromosome” to refer to entire chromosomes that remain heteropycnotic during most of the cell cycle (reported in Heitz 1928, see Passarge 1979 for a review of Heitz's important discoveries in English). The differential staining of various chromosomal elements emerged from earlier investigations on the nature of sex- differentiation in insects. Working in the early 1900's with insects of the order *Orthoptera*, McClung (1902) determined that a chromatin body that was known to stain darker than other chromatin threads, and existed in the nucleolus region of the cell, which would only be transmitted to half of the daughter cells during meiosis, was responsible for determining the sex of offspring. This chromatic body, which stained dark even when other chromosomes were weakly stained, was known as the “accessory” chromosome. It later came to be known as the X chromosome from a figure in which Henking (who also knew it as the accessory chromosome) labeled it with an “X” (McClung 1902).

The terminology started to change with Nettie Stevens's use of “heterochromosome” in 1905 to describe XX/XY type sex determination (Richardson 2013). Stevens, a cytologist and student of Thomas Hunt Morgan's at Bryn Mawr

College, discovered a small chromosome in males that paired with the large accessory chromosome, which later came to be known as the Y chromosome. The pairing of the unequally sized chromosomes responsible for sex determination is why she preferred the term “heterochromosome”, a term coined by TH Montgomery in 1904 (Richardson). Stevens's recognition of a heterogeneous pairing unit of chromosomes in various insect species starting with the mealworm (Genus: *Tenebrio*) and including, of course, the fruit fly (*Drosophila melanogaster*), led the way for “heterochromosome” to displace “accessory chromosome” and other competing terms when discussing the sex chromosomes (Carlson 2004).

By the time Emil Heitz was studying chromatin structure in mosses, “heterochromosome” had already taken firm hold in the lexicon, and from it he derived “heterochromatin” to denote those parts of the chromosome that he observed to remain condensed throughout interphase. He was the first to describe longitudinal differences in chromosome structure during different phases of the cell cycle, and defined “heteropycnosis” as “the differential behaviour of a whole or part of any chromosome at prophase and telophase during the entire development of an individual or during a certain stage of development” (Passarge 1979). Out of his cytological studies on mosses, he recognized that not only could entire chromosomes be heteropycnotic, but that autosomes could also show longitudinal differentiation between parts of the chromosome that stained heavily throughout the cell cycle, and those that became invisible at late prophase (Heitz 1928, reviewed in Passarge 1979). Following the chromosomes throughout the cell cycle with a staining method he developed (using

carmine- acetic acid), Heitz was able to reproducibly demonstrate that there were specific regions of chromosomes that remained heterochromatic. Recognizing the dark staining regions as cytological traits, and not artifacts of his preparations, Heitz (1928) says "The cause of heteropycnosis can only lie in the concerned chromosomes themselves (translation from German found in Passarge 1979)." In those very early days of heterochromatin research, Heitz recognized the two types of heterochromatin that we refer to today as "facultative" and "constitutive" heterochromatin. Facultative heterochromatin refers to regions of chromosomes and entire chromosomes that are selectively inactivated in certain cells and tissues of the organism, but not in others, whereas constitutive heterochromatin refers to regions of the genome that are invariably heterochromatic throughout cell and tissue types, and often serve as a structural element of the chromosome. My research is focused on constitutive heterochromatin, and unless specifically noted, my use of the term "heterochromatin" is used in reference to the constitutive type of heterochromatin as defined by Brown (1966).

1.2 Cytological properties of heterochromatin

Constitutive heterochromatin is a feature that is ubiquitous in all multicellular eukaryotic genomes studied thus far (Renauld and Gasser 1997), and there are many distinctive cytological characteristics that distinguish heterochromatin from euchromatin. The most obvious determining characteristic, as was noticed by Heitz back in the late 1920s and early 1930s is the darker appearance of heterochromatin compared to euchromatin that is seen when using appropriate stains. Such deeper staining is indicative

of a higher degree of compaction, as euchromatin shows similar staining at various points of the cycle as it goes through stages of condensation and decondensation. As the cell readies itself for mitotic division at prophase, the chromosomes (including euchromatic regions) condense and, when stained, appear darker than they do during interphase. They remain condensed until telophase when they seem to "disappear". Unlike euchromatin, heterochromatic regions of the chromosomes remain highly compacted through all stages of the cell cycle (Grewal and Jia 2007), including interphase, and therefore display dark staining throughout.

Another defining characteristic of heterochromatin is its ability to form chromocenters, darkly staining aggregates of centromeric heterochromatin from which the rest of the chromosome appears to radiate (Lu et al. 2009). This only takes place in certain cells, and is most easily seen in the giant polytene chromosomes of *Drosophila*. In *Drosophila melanogaster* larval salivary gland cells, all chrommosomes undergo polytenization, which is a replication of genetic material without cellular division. During polytenization, chromosomes go through 10 rounds of replication without dividing, resulting in a giant stack of 1024 copies of each major chromosome. In the polytene chromosomes, there is still longitudinal differentiation of heterochromatic and euchromatic regions of chromosomes, as heterochromatin and euchromatin show different characteristics when undergoing polytenization. The euchromatic segments show a copy number of around 1024n, while the heterochromatic segments tend to end polytenization with a copy number closer to 2n. The heterochromatin of the pericentromeric regions of the chromosomes coalesce into a structure known as the

chromocentre.

1.3 Molecular properties of heterochromatin

The most striking cytological feature of heterochromatin, its heavy staining, is a property of its molecular structure. The nucleosome, the basic unit of genome compaction in the nucleus, is coiled in a much tighter conformation for heterochromatin than it is for euchromatin. In eukaryotic cells, an octomer of 4 histone proteins (H2A, H2B, H3, H4) form the core of a nucleosome, around which 146 nucleotide pairs of genomic DNA are wrapped, forming a "bead" chromatin. Between one nucleosome and another are up to about 80 nucleotide pairs, after which another bead is formed, continuing in this fashion until an entire chromosome is packaged. Molecular modifications of the histone core proteins allow for a much tighter packaging of heterochromatic areas. The most well known of these modifications is dimethylation of lysine 9 on histone H3 (H3K9me2) (Boros 2012). In *D. melanogaster* H3K9me2 recruits a heterochromatin specific protein, heterochromatin protein 1 (HP1), which recruits the H3K9 methyltransferase Supressor of Variegation 3-9 (Su(var)3-9), leading to further H3K9 methylation, creating a feedback loop to heterochromatinize a chromosome segment. HP1, a *Drosophila* protein which shares sequence and function homology with the human protein Heterochromatin Protein 1 (Hsalpha) ($HP1^{hs\alpha}$) (Eissenberg and Elgin 2000), is highly enriched at heterochromatic regions of the genome, and considered to be a hallmark of heterochromatin (James et al. 1989).

HP1 was first discovered in *D. melanogaster* by researchers looking into the phenomenon of Position Effect Variegation (PEV, to be further discussed below), a

phenomenon in which euchromatic genes, when placed in a small block near to a large block of heterochromatin are variably silenced. The silencing can be reversed by replacing the gene back into a more euchromatic environment. HP1 was originally identified as a modifier of PEV (Eissenberg and Elgin 2000), and it is encoded by the gene *Su(var)2-5*. The HP1 protein has 2 prominent structural motifs: a chromodomain for binding to chromatin, and a chromoshadow domain necessary for protein-protein interactions (Aasland and Stewart 1995). The current model for heterochromatin formation posits that Su(var)3-9 methylates histone H3 at K9, which creates a binding site for HP1's chromodomain, while still interacting with HP1 via its chromoshadow domain. The complex of chromatin, histone H3 and HP1 forms a specialized, higher order conformation associated with its cytologically recognizable more compact appearance when compared to euchromatin. Such tight conformation is often associated with gene silencing (Fanti and Pimpinelli 2008).

Not only are there characteristic proteins that define heterochromatin, but there also exist nucleotide sequences that are generally thought to be characteristic of heterochromatin. The nucleotide composition is believed to play a large part in establishing the heterochromatic environment, as well as providing structural support. The most highly characterized of these special nucleotide regions are the satellite sequences. Satellite sequences, so-called because of their having been found separate from the major DNA band in a cesium-chloride gradient, are long tandem repeats of varying length found in heterochromatic regions of the genome. The length of the repeat can be as short as 2 base pairs such as the $(AT)_n$ repeat found in the crab *Cancer borealis*

(Gray and Skinner 1974) or as large as hundreds of base pairs such as the 359 base pair repeat found in *Drosophila* (Hsieh and Brutlag 1979). The presence of satellite sequences is highly conserved among higher eukaryotes, but neither the sequence nor the number of tandem repeats is conserved and varies widely even among closely related species (see Hilliker and Taylor-Kamall 2013 for a commentary). Highly repetitive DNA is one of the main factors behind the C-value paradox, wherein less complex organisms can show a greater genomic content than more complex ones, or closely related species with similar numbers of genes can have wildly varying DNA content (Freeling et al. 2015). It is currently thought that the highly conserved nature of their presence, but not their sequence, and the fact that they are untranscribed throughout an organism's life points to their importance in establishing an environment for proper functioning of genes that lie in heterochromatin. Another possible reason for their importance is to establish enough distance for euchromatic genes to avoid the centromere effect, which is a reduction in genetic recombination seen as the distance to the centromere decreases. This notion is based on the fact that highly repetitive DNA is so ubiquitous in the heterochromatic regions of higher eukaryotic genomes.

Differential replication of heterochromatin and euchromatin is another hallmark that defines heterochromatin. Heterochromatic regions of the genome are known to replicate much later than euchromatin during mitosis. Analyzing tritium incorporation into replicating genomes, Lima-de-Faria (1959) showed that the heterochomatic sex chromosomes of grasshoppers (*Melanoplus differentialis*) were synthesized later than the euchromatic autosomes. Extending these findings to nuclei of rye leaves, Lima-de-Faria

once again found the timing of heterochromatic and euchromatic DNA to be asynchronus in the same chromosome. Taylor (1960) confirmed these findings in cultured cells of the Chinese hamster by following incorporation of thymidine-H^3 into rapidly growing cells. Late replication, chromocentre formation, and hypercondensation are all cytological properties that differentiate heterochromatin from euchromatin, and there has been much work done in recent years to elucidate the molecular basis of these differences.

1.4 Genetic properties of heterochromatin

For many years since its discovery, it was believed that heterochromatin was simply a means of silencing transcription, but it is now known that there are genes within the heterochromatin of some species, and that the heterochromatic environment is required for their proper functioning. For example, in *D. melanogaster*, the entire Y chromosome is heterochromatic, yet an *XO* male, while being phenotypically normal will be sterile (reviewed in Hilliker et al. 1980). The current estimate for the number of heterochromatic genes in *D. melanogaster* now stands at around 450. Beginning with deletion analysis in the late 1920s, and built upon by genome sequencing methods of today (reviewed inYashuhara and Wakimoto 2006), the number of genes that have been consigned to heterochromatin has shown that heterochromatin can no longer be regarded as merely involved in silencing (Hoskins et al. 2002).

One of the earliest projects to definitively map genes to heterochromatic regions of the autosomes was the 1976 undertaking of Hilliker using deletions of the left and right arms of *D. melanogaster* chromosome 2 (2L and 2R) to find essential genes by

ethylmethanesulfonate (EMS) mutagenesis. One of the properties uncovered in this experiment, apart from the fact that there certainly are genes residing in heterochromatin, was that genes in heterochromatin are at a much lower density than euchromatic genes. Since then, many more genes have been found by genetic methods, defining around 30 loci (Hoskins et al. 2002), but it was not until the advent of large genome projects which attempt to sequence and annotate entire species's genomes, that the number of putative genes in *Drosophila* heterochromatin started to skyrocket.

The announcement of the complete euchromatic genome for *D. melanogaster* in 2000 (Adams et al.) was a first step to finally elucidating the genetic content of the heterochromatic regions. Due to the highly repetitive nature of genomic heterochromatin, it was understandably a more difficult task. With heterochromatin being approximately 30% of the *D. melanogaster* genome, the *Drospohila* Heterochromatin Genome Project (DHGP) was started. Beginning with analysis of sequences generated by whole genome shotgun sequence assembly for the original euchromatic genome project, 297 protein-coding and six non-protein-coding genes (including previously known genes) were predicted to reside in centromeric heterochromatin (Hoskins et al. 2002). By 2007, the DHGP had definitively concluded that there were at least 230 unique sequence genes, as well as 32 pseudogenes and 13 non-coding RNAs residing within centromeric heterochromatin (Smith et al. 2007).

Most of the genes found in heterochromatin are not typical compared to euchromatic genes. One of the foremost differences is in their exon/intron makeup. Heterochromatic genes can have extremely large introns between their relatively small

exons (Hoskins et al. 2002), and these introns often having transposable elements embedded within. Because of differences in molecular makeup, it is not surprising that heterochromatic genes do not fare well when rearranged such that they end up in close proximity to euchromatin. In 1993, Eberl et al. showed that the heterochromatic environment was necessary for the functioning a prototypical heterochromatic gene, *rolled*, originally determined to be heterochromatic in Hilliker's 1976 deletion mapping. It was discovered that when relocated in a sufficiently small block of heterochromatin into euchromatin, *rolled* no longer functioned normally, and showed the characteristic rolled down wings of a *rolled* mutant of *D. melanogaster*. When these small heterochromatic sections were brought closer to large sections of heterochromatin, this position-effect was reverted. Such position effects, as opposed to mutation, were originally why heterochromatin was thought to be merely a means of genetic silencing. Although the early position effects were discovered by moving genes within small blocks of euchromatin into close proximity with heterochromatin, there is now an abundance of evidence highlighting the importance of the molecular environment

As has been previously mentioned, the phenomenon of PEV was an early reason that heterochromatin was thought to have only a silencing effect in the genome. As a basic definition, PEV refers to the mosaic expression of a gene lying near a breakpoint of a chromosomal rearrangement (reviewed in Spofford 1976). In some cells, the gene will function normally, while in others its function and ultimately its expression, will be adversely affected. This is most readily seen in cell-autonomous phenotypes such as eye colour in *D. melanogaster* where each separate facet of the compound eye contains a

number of pigment cells. The first locus found in *D. melanogaster* to display PEV was the *white* gene, mutants of which show white eyes instead of the normal orange-red eye. Each facet of the compound eye, known as an ommatidium, has 12 pigment cells, which allows for very fine grain analysis of variegation. Flies showing position effects at *white* were originally isolated by Muller in the early 1930s during his experiments with X-rays (Demerec and Slizynska 1937), and were originally described as having a "mottled" phenotype to describe the pepper-and-salt nature of the eye colouring. Since that time, all genes that have been specifically analyzed for euchromatic position effect have been shown to be susceptible (Spofford). While most of them are recessive phenotypes, there is also a well known dominant PEV phenotype, that of the *brown* locus. The euchromatic position effect has been well established, but it has long been known that a similar effect happens when normally heterochromatic genes are moved in small blocks of heterochromatin into a euchromatic environment. Hessler (1958) was able to induce position effects at the *light* locus (*lt*), which resides within heterochromatin. *lt* is another contributor to eye colour phenotype, classical mutants of which show an eye colour of a lighter orange than the wild-type red-orange. Using X-rays, Hessler was able to rearrange *lt,* which normally sits in distal heterochromatin, to lie closer to euchromatin via rearrangements that had at least one break in 2L proximal heterochromatin and another in the distal three fourths of chromsomes 2, 3, or X. Because *lt* is an eye colour gene, rearrangements were readily picked up phenotypically as showing either "dark mottled" when the eyes would show a generally wild type phenotype with a sprinkling of darker ommatidia, or "pale mottled" when they eyes would appear as a mixture of light and

wild-type ommatidia. Eberl et al. (1993) were able to revert heterochromatic position effects at the *rolled* locus by radiation induced rearrangements that brought *rolled* into close enough proximity with a large block of heterochromatin.

The border between heterochromatin and euchromatin is still somewhat mysterious. From the perspective of the histones, the border region seems to be rather abrupt, as a sharp transition from the H3K9me2 enriched domain to the H3K9me2 non-enriched domain (Yasuhara and Wakimoto 2008, Noma et al. 2001). From a genetic perspective, the border region is a demarcation of where meiotic recombination stops taking place: on the euchromatic side, crossing over is possible, but on the heterochromatic side, it is suppressed to the point of silence. The point at which the recombination effect switches over is not very well understood, but Westphal and Reuter (2002) demonstrated that mutations that suppressed PEV were able to induce recombination in heterochromatin of *D. melanogaster*, signifying the importance of the proteins that establish heterochromatin conformation in suppressing recombination within heterochromatin.

The aims of this my work were two pronged: 1) to find out if the junction of euchromatin and heterochromatin as defined by histone content corresponds to the site at which inhibition of recombination is seen, and 2) to determine if *lt* behaves like *rolled* in the necessity of the heterochromatic environment for proper expression. In pursuit of the first aim, I searched for recombination around the histone defined border with the hypothesis that I would see no recombination in heterochromatin or very near to the histone-defined border region or in heterochromatin, but it would be increasingly present

in the euchromatic direction. For aim 2, the working hypothesis was that *lt* would behave in a similar fashion to *rolled*, in that if I could find position effects at *lt*, I would be able to revert those effects by chromosomal rearrangement, hopefully leading to a more general picture of the necessities for heterochromatic gene action.

CHAPTER 2

Effects of Heterochromatin on Recombination at the Border of Euchromatin and Heterochromatin

2.1 Introduction

It is still not yet known with certainty what determines the euchromatin-heterochromatin border, but where the epigenomic border is established has now been defined with fairly high certainty (Riddle et al. 2011). I am interested in learning what the effects of the presumed border are on genetic phenomena, particularly meiotic recombination. It has long been known that there is very low frequency of recombination for heterochromatic areas, and areas immediately adjacent (Carpenter and Baker 1982), so this work attempts to resolve the proximity of the genetic border, as defined by meiotic recombination, to the histone-defined genomic border of euchromatin and heterochromatin. This is necessary work to determine how far the suppression of genetic effects by heterochromatin extends into adjacent euchromatin in order to further understand the molecular genetic landscape of differing chromosomal conformations. In order to further elucidate the genetic effects of the molecular landscape, I used transgenic insertions carrying a *mini-white* insertion to flank the border region based on the hypothesis that I will see evidence of meiotic recombination on the euchromatic side of the histone-defined border, but not on the heterochromatic side. Exactly where or how close to the border I would see evidence for recombination was unknown, as I was attempting to determine where the genetic border lies.

Mini-*white* P elements

In order to investigate the effect of the euchromatic-heterochromatic border region on meiotic recombination, I chose fly stocks that had markers on either side of the putative border of chromosome 2L. Fly stocks were generated as part of the Gene

Disruption Project, an offshoot of the Berkeley Drosophila Genome Project (BDGP), which is an attempt to disrupt each gene in the genome of *D. melanogaster* with a single transposable element (TE). TEs, particularly the *P*-transposable element (*P element*), have been widely used for many years to disrupt the *D. melanogaster* genome (Ryder and Russell 2003). The *P* element commonly used for mutagenesis is an engineered derivative of a naturally occurring TE that contains the *transposase* gene and inverse terminal repeats which are recognized by the transposase protein (O'Hare and Rubin 1983). Transposase, upon recognizing the terminal repeats, is able to excise the *P element*, and translocate it to another region of the genome (reviewed in Ryder and Russell 2003). For use in directed mutagenesis, *transposase* is removed from the *P element* to be inserted. This *P element* construct is then co-injected with an element that produces Transposase, but is itself stabilized by having damaged ends, thus eliminating the recognition sites for Transposase to excise it out. The *P element* being used for insertional mutagenesis is stable, as it has no transposase activity itself, as is the transposase carrying element since it has no transposase recognition sites.

The transgenic *D. melanogaster* stocks used in this study were created by using the *suppressor P element* (*SUPorP*), a composite transposon that combines the mutagenic efficiency of the *gypsy* TE, necessary for reliable insertion into heterochromatin (as other TEs do not reliably insert into heterochromatin), with the highly controllable and well characterized mobilization of the *P element* (Roseman et al. 1995). The *SUPorP element* flanks a mini-*white* reporter gene with binding sites for the suppressor of Hairy wing [Su(Hw)] protein, borrowed from the *gypsy* retrovirus's method of insulating its

retrotransposon from chromosomal location effects such as the suppression seen in PEV. Mini-*white* is a truncated version of the *white* eye colour gene that can be reliably expressed in transgenes, which can rescue the eye phenotype in flies on a mutant *white* genetic background.

To investigate meiotic recombination frequencies around the heterochromatic border region, flies of a mutant *white* background with the *SUPorP element* inserted near to the putative euchromatic – heterochromatic border region of the left arm of *D. melanogaster* chromosome 2 (2L) were chosen. Recombination frequencies for these areas were determined by mating two different lines bearing the *SUPorP element* to each other, then mating female progeny (as males do not undergo meiotic recombination) of that cross to *white* mutant flies. If a recombination event takes place in the fly heterozygous for the *SUPorP elements* that involves the *SUPorP element*, half of her progeny descending from that oocyte, when crossed to a *white* mutant, will show white eyes, as the mini-*white* gene is no longer present in the genome (Fig. 1). *SUPorP* fly lines were chosen based on their proximity to the histone-defined border region, heterochromatin being proximal on the centromeric side of *CG3635*, and euchromatin being distal (Yasuhara and Wakimoto 2008), as well as their ability to survive as unbalanced homozygotes. My hypothesis before starting this study was that recombination would only be seen in the euchromatic insertions, and that their distance from the putative border would define its recombination rate, as opposed to their distance from the heterochromatic insertion.

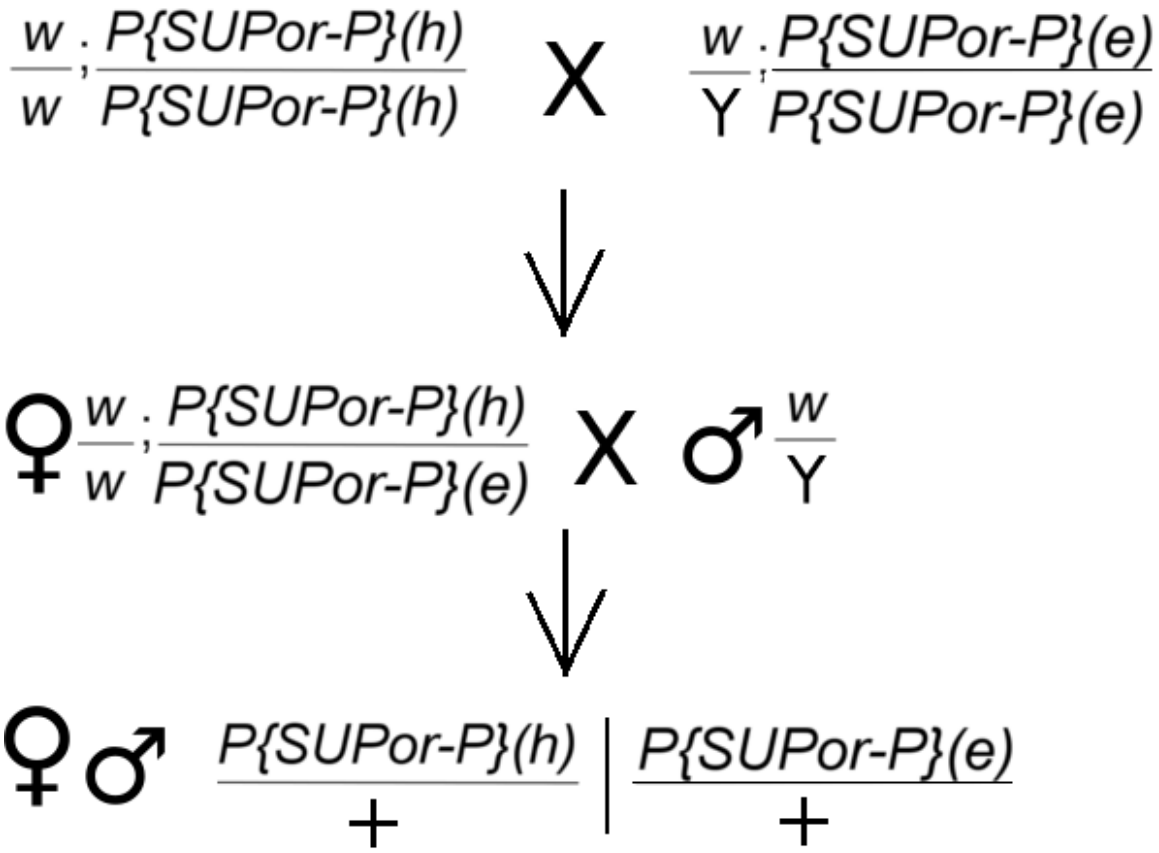

Figure 1. Simplified cross to investigate recombination around the 2L euchromatic - heterochromatic border region. Flies with a mini-*white* insertion (contained in the *SUPorP element)* proximal to the border (heterochromatic) are mated to flies with a mini-*white* insertion distal to the border (euchromatin) (P), and their progeny (F1) are mated to white eyed flies. Flies in the F2 generation are screened for white eyes, indicating that a recombination event has taken place, due to the insertion no longer being present to express the mini-*white* gene. Transgenic flies were developed on a mutant *white* genetic background, signified by "*w*" homozygous on the X chromosome. Mutant *white* male flies used to cross to the F1 are signified by the "*w*" on the X chromosome heterozygous with a normal Y chromosome.

2.2 Methods and Materials

Fly stocks and care

Transgenic flies with a mini-white insertion near the heterochromatic – euchromatic border region were ordered from the Bloomington *Drosophila* Stock Center at Indiana University, available through http://flystocks.bio.indiana.edu/. For the sake of simplicity, I have renamed the fly stocks based on their position of insertion into the genome, using H for heterochromatic insertions and E for euchromatic insertions. The naming is based on the putative border region identified by modifications of histones, which is the putative *D. melanogaster* gene CG3635, lying at nucleotide position 22175415 of chromosome arm 2L (Yasuhara and Wakimoto 2008). Insertion sites are shown in Tables 1 and 2 and figure 2. Flies carrying the transgenic insertion are of the genotype w^{1118}; *P{SUPor-P}*, signifying a loss of function mutation at *white*, which is on the X chromosome, and the insertion of the mini-*white* gene contained in the *P{SUPor-P} element* on the second chromosome.

Males carrying the heterochromatic insertion were crossed to virgin females carrying the euchromatic insertion to generate flies heterozygous for the different insertions. Female progeny of that cross were crossed to w^1 males, which also carry a loss of function mutation at the *white* locus. Heterozygotes were mated to w^1 on standard *Drosophila* medium for 2-3 days, after which they were placed on fresh food (rebrooding). Progeny of the flies heterozygous for the insertions and the w^1 flies were scored for eye colour phenotype, as a white eyed fly would not be carrying the *P{SUPor-P} element*, signifying that a recombination event had taken place.

Genomic DNA of the putative recombinants was analyzed using PCR with primers specific for the *P{SUPor-P} element* to verify that it had been removed. PCR amplification was also used to confirm the presence of genomic DNA by constructing primers directed to the essential functional domain at the 3' end of the chromosome 2 euchromatic gene *crumbs* (Coulthard 2010) as a positive control. The control primer pair was designed to produce a 1383 bp product, and the primer pair specific for the insertion was designed to produce a 208 bp product. The primers are listed in Table 3. PCR primers were designed to have a melting temperature between 60º C and 64º C. Using these primers, the steps in the PCR were an initial DNA melting at 95º C, followed by 35 rounds of 1) melting at 95º C for 30 seconds, 2) primer annealing at 58º C for 30 seconds, and 3) polymerase extension at 72º C, followed by a final extension at 72º C for 10 minutes. PCR products were then run on a 1% agarose gel stained with ethidium bromide.

Heterochromatic Insertions

Insertion Name	Insertion Site	Distance from putative border (base pairs)
H1	22 654 978	479 563
H3	22 239 540	64 125
H5	22 178 766	3 351

Table 1. Heterochromatic insertion sites of the *P{SUPor-P}* transgenic element, and their distances from the histone defined euchromatic – heterochromatic border region.

Euchromatic Insertions

Insertion Name	Insertion Site	Distance from putative border (base pairs)
E2	21 795 549	379 866
E7	22 078 760	96 655
E10	22 117 782	57 633
E11	22 132 728	42 687

Table 2. Euchromatic insertion sites of the *P{SUPor-P}* transgenic element, and their distances from the putative euchromatic – heterochromatic border region.

Primers Used to Verify Recombinants

Primer	Left Primer	Right Primer
P{SUPor-P}	5'- tttttgtgcatctaggatag	5'- aactgcgacccacaaaaatc
Control	5'- ggcaactgcacggatcttat	5'- gggcggtacgtatgtcatct

Table 3. Primers designed for PCR amplification of *P{SUPor-P} element* and a segment of the *crumbs* gene. Amplification of *P{SUPor-P} element* was used to verify the absence of the element, and amplification of the *crumbs* gene was used as a control to verify that genomic DNA was present.

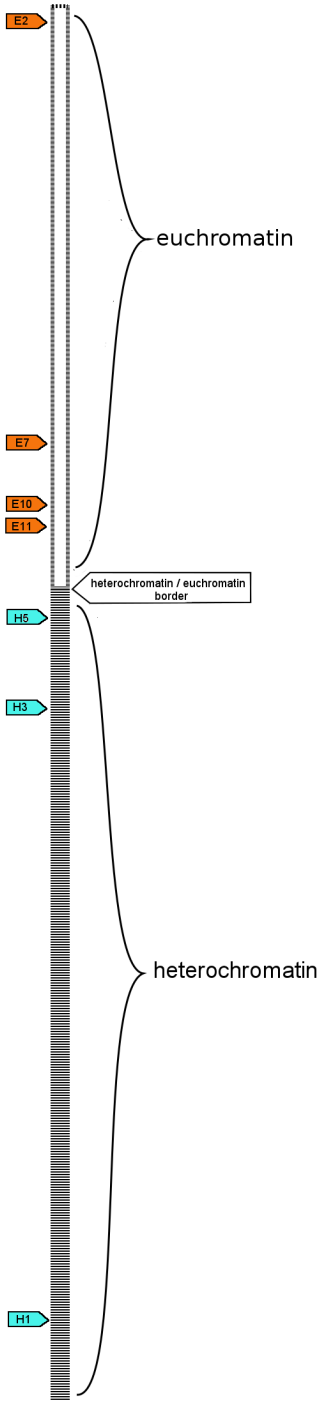

Figure 2. Approximate scale map showing locations of *P{SUPor-P} element* insertions near the border region of pericentric heterochromatin and euchromatin for the left arm of chromosome 2.

2.3 Results of the recombination assay

Evidence of meiotic recombination was seen when the euchromatic insertion was sufficiently distal to the histone defined heterochromatin – euchromatin border. As summarized in Table 4, only crosses that involved at least one insertion on the euchromatic side of the border displayed evidence of meiotic recombination. Insertions E10 and E11, inserted 39 022 bp and 53 968 bp closer to the border than E7, respectively, did not show evidence of recombination, whereas E7 was the most proximal of insertions for which recombinants were found. There was no evidence of recombination when the heterozygous insertions were both in heterochromatin. When fly line E7 was initially tested, flies of varying eye phenotypes were seen, with some flies showing wild-type eyes, and some flies showing extreme variegation, as shown in figure 3. This is most likely due to the insertion's placement within heterochromatin, and is most likely a position effect. The original E7 lines that showed evidence of recombination (103 410 flies scored) were not confirmed with PCR, so the cross was repeated (75 982 flies scored), and all future putative recombinants were verified by PCR. An example PCR gel displaying the bands corresponding to the primers for the *P{SUPor-P} element* and the control segment is shown in figure 4.

Recombination events for insertion heterozygotes

Parental Strains	Number of Progeny Scored	Recombinants Scored	% recombination	95% confidence limits
H1 and H3	92 749	0	0	$0 – 4.0x10^{-3}$
H1 and H5	148 997	0	0	$0 – 2.5x10^{-3}$
H1 and E11	151 560	0	0	$0 – 2.4x10^{-3}$
H1 and E10	153 349	0	0	$0 – 2.4x10^{-3}$
H1 and E7*	103 410	16	0.015	$8.9x10^{-3} – 0.029$
H1 and E7	75 982	13	0.017	$9.1x10^{-3} – 0.029$
H1 and E2	99 178	15	0.015	$8.5x10^{-3} – 0.025$
H3 and E7	59 058	7	0.012	$4.6x10^{-3} – 0.024$
E7 and E7	51 296	0	0	$0 – 6.2x10^{-3}$

Table 4. Results of scoring flies heterozygous for heterochromatic (H) and euchromatic (E) insertions of the *P{SUPor-P} element* mated to flies with a mutant allele of *white*. Recombinant scoring was based on eye colour, as flies with white eyes have lost the *P{SUPor-P} element* containing the mini-*white* gene. 95% confidence limits were calculated using the method described by Stevens (1942).

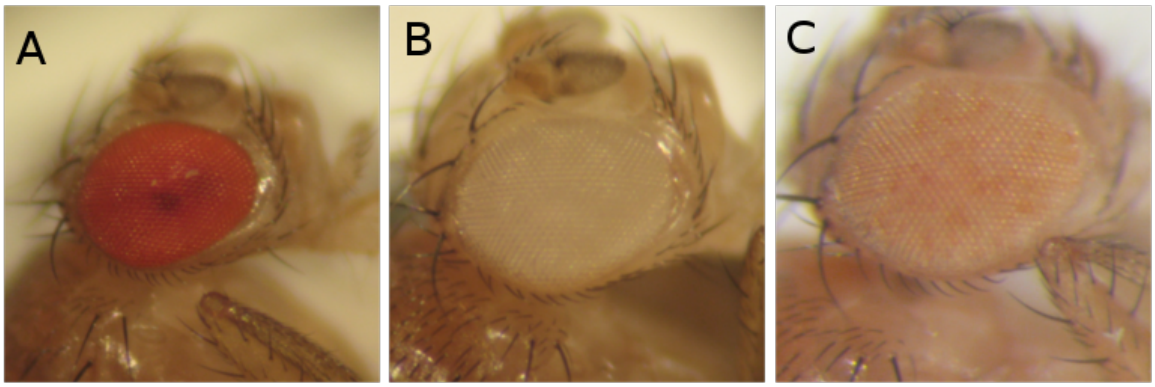

Figure 3. Scoring classifications used to determinine recombinants. A red-orange eyed fly (ry^{+5}) displaying the wild type phenotype (A), a mutant *white* fly (w^1/w^1) (B), and a variegating mini-*white* bearing fly (*w*; *P{SUPor-P}*). The variegated phenotype was shown by many flies with a heterochromatic insertion of the *P{SUPor-P} element*. Flies with the *P{SUPor-P} element* in heterochromatin show phenotypes in between wild type and the extreme variegated phenotype shown in C, while flies with the *P{SUPor-P} element* in euchromatin show a wild-type eye phenotype.

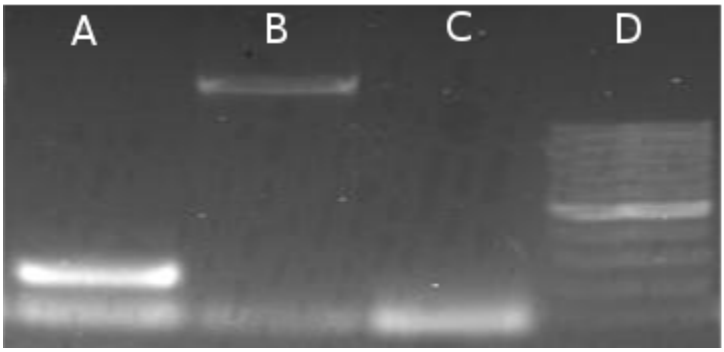

Figure 4. PCR products visualized on a 1% agarose gel. Putative recombinants, i.e., flies in which a recombinant event between the transgenic insertions has taken place, were homogenized and their genomic DNA was used for PCR amplification. Products shown are a 208 bp product (A) representative of the *P{SUPor-P} element*, a 1383 bp product (B) representative of the control primers directed at the *crumbs* gene, and a 100 bp DNA ladder (D). Lane C shows a reaction in which there was no genomic DNA.

2.4 Discussion of recombination results

The experimental results examining the properties of heterochromatin relative to meiotic recombination are in accordance with the placement of the euchromatin-heterochromatin border defined by Yasuhara and Wakimoto (2008). That there were no recombination events scored when the insertions were both in heterochromatin is in accordance with many previous studies that recombination is suppressed in heterochromatin (Carpenter and Baker 1982), and also lends credence to the assumption that those inserted elements were indeed located in heterochromatin. Also, it should be noted that since reversions to the white-eyed phenotype were not seen in the control cross with flies homozygous for the E7 insertion, the insertions appear to be stable.

For the euchromatic insertions near the border region that did not show any recombination events, it appears that the border region may not be a distinct and well defined transition, and that heterochromatin may be attenuating recombination of DNA near to, but not necessarily in, heterochromatin. Riddle et al. (2011) found that the euchromatin - heterochromatin border, once again defined by histone modifications, was different for various stages of development and for different cell types. Using chromatin immunoprecipitation (ChIP), Riddle's team assayed various stages of development and cell types for classical “silencing” marks such as H3K9me2, and “active” marks such as H3K4me3, and their border varied from 22 040 000 in larvae to 22 900 000 in BG3 cells. In adults, which were tested using fly heads, the border was located at 22 160 000, but in newly developing embryos (2-4 hours old), the border was located at 22 150 000. It is no surprise that meiotic recombination on the heterochromatic side of the presumed border

was found to be non-existent, but abruptly increased as the insertions became more distal. This is very much in line with previous studies which showed very low recombination frequencies per unit physical length for areas adjacent to to centromeric heterochromatin compared to to the high and uniform frequency of exchange along the rest of the chromosome (Carpenter and Baker 1982). For the insertions that did show evidence of recombination, the percent recombination frequencies were all very close to each other (0.012 – 0.017), such that it cannot be said with any certainty that the frequencies are dictated by the distanec of the insertion from the border. Instead, it can be said that there is an abrupt increase in recombination somewhere in the neighbourhood of 96 kilobases from the putative border on the euchromatic side. The presumed border region between euchromatin and heterochromatin, as demarcated by the abrupt transition between low levels and enrichment of H3K9me2, appears to be the boundary for the inhibition of recombination associated with heterochromatin. My results are in line with the modified histone defined euchromatin – heterochromatin border, and the genetic and molecular borders show agreement with each other.

From the current study on recombination frequencies for transgenic insertions near the border of euchromatin and heterochromatin, I can say that previous studies which defined the border based on histone modifications are in line with the genetics. The genetic effects of the border region are what I expected, and support the notion that functionally, the transition from euchromatin to heterochromatin is defined by the transition in H3K9me2 enrichment. This study has shown the importance of, and with some degree of accuracy, the distance from heterochromatin necessary to display genetic

recombination. Further studies would use similar methods on other major chromosomal arms, 2R, 3L, 3R, and X to verify and compare just how far along a chromosome arm heterochromatin is able to inhibit meiotic recombination to determine if the distance we found is a general feature or if there are other factors involved. Another study that could be done would be to determine if there is a critical size of the block of heterochromatin for inhibition of recombination. That can be done using rearrangements that move parts of the centromeric heterochromatin into euchromatin, and placing mini-white bearing insertions at varying distances from those.

CHAPTER 3

Position Effects at the *light* Locus

3.1 Introduction

Thus far, it has been shown that heterochromatic genes depend on the heterochromatic environment for proper function (Eberl et al.). In order to learn more more about position effects on heterochromatic genes, it would be useful to determine if they all act in a similar manner and if PEV at all heterochromatic genes can be reverted. As Eberl et al. showed, PEV of one heterochromatic gene, *rolled*, was able to be reverted by relocating the displaced genes close enough to large blocks of heterochromatin. The objective of this project was to determine if the 2L heterochromatic gene *light* (*lt*) could similarly be induced to variegate (as shown by Hessler in 1958 and Wakimoto and Hearn in 1990), then reverted to rescue the phenotype. Since reversion of PEV of heterochromatic genes has been shown for only one gene, *rl*, thus far (Hilliker 1976), more work must be done to determine if there are general principles that apply across the board, or if there are other factors that influence revertibility beyond proximity and size of the closest heterochromatin block. This information will enable further insight into the particular requirements necessary for expression of heterochromatic genes. Furthermore, an increased understanding of heterochromatic gene requirements will also build on the knowledge of euchromatic gene expression, such as how far from a large block of heterochromatin must a euchromatic gene be to avoid PEV.

PEV at the *light* locus

The *lt* gene, a recessive gene that shows strong homology to the yeast gene VPS41, thought to be involved in intracellular protein trafficking (Warner et al. 1998), is

one of the genes responsible for wild-type eye colour in *D. melanogaster*. Variegation of the *lt* gene, which normally lies in the heterochromatic region of 2L and spans the molecular location 22 924 795 to 22 943 135, was first studied in detail by Hessler (1958). In Hessler's studies, 35 lines showing variegation at *lt* induced by X-ray irradiation were all associated with a breakpoint near to *lt* and a breakpoint in euchromatin. To my knowledge, the phenotype has never been rescued by further chromosomal rearrangement.

Very few of Hessler's original *lt* rearrangements are still extant, and *D. melanogaster* lines that show variegation at *lt* are not readily available, so new variegators had to be made, after which they could potentially be reverted to the wild-type phenotype. My hypothesis before beginning this experiment was that *lt* variegators would be revertible by rearrangements that would bring *lt* back into heterochromatin or in close enough proximity to be influenced by the heterochromatic environment to which it is native.

3.2 Materials and methods

2-3 day old male wild type flies of the strain $rosy^{+5}$ (ry^{+5}) were exposed to 60 Gy of X-ray radiation, then mated to virgin female flies carrying a balanced mutation of *lt*. Progeny of the wild type flies and *lt* mutants that had the balanced mutation of *lt* were readily isolated due to the dominant marker *Curly* (*Cy*) being on the second chromosome balancer carrying lt^{16} and they were screened for variegation of *lt*. Putative *lt* variegators were tested for dominant variegation, which would have been indicative of their actually

being variegators of *brown* (*bw*), and for variegation with lt^{l}, a classical mutant allele of *lt* to ensure that they were indeed created by the X-ray radiation, and not due to some unexplained effect of the balancer chromosome (Fig 5).

Following similar protocol to Hessler's 1958 experiments, 20 irradiated males and 30 virgin females carrying the balancer chromosome were placed in culture bottles on standard yeast/sugar/agar *Drosophila* medium. These flies were transferred to fresh medium after three days, then to fresh medium three days after that, after which males were discarded and females transferred to fresh medium for a further three days of egg laying, then discarded. Progeny were examined for eye colour variegation soon after they emerged, as the eyes tend to darken as flies age, making *lt* variegation more difficult to detect.

Flies that were confirmed to variegate at *lt* were cytologically studied by preparing squashes of larval salivary glands to expose the giant polytene chromosomes. Chromosomes were stained using 2% aceto-lacto-orcein as described by Hilliker (1975) and observed using a Zeiss Standard microscope equipped with phase contrast optics. Stained chromosomes were compared to standard cytological maps (Lefevre 1976, Lindsley and Zimm 1992) to determine where the breakpoints lay.

Male flies of the newly established variegating lines, which were stable due to the balancer chromosome, were then irradiated and back-crossed to balanced flies carrying the rearrangement in the hope of isolating revertants of the *lt* position effect. Progeny of this cross that displayed wild-type eyes were presumed to be revertants, and they were tested against lt^{l} to ensure that the rescue of the wild type eye phenotype was due wholly

to the further rearrangement of the initial X-ray induced chromosomal rearrangement that caused rearrangement in the initial variegators.

The wild type flies, ry^{+5}, are a commonly used wild-type stock, and the stock has been in our lab for many generations. The balanced flies carrying the *lt* mutation, *l(2)40Fc*^{1}/SM1 lt^{16}, were ordered from the Bloomington Drosophila Stock Center at Indiana University.

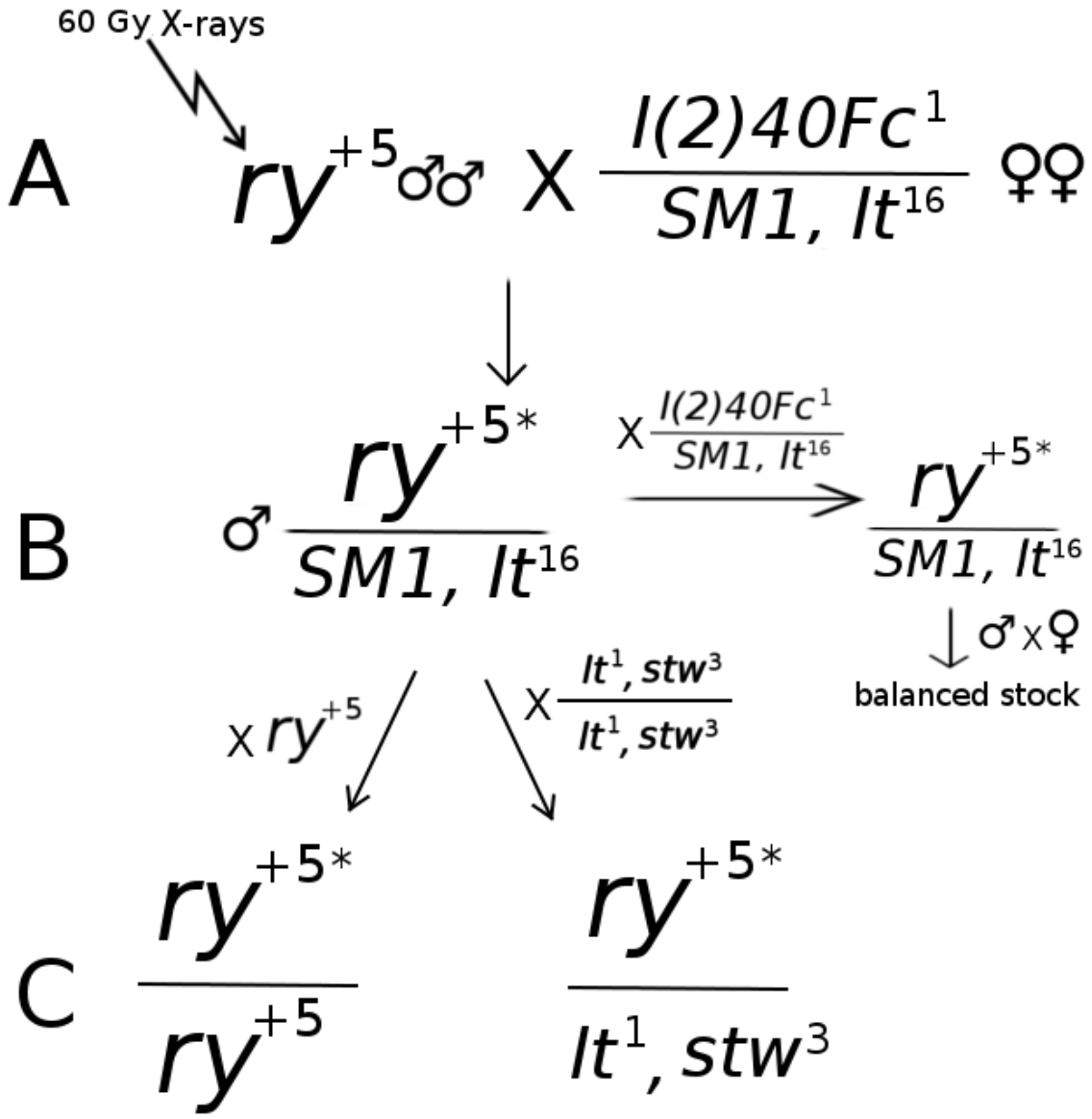

Figure 5. Mating schemes for generating *lt* position effects. Crosses are shown for the generation of chromosomal rearrangements in which the *lt* gene is position affected (A) with an asterisk signifying the X-ray induced chromosomal rearrangement, as well as making balanced stocks carrying those rearrangements (B). C shows a test to verify that the position effects were at the *lt* locus, as variegation with ry^{+5}would indicate variegation at *bw*, and variegation when crossed to lt^{1}, stw^{3} would confirm variegation at *lt*. The dominant marker *Cy* on the balancer chromosome SM1 shows a curly-wing phenotype, allowing for selective scoring. ry^{+5} denotes the wild-type *D. melanogaster* strain, *rosy* that was used for initial irradiation and mated to a fly carrying a lethal allele (signified by *l(2)*) in 2L heterochromatin balanced of SM1. The presence of the *lt* allele, lt^{16}, on the balancer chromosome was necessary to detect new alleles of the recessive gene *lt*.

3.3 Results of the search for variegators at *lt*

After the initial search (roughly 20 standard medium bottles with between 100 and 500 flies each) for X-ray induced variegation at *lt*, 5 putative variegators were isolated. I refer to them as *light-* variegator 1 – 5, or lt^{vr1} – lt^{vr5}. Upon back crossing the hybrids heterozygous for the balancer second chromosome *SM1* lt^{16} and the presumed rearranged chromosome to the parental wild type strain ry^{+5}, it was found that lt^{vr1} and lt^{vr3} both displayed dominant variegation, so they were ruled out for most likely being variegators of *brown* (*bw*). Two of the remaining 3 putative variegators, lt^{vr2} and lt^{vr4} displayed the variegated eye phenotype described by Hessler as "dark mottled", where the eyes appear to be essentially wild type with a sprinkling of occasional darker ommatidia (Fig 6), and the final one, lt^{vr5} displayed the phenotype referred to by Hessler as "pale mottled", in which a mixture of light and wild-type ommatidia are present with no sharp segregation. lt^{vr2} phenotypically displayed the characteristics of position effect variegation in the eye itself (Fig. 7A) as well as in the colourless ocelli (Fig. 7b).

It was cytologically determined that lt^{vr2} had a chromosomal breakpoint in distal (to the centromere) heterochromatin, and another one in distal euchromatin between cytological bands 32F and 33A which inverted a section of the chromosome so that a small block of heterochromatin containing *lt* was now in the euchromatin of 2L (Figs. 8 and 9). The cytological map is arranged such that each of the major chromosomal arms (X, 2L, 2R, 3L and 3R) are divided into 20 numbered segments, and each of those segments is divided into 7 lettered segments (A-F).

Attempts were made with lt^{vr2}, lt^{vr4}, and lt^{vr5} to derive revertants of the mottled

phenotype, and the only revertant found was a derivative of lt^{vr2}, and is named *light-variegator 2 derivative 2*, or $lt^{vr2\text{-}D2}$. The reversion of position effects at *lt* can be seen in the eyes and ocelli of $lt^{vr2\text{-}D2}$ (Fig. 7 C and D). Cytological analysis determined that there was a new breakpoint in the small block of heterochromatin containing *lt*, and another one between the euchromatic sections 36E and F (Figs 8 and 9).

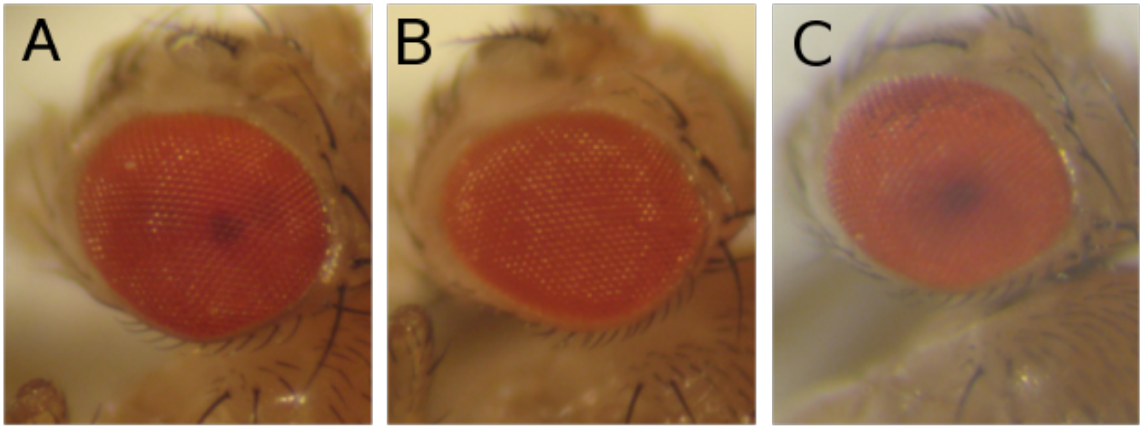

Figure 6. Eye phenotypes used to investigate *lt* position effects. A wild-type eye (A), a heterozygous lt^{1} mutant (B), and a variegator of *lt* (C). Variegation is of the "dark mottled" type, as the eye appears essentially wild-type with a sprinkling of darker ommatidia near the top of the image.

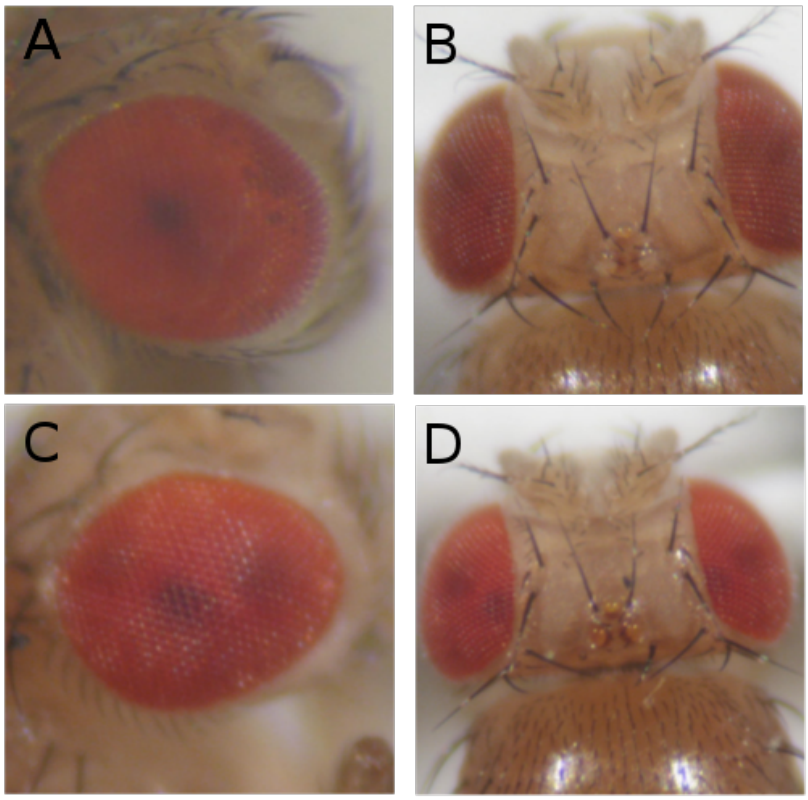

Figure 7. Eye and ommatidia phenotypes demonstrating position effects at *lt*. Position effects can be seen in the "dark mottled" phenotype the eye of lt^{vr2} (A), as well as the ocelli (B, three bumps on the dorsal surface of the head) which are colourless as in classical *lt* mutants. Reversion of the variegated phenotype is shown in the wild-type appearance of the eye (C) and ocelli (D) of $lt^{vr2\text{-}D2}$.

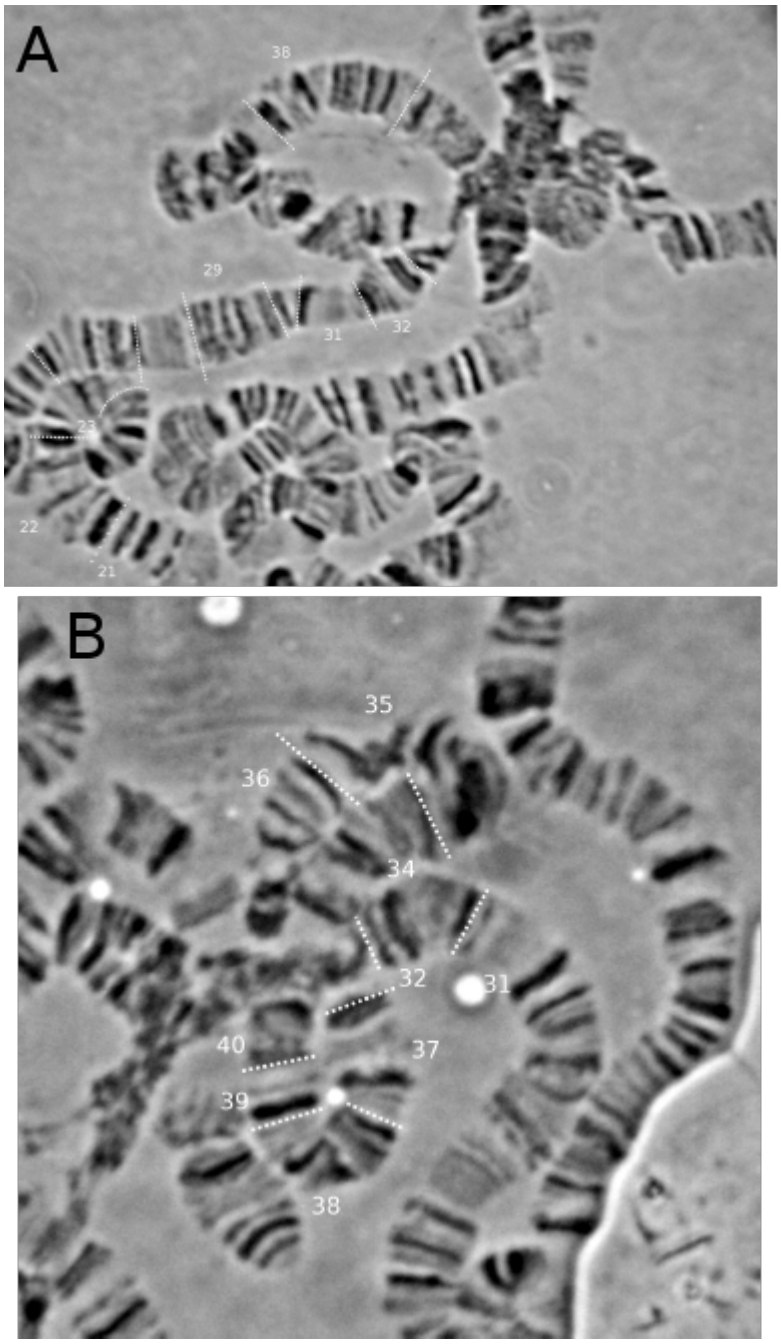

Figure 8. Polytene chromosomes of lt^{vr2}(A) and $lt^{vr2\text{-}D2}$(B). Breakpoints induced by X-ray radiation are distinguished by association with the chromocentre upon comparison with established polytene maps. lt^{vr2} shows a euchromatic breakpoint between sections 32 and 33 which (along with a break in heterochromatin) moved a heterochromatic block containing *lt* into distal euchromatin. $lt^{vr2\text{-}D2}$ shows another euchromatic breakpoint at the proximal end of polytene section 36 which brought the *lt* locus within close enough proximity to the large block of centromeric heterochromatin to rescue the phenotype.

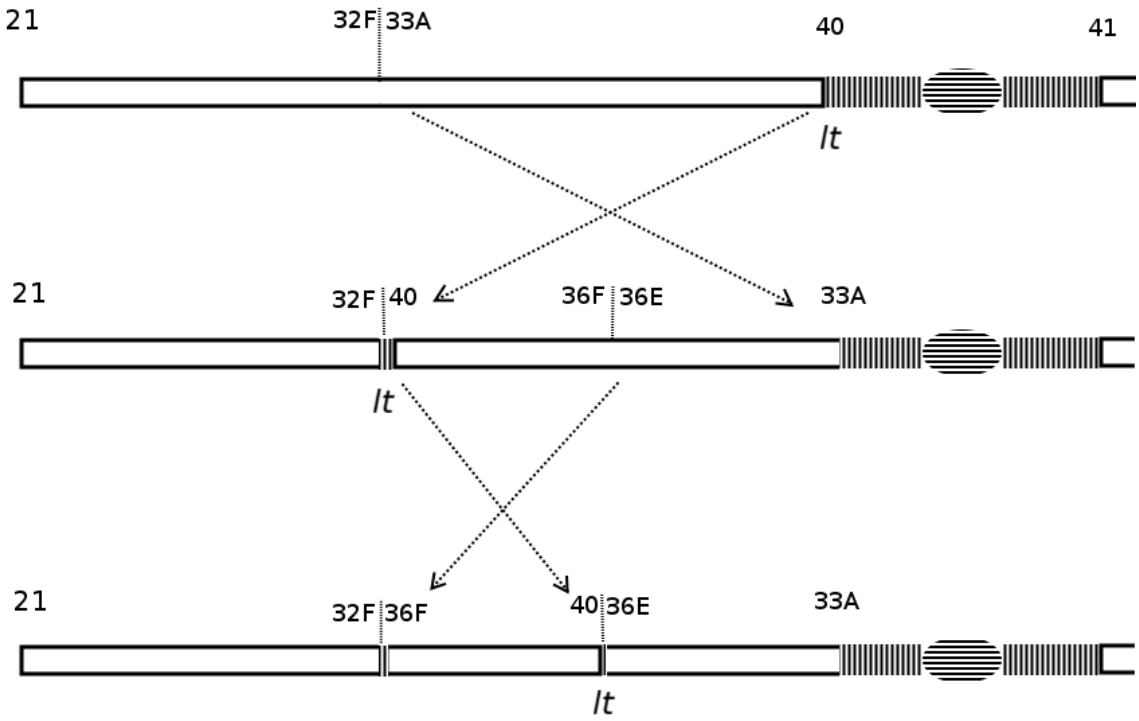

Figure 9. X-ray induced rearrangements of the left arm of chromosome 2. The initial rearrangement that generated a position effect variegation at *lt* (lt^{vr2}) had breakpoints in distal heterochromatin near the *lt* locus and another between cytological bands 32F and 33A. That initial inversion was further irradiated to revert the *lt* phenotype ($lt^{vr2\text{-}D2}$), and it had a break at the new location of the *lt* locus and another in proximal euchromatin between cytological bands 36E and 36F. Euchromatin is symbolized by empty outlines, heterochromatin by vertical lines, and the centromere by horizontal lines.

3.4 Discussion of *lt* variegation results

For the initial flies that were irradiated with 60 Gy of X-rays radiation and showed variegation at *lt*, the cytological analysis was in line with Hessler's results. The rearrangement that created lt^{vr2},which I now refer to as In(2L)lt^{vr2} in the interest of clarity of nomenclature to denote an inversion only involving chromosome arm 2L, had similar breakpoints to one of Hessler's lines, In(2L)lt^{m20}, which had a breakpoint in distal heterochromatin, and another one at cytological band 32C. This is very similar to my In(2L)lt^{vr2}, which had a breakpoint in heterochromatin and another one at cytological band 32F, so it is completely reasonable that I would find flies with this rearrangement to show variegation for *lt*. Eberl et al. needed at least two breaks in heterochromatin to induce position effects at *rl* (and other genes near to it), due to its position deep within the heterochromatin of 2R, but only one break in heterochromatin was needed to see *lt* position effects. This is due to the fact that *lt* lies in distal heterochromatin near enough to the junction with euchromatin that a single break in heterochromatin was enough to locate it in a small block of heterochromatin which was susceptible to the position effect. All that was needed to see position effects at *lt*, and probably genes close to *lt*, was a simple two-break rearrangement.

For the revertant line, which I now refer to as In(2L)$lt^{vr2\text{-}D2}$ signifying that it is an inversion only involving chromosome arm 2L, and derived (D) from an earlier rearrangement, it is the first time to my knowledge that variegation at *lt* has been reverted to wild-type phenotype. However, it is not the first time that heterochromatic position effects have been reverted, and there is precedent for the cytological location of the area

in which *lt* settled. Although the block of heterochromatin in which *lt* resided for In(2L)$lt^{vr2\text{-}D2}$ was probably even smaller than the block in which it resided for In(2L)lt^{vr2} based on cytological analysis, the position effect was still able to be reverted by moving the heterochromatic block containing *lt* closer to a large block of heterochromatin. This is similar to the findings of Eberl et al., as they were able to revert position effects at *rl* by moving small blocks of heterochomatin containing *rl* close to large blocks of heterochoromatin, even if they were not in heterochromatin. When *rl* was in small blocks of heterochromatin, they were able to find revertants that placed this block as far as 7 polytene sections away from the nearest large block of heterochromatin. With In(2L)$lt^{vr2\text{-}D2}$ placing *lt* between 3 and 4 polytene sections (36E to 33A in Fig. 7), it shows that position effects at *lt* are similar to position effects already shown for other heterochromatic genes. That I was able to revert the initial variegated phenotype indicates that is was a position effect, and not a mutation which would not be so easily reverted. These results are also further proof of the importance of the heterochromatic environment on the expression of heterochromatic genes, since *lt* was unable to express properly unless it was within (wild-type) or in close proximity (In(2L)$lt^{vr2\text{-}D2}$) to a large block of heterochromatin.

A possible explanation for the requirement of lt to be in close proximity to the centromeric heterochromatin for proper expression lies in the molecular environment of heterochromatic regions themselves. As has been speculated by Eberl et al. (1993), it is probably the highly repeated satellite sequences, which make up the bulk of heterochromatic regions (reviewed in Grewal and Jia, 2007), which confer the

characteristic qualities of heterochromatin to the regions that contain them. Eberl et al. (1993) found that quite a large amount of highly repeated DNA was required for the expression of *rl*, which led them to speculate that large aggregations of heterochromatin were necessary to provide a "functional milieu" for native heterochromatic genes. Furthermore, they speculated that this functional aggregation was due to the ability of the regions containing highly repeated sequences to be extensively misaligned in their pairing, creating a three dimensional complex of aggregation. Turning to my work on *lt*, the further the block containing it was away from the main block of centromeric heterochromatin, the more difficulty it would encounter in joining the large aggregate. As it is moved closer to the pericentromeric heterochromatin of 2L, it would be more likely to be included in a large chromocentral aggregate, and thus, more likely to be in an environment more conducive to its proper expression. This is likely why position effects involving *lt* show a mottling effect on the eye. In some cells, *lt* was able to join the large aggregate and express properly, whereas in other cells it was not able to join the aggregate. However, once *lt* is moved to a position in which it has a high chance of being included in the chromocentral aggregate, the expression is normal in a far higher number of cells, providing for a wild-type phenotype.

The *light* locus of *D. melanogaster* shows a similar pattern of effects of chromosomal rearrangements as a previously studied heterochromatin gene; its functioning was hindered by being within a small block of heterochromatin inside of a large block of euchromatin, but was restored by relocation near to a large block of centromeric heterochromatin. This further supports the notion that the heterochromatic

environment is necessary for the proper function of heterochromatic genes. To further expand on the knowledge gained by this work, quantative expression may be investigated using real-time PCR (RT-PCR) to compare expression levels of the *lt* gene for phenotypical variegators and revertants. RT-PCR can also be used for genes near to *lt* to give a clearer picture of PEV of heterochromatic genes of 2L. Heterochromatic genes on other chromosomal arms would be well suited for further analysis, particularly RT-PCR for those genes that do not display a readily distinguished phenotype. The current work shows, once again, the importance of the proper molecular environment for the expression of heterochromatic genes.

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