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Characterizing *P* element dependent silencing in
Drosophila melanogaster

by

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Abstract

Transposable elements are DNA segments that replicate by inserting themselves into new genomic locations. Controlling this replication is important because these new insertions cause spontaneous mutations that can be lethal to the host. Silencing expression from the transposable element can prevent replication because transposable elements often encode the enzymes that catalyze their mobilization. This silencing involves interactions between the host and the transposable element. This thesis studies the interactions that occur between the *P* transposable element and *Drosophila melanogaster*.

P{lacW}ci^{Dplac} is a transgenic insert that occurs in a region between *cubitus interruptus* (*ci*) and *ribosomal protein S3a* (*RpS3a*). Within this *P* element construct there is a w^+ transgene, which is uniformly expressed throughout the eye in the absence of other *P* elements. Previous work has shown that when other *P* elements are present in the genome, variegated expression occurs from the w^+ transgene within the *P{lacW}ci*^{Dplac} insert. This variegated expression results from random silencing in different cells in the eye and resembles heterochromatic position effect variegation. In a genetic screen, I found mutations in *Su(var)2-5* and *Su(var)3-7* that suppress this P element dependent silencing (PDS). Further tests using these mutations, suggest that *P* elements enhance heterochromatic silencing in this region rather than

recruiting these factors or causing heterochromatin formation *de novo* in this region.

I also tested what sequences within the *P* element contribute to PDS at *P{lacW}ci*^{Dplac}. Previous analysis divides non-autonomous *P* element that modify *P* dependent phenotypes into two types: Type I and Type II. I found that both types of elements cause silencing at *P{lacW}ci*^{Dplac}. Intermediate elements that belong to neither type did not cause silencing of the w^+ transgene at *P{lacW}ci*^{Dplac} or modify the other *P* dependent phenotypes tested. These experiments demonstrate that the protein encoding sequences within the *P* element contribute to the ability of an insert to act in *trans* and modify *P* dependent phenotypes such as PDS. Together, these results indicate PDS at *P{lacW}ci*^{Dplac} behaves very similarly to other *P* element dependent phenomena.

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1 Introduction

All the genomes currently characterized have transposable elements (TEs). A TE is a sequence of DNA that can replicate itself through either a DNA or RNA intermediate and insert itself into the genome. These insertions can occur in genes necessary for the normal functioning of the cell. Consequently, if TE replication goes unchecked it can kill the host. About 10% of the *Drosophila* genome (LABRADOR and CORCES 1997) and 45% of the human genome is composed of interspersed TE sequences (LANDER *et al.* 2001). In *Drosophila,* the *P* element is one of the best-characterized TEs. A *P* element is similar to other TEs in that it encodes proteins that interact with the host's proteins to catalyze and regulate transposition. By using recombinant DNA techniques to remove internal DNA sequence within the *P* element and replacing them with sequence from other genes, researchers have been able to transform *D. melanogaster* with *P* element constructs that contain genes of interest.

Within genomes, TEs tend to cluster within heterochromatic regions. These heterochromatic regions appear as darkly staining chromatin (DNA and protein) during periods of the cell cycle when DNA is usually diffuse throughout the nucleus. This dark staining is due to the unique chromatin structure found in these regions. In the nucleus, DNA is wrapped around nucleosomes and within these darkly staining regions, these nucleosomes are specially modified to maintain a compact chromatin structure. Due to the simultaneous co-existence between these biological components, researchers have often postulated that there is a relationship between heterochromatin and TEs. This thesis explores a unique interaction between heterochromatin and a *P* element insert in *Drosophila melanogaster.*

1.1 *P* element phenotypic effects

The first trait associated with *P* elements was hybrid dysgenesis, which occurred in crosses between wild stocks and old laboratory stocks (reviewed in PINSKER *et al.* 2001). When wild males, which have *P* elements (*P* strains), are crossed to females from laboratory stocks, which do not have *P* elements, (M strains) hybrid dysgenesis occurs in the germ line of their progeny (ENGELS 1996; ENGELS and PRESTON 1980). Hybrid dysgenesis includes a high mutation rate, chromosomal rearrangements, male recombination, and thermosensitive, agametic sterility referred to as gonadal dysgenesis. In the reciprocal cross between *P* strain females and M strain males, hybrid dysgenesis does not occur. The laboratory stocks do not have *P* elements because they were isolated from wild populations early in the 20th century before *P* elements spread through the wild *Drosophila melanogaster* populations (ANXOLABEHERE *et al.* 1988; PINSKER *et al.* 2001). As well as hybrid

dysgenesis, *P* strains can modify certain *P* element dependent phenotypes in the soma. Previously, these phenotypes include sn^{w}, $vg^{21\text{-}3}$ and somatic mosaicism and will be discussed later (ROBERTSON and ENGELS 1989). This thesis adds to this list and studies the phenotypic effects *P* element have on *P* constructs inserted in the distal regulatory region of *cubitus interruptus* (*ci*). *P* elements cause variegated expression from w^{+} transgenes in *P* constructs inserted in this region and suppress the phenotypic effects these insertions have on *ci* and *Ribosomal protein S3a* (*RpS3a*) expression. When inserted in other regions, variegation does not occur from these w^{+} transgenes when *P* elements are present.

1.1.1 P cytotype, P repressor, and alternative splicing

The massive mobilization of *P* elements and their random insertion into necessary genes leads to hybrid dysgenesis (KIDWELL and KIDWELL 1975). Hybrid dysgenesis only occurs when *P* elements are inherited paternally. If inherited maternally, a cytoplasmic condition, referred to as P cytotype, prevents *P* element mobilization. A P factor(s) in this state could inhibit transposition either by repressing expression from *P* elements or inhibiting the transposition process. Since the ability to mobilize *P* elements and produce P cytotype segregates with *P* elements, both qualities must result from the presence of *P* elements (SVED 1987). *P* elements produce a transcript in the germ line that is composed of four exons labeled 0-3. This transcript encodes a transposase that catalyzes the excision of *P* elements, as DNA intermediates, and pastes them back into the genome. Replication occurs when the sister chromatid is used as template to repair the gap left by the *P* element excision. Transposition does not occur in the soma, because an alternative transcript is produced that retains the intron between exons 2 and 3, which is spliced out in the germ line. The somatic transcript encodes a truncated polypeptide that lacks transposase activity. I will refer to this truncated protein as the P repressor because it is hypothesized to repress transcription from the *P* promoter (MISRA and RIO 1990). However, as discussed below, although the *P* repressor is associated with P cytotype, it is not sufficient nor necessary to reproduce P cytotype effects (MISRA *et al.* 1993; RONSSERAY *et al.* 2001; RONSSERAY *et al.* 1998).

1.1.2 Previously described somatic *P* element dependent phenotypes

Previously described somatic *P* dependent phenotypes depend on expression from the *P* promoter. The most direct assay to measure expression from the *P* elements uses *P* constructs that have the *lacZ* gene fused to the *P* promoter (*P-lacZ*) (LEMAITRE and COEN 1991). Originally, these constructs acted as enhancer traps, which detect nearby transcriptional enhancers that activate the *P* promoter. *P* promoter activity results in *lacZ* expression and β-galactosidase activity. Consequently, expression from the *P-lacZ* insert would mimic the expression pattern from the endogenous gene on which the enhancer usually acts. However, it was discovered that crosses to *P* strains would

repress expression from the *P-lacZ* reporter at all genomic locations in somatic tissues. Furthermore, *P-lacZ* expression could be studied in both the somatic and germ tissues. This provided a means to study *P* element expression directly in both tissue types, whereas previous assays using hybrid dysgenesis only studied suppression in the germ line.

Parental origin results showed that *P* element regulation in the germinal cells differs from somatic cells. In the soma, both parental origins repressed expression from these enhancer traps (LEMAITRE and COEN 1991; LEMAITRE *et al.* 1993). In the germ line, only the maternally inherited P cytotype completely silences expression from *P-lacZ* expression (LEMAITRE *et al.* 1993), while paternally inherited *P* elements do not repress expression from the *P-lacZ* reporter construct. These results demonstrate that the P cytotype model only applies to the germ tissues and does not apply to *P* dependent phenotypes in somatic cells.

Other *P* dependent phenotypes such as *vestigal*$^{21\text{-}3}$ (*vg*$^{21\text{-}3}$) (WILLIAMS *et al.* 1988), and certain *singed*^{w} (*sn*^{w}) alleles, involve *P* elements inserted within genes whose mis-expression causes the mutant phenotype. In the case of *vg*$^{21\text{-}3}$, a transcript initiates from within the *P* element and elongates into the *vg* gene sequence. This transcript is postulated to inhibit normal expression from *vg*, thus causing the vestigial phenotype (HODGETTS and O'KEEFE 2001). Loss of *vg* expression causes small malformed wings that are easy to identify. The presence of other *P* elements can repress expression from the *P* insert in the *vg*$^{21\text{-}3}$ allele and suppress the vestigial phenotype. When *P* elements are present, homozygous *vg*$^{21\text{-}3}$ flies have wings that have almost a wild type phenotype and this contrasts the small malformed wings found when *P* elements are absent.

Another *P* dependent test involves suppressing somatic mosaicism that is caused by transposase production in somatic cells (MISRA and RIO 1990). By removing the intron between exons 2-3 (*Δ2-3*) a transcript, which encodes transposase, can be produced in somatic cells. Transposase activity in the soma causes random *P* element transposition. The loss or gain of a *P* element insert in somatic tissues results in a mosaic phenotype. For instance, the *singed-weak* (*sn*^{w}) allele has two non-autonomous *P* elements inserted near *sn* causing a weak singed bristle phenotype (ROBERTSON and ENGELS 1989). Excision of one of these elements causes a strong singed phenotype, while excision of the other *P* yields wild type bristles. Expressing *Δ2-3* in somatic tissues can result in transposition of either *P* element and this produces a mosaic-singed phenotype. The presence of *P* elements can repress transposition in somatic and germ line cells. When transposition is repressed in somatic lines, all the 44 macrochaetae have the same bristle morphology that is affected with the *sn*^{w} allele.

In another assay, *Δ2-3* expression causes the loss or gain of a *P{lacW}* insert (MISRA and RIO 1990). Those ommatidia without the *w*$^{+mC}$ transgene are white whereas ommatidia cells expressing *w*$^{+mC}$ become colored, resulting in a mosaic pattern when *Δ2-3* is expressed. When *P*

elements are present, transposition is represented and the eye is uniformly colored rather than variegated.

1.1.3 *P* element dependent phenotypes at *ci* are different from previous examples

The *P* inserts at *ci* occur in a region of AT-rich repetitive DNA, which is 3 kbp upstream from the first *ci* exon and 6 kbp downstream from the last exon in *RpS3a* (Figure 1.1.1) (LOCKE *et al.* Submitted). Given the distance and orientation between these *P* insertions and the adjacent genes, transcription from the *P* promoter is not expected to interfere with the normal transcription of these genes. However, these insertions can affect the expression from the proximal genes. A second item of interest is that the presence of *P* elements causes variegated expression from the w^{+mC} within the single *P* inserts at this location. *P* elements do not affect expression of w^{+mC} when inserted at other locations. Therefore, the *P* element dependent silencing (PDS) is limited to the *P* inserts within this region near *ci* and does not represent a general phenomenon that occurs at all the *P* inserts.

1.1.3.1 Minute and ci phenotypes associated with *P* inserts

The *P* insertions in region between *ci* and *RpS3a* can affect the normal expression from these proximal genes. As well, other mutations, such as ci^{1}, ci^{57g}, and ci^{36l} within the *ci* distal regulatory region cause mutant phenotypes associated with mis-expression of the *ci* gene (SCHWARTZ *et al.* 1995). In the following cases, the mutant phenotypes caused by the *P* inserts in this region are suppressed by the presence of *P* elements.

The *ci* gene is necessary for appropriate anterior: posterior boundaries forming in the embryo and imaginal discs. Within each compartment, *ci* is expressed only in the anterior compartment. Amorphic mutations, which knockout *ci* function, are recessive lethal. Another recessive phenotype, which affects the adult wing veins, occurs when *ci* is ectopically expressed in the posterior compartment. This *ci* phenotype is recessive because a wild type ci^{+} allele in *trans* can repress expression from alleles such as ci^{1} or ci^{57g} (LOCKE and TARTOF 1994). I refer to these recessive *ci* alleles as Dubinin alleles (DUBININ *et al.* 1935). When homozygous, no *trans* repression occurs and these Dubinin alleles ectopically express *ci* in the posterior compartment. Due to ectopic *ci* expression, the wings have an interruption in the L4 wing vein. In addition, ectopic expression occurs from the Dubinin alleles when rearrangements, such as $In(1;4)w^{m5}$, prevent pairing between chromosome 4 homologues. This phenomenon is referred to as transvection and occurs at other loci such *yellow* and the *bithorax* complex (HENIKOFF 1997; HENIKOFF and COMAI 1998).

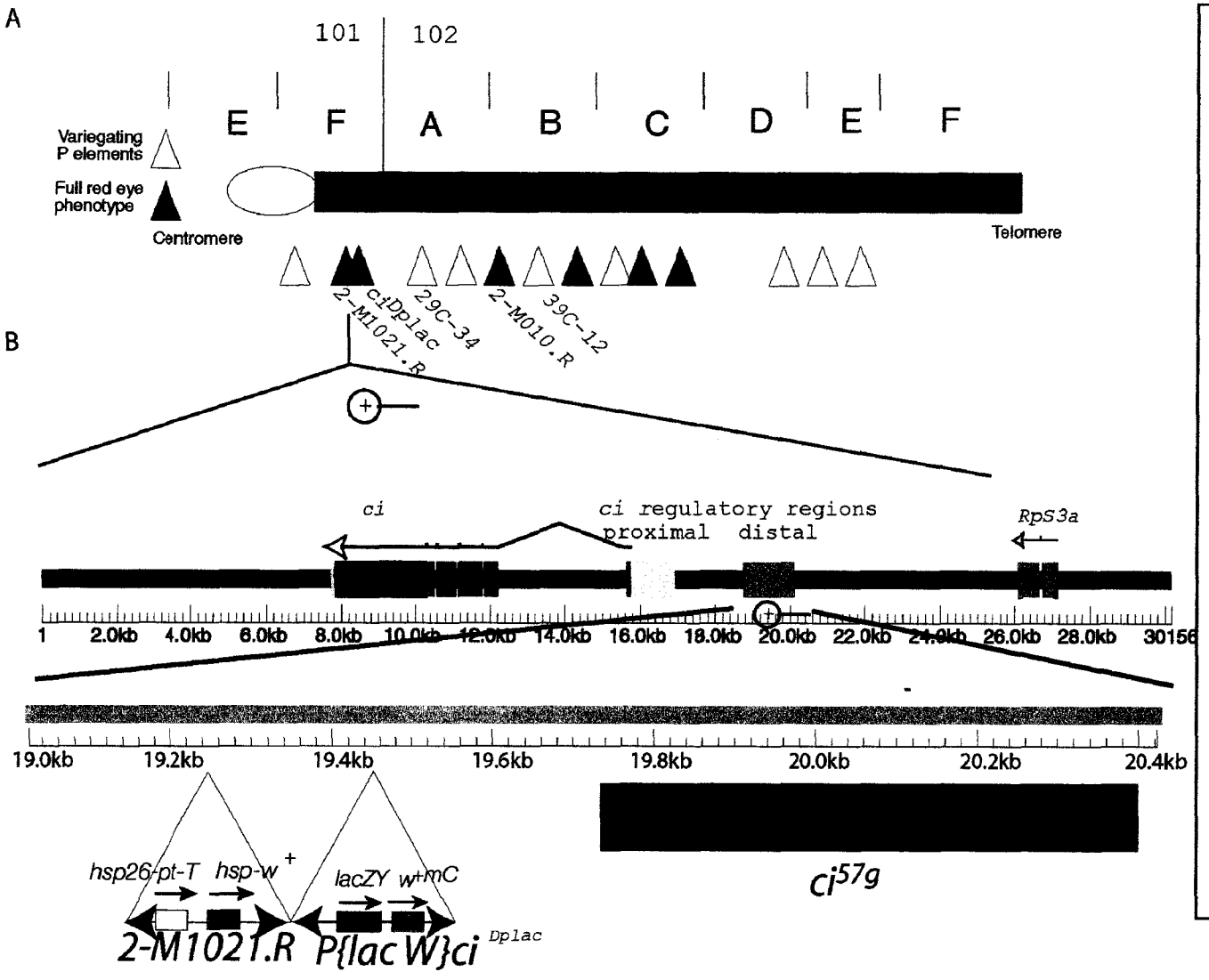


Figure 1.1.1 - *P* w+ inserts on chromosome 4. **A.** Diagram describing where the different inserts occur along chromosome 4. The *w*+ transgenes that are uniformly expressed are represented by solid red triangles. The *w*+ transgenes that variegate are represented by open triangles. Only the *w*+ transgenes described in this thesis are abbreviated in this diagram. This diagram is based on a diagram originally made by Sun *et al* (2000) **B**. The *ci* distal regulatory region. The *ci* gene is in red and the *RpS3a* gene is pink. The ci^{57g} mutation is a deletion of 646 bp and an insertion of 29 bp.

The *P{hsp26-pt-T}ci*$^{2\text{-}M1021.R}$ is a w^+ transgene insert that occurs 140 bp closer to *ci* compared to *P{lacW}ci*^{Dplac}. Similarly to *P{lacW}ci*^{Dplac}, this w^+ transgene variegates when other *P* elements are present. The insert *P{hsp26-pt-T}ci*$^{2\text{-}M1021.R}$ also interrupts the ability to *trans*-repress expression from the Dubinin alleles. The expression from *ci*$^+$ on this chromosome is normal because *P{hsp26-pt-T}ci*$^{2\text{-}M1021.R}$ homozygotes have a wild type wing vein phenotype. However, when *P{hsp26-pt-T}ci*$^{2\text{-}M1021.R}$ is heterozygous with *ci*^{1}, there is a gap in the L4 wing vein suggesting that there is ectopic expression from *ci*^{1} (HANNA S. PERSONAL COMMUNICATION). In the results, I provide evidence that demonstrates that the presence of *P* elements restores *trans*-repression. Although *P{lacW}ci*^{Dplac}, does not produce a ci phenotype under similar circumstances, *P{lacW}ci*^{Dplac} is a cryptic *ci* allele and in combination with *engrailed* mutants produces a weak *ci* phenotype (LOCKE and HANNA 1996). Therefore, these *P* insertions in the *ci* distal regulatory region can affect expression from *ci*.

If the *P* inserts within the *ci* distal regulatory also reduce expression from *RpS3a*, they should produce a dominant Minute phenotype (HANNA S. PERSONAL COMMUNICATION; REYNAUD *et al.* 1997). Mutations in any of the 40 genes that encode ribosomal proteins cause the Minute phenotype, which is characterized by small narrow bristles, small body size, and delayed development (KAY and JACOBS-LORENA 1987). Although none of the original *P* insertions in this region cause the Minute phenotype, imprecise excision mutations of *P{lacW}ci*^{Dplac}, induced by Δ2-3 mutagenesis, have produced a Minute phenotype. These Minute alleles fail to complement known *RpS3a* mutations indicating they affect this gene. Based on PCR analysis, *Δ2-3* mutagenesis caused imprecise excisions within the *ci* distal region and did not delete the *RpS3a* gene. Therefore, the phenotype must result from silencing of *RpS3a* expression. This is interesting because the original *P{lacW}ci*^{Dplac} does not cause silencing of the adjacent genes in the absence or presence of *P* elements. Evidence in this thesis confirms these results and I show that the *Δ2-3* induced internal deletions within the *P{lacW}ci*^{Dplac} insert. I conduct a genetic screen that looked for suppressors of the *Minute*^{F2} (*M*^{F2}) allele but do not find any single mutations that suppressed the Minute phenotype. However, the presence of *P* elements suppresses the Minute phenotype caused by these Minute *P{lacW}ci*^{Dplac} derivatives.

1.1.3.2 *P* elements cause variegated expression from w^+ transgenes within the *P* inserts in this region

P elements cause variegated expression of the w^+ transgene in *P{lacW}ci*^{Dplac} and *P{hsp26-pt-T}ci*$^{2\text{-}M1021.R}$, which are inserted in *ci*'s distal regulatory region (Figure 1.1.1) (LOCKE *et al.* Submitted). In M type flies, which have no *P* elements, expression from either w^+ transgene produces eyes that uniformly accumulate pigment in each ommatidia. When crossed to a *P* strain, which has many *P* elements throughout its genome, expression from these w^+ transgenes produce a variegated eye phenotype with a mosaic of white and colored ommatidia. This

variegated phenotype results because w^+ is stochastically silenced in some ommatidia (white) but expressed in others (colored). This variegated silencing resembles position effect variegation (PEV) where changes in local chromatin structure can silence gene expression.

1.1.3.3 A change in local chromatin structure best explains PDS silencing at *P{lacW}ci^{Dplac}*.

The arguments supporting this statement are divided into two parts. First, changes in chromatin structure can affect any gene regardless of its sequence. *P{lacW}ci^{Dplac}* includes the *mini-white$^+$* (w^{+mC}) gene that uses portions of the original w^+ promoter, while *P{hsp26-pt-T}ci$^{2\text{-}M1021.R}$* has the promoter from *hsp70$^+$* fused to the w^+ transgene. Since silencing affects different *P* constructs with different promoters, a specific DNA promoter sequence is not required to induce silencing. Secondly, chromatin structure within a region depends on its chromosomal location. Experiments in yeast, describe a chromatin structure where specific modifications to nucleosomes can actually be detected as a gradient along the chromosome (KIMURA *et al.* 2002). Changing a gene's position within this gradient will change its expression pattern. The extent to which *P{lacW}ci^{Dplac}* is silenced when *P* elements are present varies with changes in chromosomal position, as shown by translocations that suppress PDS. In addition, w^{+mC} expression from *P{lacW}* inserted at other locations is not silenced by the presence of *P* elements (LOCKE *et al.* Submitted). *P* elements could cause w^+ silencing within the *P* inserts in this region because of the unique chromatin structure within this region. Changing the chromosomal location changes the chromatin structure and PDS no longer occurs.

PEV is seen with chromosomal rearrangements or insertions that place genes next to telomeres and centromeres, which are sites where heterochromatin forms. The *ci* gene is located next to the centromere on chromosome 4. Therefore, transgene inserts at this locus could behave similarly to PEV caused by heterochromatin formation at centromeres. Modifiers that suppress or enhance PEV have been tested for their affect on PDS at *P{lacW}ci^{Dplac}* (LOCKE *et al.* Submitted). Surprisingly, none of the modifiers tested had a significant affect on w^{+mC} expression from *P{lacW}ci^{Dplac}*.

Many modifiers of PEV act dose-dependently to either enhance or suppress silencing. In *Drosophila*, mutations in *Su(var)2-5* suppress telomeric PEV (tPEV) on chromosome 4 and heterochromatic PEV (hPEV) that occurs at centromeres (CRYDERMAN *et al.* 1999b). However, *Su(var)2-5* does not affect tPEV on chromosomes 2 and 3, which are suppressed by *Su(z)2* (CRYDERMAN *et al.* 1999a). *Su(z)2* is a member of the polycomb group which binds at polycomb response elements (PRE) and these sequences are necessary for maintaining gene silencing at certain genes during development (PIRROTTA and RASTELLI 1994). *P* inserts that contain PRE sequences display a variegated phenotype similar to the phenotype of inserts located within heterochromatic regions at centromeres (CHAN *et al.* 1994). Although PEV occurs at

centromeres and telomeres, the modifiers that enhance or suppress expression from these locations are not limited to a single chromosomal position.

Parallels between the silencing phenotype caused by *P* elements at *P{lacW}ci*^{Dplac} and classical hPEV suggests that these phenotypes result from similar changes in chromatin structure. This thesis describes a genetic screen that recovered mutations in *Su(var)2-5* and *Su(var)3-7* that suppress PDS at *P{lacW}ci*^{Dplac}. Since these mutations occur in heterochromatic modifiers, these results confirm that changes in chromatin structure cause the silencing. Consequently, I will give a brief description of heterochromatin and position effect variegation.

1.2 Heterochromatic position effect variegation

A condensed chromatin state during interphase, such as the chromocenter of polytene chromosomes or Barr body caused by X chromosome inactivation in human females, cytologically defines heterochromatic regions (PIRROTTA and RASTELLI 1994; WEILER and WAKIMOTO 1995). Chromosomal rearrangements that place euchromatic genes in heterochromatic environments result in random silencing of the euchromatic gene and this causes a variegated phenotype. This variegated phenotype describes a mosaic tissue displaying two different phenotypes because the gene is turned off and on randomly in the tissue. The chromosome inversion, *In(1)w*^{m4}, *w*^{m4}, referred here after as *w*^{m4}, places the *w*$^{+}$ gene next to centromeric heterochromatin causing variable silencing and a variegated eye phenotype, with white and colored ommatidia. The *w*$^{+}$ gene is necessary for red and brown pigment accumulation in individual cells and when it is turned off the ommatidia are white. Genetic screens using *w*^{m4} have identified over 150 heterochromatic suppressors (*Su(var)*) and enhancers (*E(var)*) (SCHOTTA *et al.* 2003). Mutations in chromatin modifiers either suppress the variegated phenotype and produce more colored ommatidia, or enhance the variegation and produce more white ommatidia. Since eye color is visible and not necessary for viability, this phenotype is advantageous to use in genetic screens.

The screens using *w*^{m4} identified genes whose product bound to these densely staining regions. One modifier identified using *w*^{m4} was *Su(var)2-5*, whose product, HP1, binds to cytologically visible heterochromatin at the centromere (EISSENBERG *et al.* 1990; EISSENBERG *et al.* 1992). As well as where these SU(VAR) proteins localize, certain variant histones, and certain chemical modifications are associated with heterochromatic regions (JENUWEIN and ALLIS 2001; TURNER 2002). Specific amino acids within nucleosomes can be acetylated, ubiquitinated, phosphorylated, or methylated. Specific modifications at certain amino acids are associated with either heterochromatic or euchromatic regions. Methylation at lysine 9 on Histone 3 (K9-H3) is associated with heterochromatic regions in *Drosophila* and mammals. These modifications provide a "histone code" which can be recognized by protein domains found in the SU(VAR)s that bind specific types of

chromatin structure (JENUWEIN and ALLIS 2001; NAKAYAMA *et al.* 2001; TURNER 2002).

Although heterochromatin typically silences expression from euchromatic genes, a heterochromatic environment is necessary for the appropriate expression from those genes that normally reside in heterochromatin. These heterochromatic regions are typically gene poor but contain a few essential genes, such as *rolled* (*rl*) and *light* (*lt*) (EBERL *et al.* 1993; WEILER and WAKIMOTO 1998). Reducing the *Su(var)2-5*$^{+}$ dosage reduces gene expression from both *rl* and *lt* but increases expression from a *white*$^{+}$*-lacZ* euchromatic reporter transgene inserted in a heterochromatic region and subject to PEV (LU *et al.* 2000). Therefore, the heterochromatic environment is generally inhibitory to euchromatic gene expression but necessary for appropriate expression from genes that normally reside within it.

The heterochromatic modifiers identified both in *Drosophila* and other species have similar protein domains, such as the SET and chromodomain (JACOBS and KHORASANIZADEH 2002). The SET domain is found in *Su(var)3-9* and this domain is necessary to methylate lysine 9 in H3 (K9-H3). Homologues to *Su(var)3-9* include *Suv39H1* and *Suv39H2* in mammals and *clr4* in *Saccharomyces pombe* (BANNISTER *et al.* 2001). Both SU(VAR)3-9 and HP1 interact to maintain histone methylation in heterochromatic regions. This mechanism works in mammals, *S. pombe*, and *Drosophila* and research in all these organisms has been used to describe this common pathway. The chromodomain in HP1 and its homologue in *S. pombe*, Swi6, has affinity for H3 tails that have a methylated K9. In turn, HP1 has affinity for SU(VAR)3-9 and recruits this protein to heterochromatic regions that have this modification (NAKAYAMA *et al.* 2001). This sets up a feedback loop that maintains the H3 methylation status. Murine tissue culture results show that the three HP1 (α,β, and γ) homologues act in a similar feedback loop with both SUV39H1 and SUV39H2 (LACHNER *et al.* 2001). Although the interactions between SU(VAR)3-9, HP1, and H3 indicate how heterochromatin is maintained, it does not indicate how heterochromatin formation is initiated. Similar feedback loops maybe occurring at other regions such as at PRE sequences. Polycomb has a chromodomain and it is necessary to maintain silencing at these regions (EISSENBERG and ELGIN 2000).

Heterochromatin formation is necessary for genomic stability. Complete loss of HP1 in *Drosophila* is lethal. In these dying embryos chromatin bridges form between nuclei and chromosomes do not completely condense during mitosis (KELLUM and ALBERTS 1995). Complete loss of *Suv39h1* and *Suv39h2* in murine cells, impairs viability, male fertility, and chromosomal stability (KELLUM and ALBERTS 1995; PETERS *et al.* 2001). Also, HP1 is necessary to prevent telomere fusion in *Drosophila* (FANTI *et al.* 1998b; SAVITSKY *et al.* 2002) and interacts with the origin recognition complex (SHAREEF *et al.* 2001). At the centromere, heterochromatin could prevent premature replication that could interrupt kinetochore assembly and chromosome condensation during

mitosis (HENIKOFF 2000). The loss of heterochromatin could directly lead to genomic instability because heterochromatin is necessary for appropriate chromosome condensation and segregation during the cell cycle. However, the change of heterochromatic to euchromatic regions could result in ectopic expression of genes that reside in heterochromatin and whose mis-expression causes genomic instability. Two different arguments favor a model where the change in heterochromatin directly causes chromosomal instability. First, in *Drosophila*, all the known lethal genes in heterochromatin are associated with abnormalities later in development than the chromosomal instability caused by loss of HP1 (KELLUM and ALBERTS 1995). Lastly, the same chromosomal instability is seen in widely divergent organisms, such as mouse and *Drosophila*. Gene locations between these organisms vary but the chromosomal instability persists. This suggests heterochromatin function is conserved and necessary for chromosomal stability.

Heterochromatin does not consist of a unique sequence to which heterochromatin modifiers bind. Instead, sequences within heterochromatin are repetitive and consists of TEs and satellite sequence (BARTOLOME *et al.* 2002; HOSKINS *et al.* 2002). The fact that heterochromatic regions appear to be graveyards for TEs indicates that the heterochromatin formation could be involved in the domestication and control of TEs. However, TEs could cluster in heterochromatic regions simply because heterochromatic regions have few genes and therefore TE insertions would be less detrimental in these regions. Insertions in gene rich euchromatic regions will frequently interrupt a gene, whereas, insertions in gene poor heterochromatin should rarely interrupt a gene. However, I-R hybrid dysgenesis experiments found that *I* element insertions within the chromosome 2 heterochromatin are frequently lethal (DIMITRI *et al.* 1997). Another reason why TEs may accumulate in heterochromatin is that ectopic recombination between TEs at different locations on homologous chromosomes will result in the loss or gain of genetic material and such changes in gene copy number could be harmful. Since heterochromatic regions have a reduced recombination frequency, this would inhibit ectopic recombination with TEs inserted elsewhere in the genome (DIMITRI and JUNAKOVIC 1999).

However, the results at *ci* suggest that *P* elements can interact in *trans* and change chromatin structure at *P* elements. This suggests that heterochromatin can interact with TEs and assist in their domestication. A link between heterochromatin and transposable elements (TE), such as presented in this thesis, provides a unique opportunity to study interactions between these biological components. As discussed throughout this thesis, mutations in certain *Su(var)*s suppress the PDS phenomenon. Also discussed are mutations in *Su(var)2-5* that can interfere with *P* cytotype under certain conditions (RONSSERAY *et al.* 1996). Lastly, TEs accumulate in regions that form heterochromatin and this may reflect an interaction between these biological components (DIMITRI and JUNAKOVIC 1999; PIMPINELLI *et al.* 1995).

1.2.1.1 What triggers heterochromatin formation?

Repetitive sequences trigger heterochromatin formation (HENIKOFF 2000) (DORER and HENIKOFF 1994). Classic hPEV involves the translocation or insertion of euchromatic genes into tandem repeated sequences that are found in centromeric and telomeric regions. Tandem *P{lacW}* repeats alone can trigger heterochromatin formation and silencing of the w^{+mC} transgenes found in the repeats. Decreasing the *Su(var)2-5* dosage suppresses heterochromatic silencing within the *P{lacW}* arrays (DORER and HENIKOFF 1994) and like hPEV at other loci, proximity to heterochromatic regions enhances heterochromatic silencing in the repeats (DORER and HENIKOFF 1997). At centromeres that consist of repetitive sequence and satellite sequences a similar event could be occurring. Such a system appears to act both in plants (WATERHOUSE *et al.* 2001) and in mammals, since even simple TA repeats trigger heterochromatic silencing (SAVELIEV *et al.* 2003). Although tandem repeats of *P{lacW}* consistently result in heterochromatic silencing, repeats of *P[ry; Prat:brown]* do not result in variable silencing or the condensed chromosome structure associated with heterochromatin (CLARK *et al.* 1998). HP1 binds both the *P[ry; Prat:brown]* and *P{lacW}* arrays (FANTI *et al.* 1998a) but possibly since *P[ry; Prat:brown]* has a housekeeping gene, it may be protected against heterochromatic silencing. Although HP1 does not bind to a specific DNA sequence, it does associate with single *P{lacW}* inserts (FANTI *et al.* 1998a).

How repeats are recognized is not understood. Work in *S. pombe* indicates that RNA transcription from sequences associated with heterochromatic silencing enters the RNA interference (RNAi) pathway, which triggers both transcriptional and post-transcriptional gene silencing. Transcriptional gene silencing triggers heterochromatin formation at the mating type locus and at the centromeres (HALL *et al.* 2002; VOLPE *et al.* 2003). Mutations that interrupt the RNAi pathway prevent heterochromatin formation but not its maintenance. Such RNA production could bind and recruit proteins like HP1 that have a chromodomain. In dosage compensation, a chromodomain in the histone acetyltransferase, MOF, interacts with *roX2* RNA and this interaction is necessary for hyperactivating transcription on the X chromosome in male *Drosophila* (AKHTAR *et al.* 2000). Therefore, RNA is a major component of chromatin formation.

The dsRNA that enters the RNAi pathway could form after transcription through repeats that are inverted relative to each other. Transgene arrays that contain inverted repeats are more strongly silenced (SABL and HENIKOFF 1996). However, in plants and *Drosophila*, expressing transgenes at a high rate can result in co-suppression and changes in chromatin structure. In *Drosophila*, this co-suppression occurs when the expression level surpasses a certain level (PAL-BHADRA *et al.* 1997). Members of the polycomb group proteins are necessary for this silencing. Therefore, tandem copies as well as individual insertion

events appear to trigger silencing through changes in chromatin structure.

The relationship between repeats and heterochromatin silencing suggests that heterochromatin could have evolved as a means to control TEs. TEs by definition replicate and this replication could lead to inverted repeats or increase gene copy number throughout a genome. Either tandem copies of a TE or insertion in repetitive sequence could trigger heterochromatin formation and silencing. As discussed later, P cytotype may be an example of such a system.

1.2.2 What P factor is acting in *trans* to modify *P* element dependent phenotypes?

Since strains that produce a strong P cytotype consistently suppressed the *P* effects in somatic tissue, the same factor was assumed to cause both. However, *P* element derivatives that modify *P* dependent phenotypes in the soma do not generate P cytotype effects in the germ line (GLOOR *et al.* 1993; ROBERTSON and ENGELS 1989). These derivative *P* elements are non-autonomous because they have a mutation that prevents the full-length transposase from being produced. Since they are non-autonomous, inserts at specific locations can be stocked and studied without being lost due to a transposition event. Certain *P* derivatives were isolated at certain locations that could modify the *P* dependent phenotypes in somatic lines. However, the same *P* derivatives could not re-produce the P cytotype effects. These non-autonomous elements weakly suppress hybrid dysgenesis when inherited from either parent (ROBERTSON and ENGELS 1989). In comparison, P cytotype produced by *P* strains strongly suppresses hybrid dysgenesis only when the *P* elements are inherited maternally. Therefore, the effect these *P* derivatives have on *P* dependent phenotypes depends on their position and their sequence (MISRA *et al.* 1993).

In terms of sequence, these *P* derivatives are divided into two *P* Types: Type I and Type II (GLOOR *et al.* 1993). Type I elements include exon 0-2 as well as the first 9 base pairs between exons 2-3. Therefore the sequence in Type I inserts encodes nearly the full length P repressor protein. Type II elements refer to non-autonomous P elements, such as KP or D50, that have a deletion that produces a truncated P repressor protein. This deletion includes the end of exon 1 and all of exon 2. There is no phenotypic difference between these different types of *P* elements. With one exception, examples of both types exist that can modify all the *P* dependent phenotypes thus far described (RASMUSSON *et al.* 1993). The exception is that there is no known Type II element that represses expression from *P-lacZ* inserts in somatic cells (LEMAITRE and COEN 1991). This probably represents an oversight in the literature and unpublished examples may exist. Other non-autonomous that have a larger or smaller deletion than what occurs in the Type I and II elements do not modify somatic P dependent phenotypes. However, *P* constructs with less P repressor coding sequence than Type II elements have been

identified that reproduce P cytotype and silence expression from *P* inserts with homologous sequence in the germ line (MARIN *et al.* 2000; ROCHE *et al.* 1995; RONSSERAY *et al.* 2001).

The most frequently used Type I construct is *P{ry⁺ SalI}89D* because of its strong phenotypic effects. In comparison, Type II have both been constructed and isolated from wild *Drosophila* strains (ANDREWS and GLOOR 1995; RASMUSSON *et al.* 1993). *KP* elements produce a truncated P repressor that could compete with transposase and poison the transposition reaction (ANDREWS and GLOOR 1995). *In vitro* evidence indicates that such a protein can bind sequences within the *P* element that occlude the promoter and are necessary for transposition (LEE *et al.* 1998; LEE *et al.* 1996). A zinc finger motif at the N-terminal is necessary for this DNA binding activity. This protein also has the leucine zipper within the P repressor that allows dimerization, which increases DNA affinity, and could bind transposase directly. Testing different *KP*-like constructs indicates that production of the protein is necessary to suppress gonadal dysgenesis *in vivo* (ANDREWS and GLOOR 1995). However, Type II elements exist, such as *SP*, which do not produce a functional protein but do suppress hybrid dysgenesis.

Researchers have consistently re-interpreted past experiments studying how *P* elements suppress hybrid dysgenesis. At the beginning, researchers assumed the same factor that modified *P* dependent phenotypes in the soma also suppressed hybrid dysgenesis. However, the single non-autonomous *P* elements, which modified *P* dependent phenotypes in the soma, do not suppress hybrid dysgenesis as strongly as *P* strains. This difference has been attributed to the high *P* element copy number in *P* strains, which results in a large amount of P repressor being produced (ROBERTSON and ENGELS 1989). Single *P* copies could not achieve this high expression level. In addition, researchers assumed that the *P* promoter was necessary to suppress hybrid dysgenesis. Tests with the Icarus *P* construct found that P cytotype could suppress transposase activity when an *hsp70* promoter is fused to the transposase gene within a *P* element construct (STELLER and PIRROTTA 1986). Since P cytotype continued to repress expression from a different promoter, they assumed the repression must occur post-transcriptionally. Based on this theory, the single *P* elements tested would not produce enough P repressor in the maternal germ line to suppress hybrid dysgenesis by poisoning the transposition reaction. However, special *P* element constructs that use germ line specific promoters to produce high amounts of P repressor in the germ line do not reproduce the maternally derived P cytotype effects (MISRA *et al.* 1993). The production of copious amounts of P repressor should interrupt transposition. Since there is no correlation between the production of P repressor protein and suppression of hybrid dysgenesis, this theory must be incorrect. Further, P cytotype is able to silence expression from *P* elements independently of the *P* promoter through a mechanism that requires sequence homology (ROCHE *et al.* 1995). This result indicates that the *P* promoter is not necessary for *P* element regulation.

A chromosome (*LK-P(1A)*) that has two autonomous *P* elements, within the 1A region can reproduce P cytotype effects (RONSSERAY *et al.* 1991; RONSSERAY *et al.* 1996; RONSSERAY *et al.* 1997). These effects are P cytotype-like, because they are maternally inherited and strongly suppress gonadal dysgenesis. No transcripts can be detected from these *P* elements or other *P* derivatives in this region, which have deletions including the *P* promoter (MARIN *et al.* 2000). These *P* derivatives and *P* constructs silence expression from *P-lacZ* reporter constructs that have nucleotide sequence homology (MARIN *et al.* 2000; ROCHE *et al.* 1995). Further, these inserts at 1A promote intron 2-3 retention (ROCHE *et al.* 1995). Possibly changes in chromatin structure at *P* elements could alter the splicing pattern (CRAMER *et al.* 1999; CRAMER *et al.* 1997; MANIATIS and REED 2002). Therefore, single *P* elements at certain genomic locations could reproduce qualities associated with P cytotype by triggering chromatin changes at other *P* elements with homologous sequence. These chromatin changes result in both silencing and intron 2-3 retention.

Only *P* inserts in the 1A region that are inserted in telomere associated sequences (TAS) repress transposition activity in the germ line (STUART *et al.* 2002). These TAS repeats have heterochromatic qualities and bind HP1. These heterochromatic qualities are necessary for the P cytotype effect, since mutations in *Su(var)2-5*, which encodes HP1, suppress the P cytotype effects (RONSSERAY *et al.* 1996; RONSSERAY *et al.* 1998). If *P* elements inserted in heterochromatin cause the P cytotype effects then other inserts in heterochromatic sequences should produce a similar effect. However, only a *P-w-ry* construct inserted in chromosome 3's (right) telomere reproduces P cytotype effects in combination with paternally inherited helper *P* elements (RONSSERAY *et al.* 1998). No other single *P* insertions in heterochromatic or euchromatic regions have been described that reproduced this effect. Thus, P cytotype is not a general phenomenon associated with *P* elements inserted within heterochromatic regions. However, thus far, only *P* elements inserted in repetitive sequence and heterochromatic regions have reproduced P cytotype-like effects.

The insertions at 1A do not repress *Δ2-3* expression (RONSSERAY *et al.* 1991; STUART *et al.* 2002) or expression from *P-lacZ* constructs in somatic tissues (ROCHE *et al.* 1995). This separates the P cytotype effects that suppress hybrid dysgenesis in the germ line from the *P* elements effects that modify P dependent phenotypes in the somatic tissues. Since the *P* elements that reproduce P cytotype do not modify somatic *P* element dependent phenotypes, the mechanism responsible for P cytotype may be independent of the mechanism that is responsible for the *P* element dependent phenotypes in somatic tissues. Although *P* insertions that cause P cytotype effects in the germ line do not cause similar effects in somatic cells, *P* insertions can cause heterochromatic changes at certain loci within somatic cells. The ability of *P* elements to enhance heterochromatic silencing at *P{lacW}ci*^{Dplac} is the major focus of this thesis and will be discussed throughout. The chromatin changes caused by P

cytotype in the germ line may be similar to the chromatin changes caused by *P* elements in the soma.

1.2.3 Heterochromatin and P cytotype

As mentioned, *P* insertions at 1A silence only *P* insertions at other locations that have homologous sequence. This silencing acts on different promoters (ROCHE *et al.* 1995) and does not require high expression levels from a *P* element insert at 1A (STUART *et al.* 2002). The *P* inserts causing the *trans*-silencing effect and their target must share between 0.800 kbp to 1.6 kbp of homologous sequence for the *trans*-silencing effect to occur. The homologous sequence can be any sequence, such as *P* or *lacZ*. However, homologous sequence must be proximal to the promoter or silencing does not occur. The mechanism mediating the silencing may be either pairing between the heterochromatic *P* element and the other *P* elements in the genome or an RNA molecule produced from the heterochromatic sequence binding to and silencing, other *P* element sequences (STUART *et al.* 2002). Current evidence favors the pairing model between ectopic sites in the germ line (STUART *et al.* 2002). This is based on work that uses recombination between ectopic pairing sites in the germ line. However, this evidence is not conclusive and although antisense RNA cannot induce P cytotype effects, dsRNA has not been tested (SIMMONS *et al.* 1996).

If P cytotype results from *P* constructs inserted in repetitive sequence, then repeated *P* elements by themselves should be able to induce this phenomenon. Testing such repeats has found that they can mimic P cytotype effects (RONSSERAY *et al.* 2001). Thus far, the strength of heterochromatic silencing in these repeats correlates with P cytotype strength. Weak heterochromatic w^{+mC} gene silencing occurs within the *BX2* array and this array does not repress hybrid dysgenesis. However, the *T1* array is the same array but is moved by a chromosomal rearrangement to a new position and it is strongly silenced. This strong heterochromatic silencing coincides with this *P{lacW}* array being able to suppress hybrid dysgenesis and silence *P-lacZ* insertions. This *P* array is reproducing P cytotype effects in the complete absence of a *P* element that encodes the P repressor. Thus, the P repressor protein is not necessary to produce P cytotype effects.

Similarly to $P\{lacW\}ci^{Dplac}$, the presence of *P* elements enhance w^{+} silencing within the *P{lacW}* arrays, such as *T1* and *BX2* (JOSSE *et al.* 2002). hPEV already occurs at these arrays and mutations in *Su(var)2-5* suppress this silencing (DORER and HENIKOFF 1994). When *P* inserts are present, such as $P\{ry^{+}\ SalI\}89D$ or *KP* derivatives, more white ommatidia form. It is assumed that *P* elements enhance heterochromatic silencing within the repeats. This result suggests that $P\{lacW\}ci^{Dplac}$ behaves similarly to the *P* insertions that cause P cytotype-like effects. The P insertions that cause P cytotype-like effects are inserted in repetitive sequence with heterochromatic qualities. $P\{lacW\}ci^{Dplac}$ is also inserted in repetitive sequence but this region is not defined as heterochromatic

because the w^+ transgene in *P{lacW}ci*^{Dplac} does not variegate (SUN *et al.* 2000).

Determining whether PDS at *P{lacW}ci*^{Dplac} and these *P{lacW}* arrays involves a similar mechanism follows two different approaches. First, a common set of heterochromatic modifiers may be necessary for silencing in the regions where PDS occurs. PDS may occur in these regions because *P* elements produce a *trans*-acting factor that enhances silencing by interacting with a common chromatin structure forming in these regions. Lastly, heterochromatic silencing already occurs within the *P{lacW}* arrays and the same could be true for *P{lacW}ci*^{Dplac}. If these chromatin modifiers can cause silencing at *P{lacW}ci*^{Dplac} in the absence of *P* elements, this would suggest that heterochromatic silencing is a property of this region. According to this theory, at the *P{lacW}* arrays and *P{lacW}ci*^{Dplac}, the presence of *P* elements enhances this heterochromatic silencing but do not cause its *de novo* formation. This thesis does not study whether *P{lacW}ci*^{Dplac} acts similarly to *T1* in the germ line and reproduce P cytotype-like effects. I am only concerned with PDS that occurs in somatic tissues.

P elements also cause w^+ silencing at the *P[w*^{d1}*9.3]19DE* insert. With *P[w*^{d1}*9.3]19DE*, the silencing induced by *P* elements is dependent on *zeste*^{1} and involves regulatory regions necessary for w^+ expression. The *mini-white* ($w^{+mC4.1}$) gene in *P{lacW}ci*^{Dplac} and in the *P{lacW}* repeats has a minimum of sequence from the original w^+ gene and expression is not affected by mutations in *zeste* (PIRROTTA 1988). Since PDS at the *P{lacW}* arrays and at *P{lacW}ci*^{Dplac} does not rely on any sequence within the w^+ gene or mutations within *zeste*, the variegation within the *P[w*^{d1}*9.3]19DE* appears to be a separate phenomenon (COEN 1990). However, in either case the same P *trans*-acting factor could be enhancing silencing although this silencing requires different chromatin modifiers.

1.2.4 Do the Type I and II *P* elements act similarly at *P{lacW}ci*^{Dplac} and *vg*$^{21\text{-}3}$?

The final portion of this thesis determines whether the Type I and intermediate *P* elements act differently on the *P* dependent phenotypes caused by *P* element insertions at *ci*. As mentioned, both Type I and Type II inserts modify the other *P* dependent alleles (GLOOR *et al.* 1993) but inserts that have sequencing ranging between these two types have no effect. The reason why a intermediate P repressor protein that ranges in size between that encoded by a Type I and Type II element has no effect is not understood. One possibility is that proteins with an intermediate length are thermodynamically unstable and targeted for degradation. This theory has not been tested.

The Type I and II classes are based on assays using the somatic phenotypes such as *vg*$^{21\text{-}3}$. However, these different types may not apply to PDS. It is necessary to show clearly that these two types have the same effect on PDS, but intermediate inserts do not. Then future research can concentrate how Type I and Type II elements mediate their effect. This research could treat silencing and other *P* element

dependent phenotypes as different phenomenon, if intermediate elements cause silencing at *P{lacW}ci*^{Dplac} but do not modify other *P* dependent phenotypes. However, if silencing at *P{lacW}ci*^{Dplac} responds similarly to other *P* dependent phenotypes then this suggests a similar mechanism. Since the difference between the two types affects the protein-coding portion of the *P* element, this suggests a protein is mediating the silencing affects. Future research can then look for a correlation between expression pattern and silencing at *P{lacW}ci*^{Dplac}. Testing w^{+mC} transgene expression from *P{lacW}ci*^{Dplac} provides a sensitive assay that can be easily quantified for comparison. In this thesis, I use a novel approach to determine if Type I and intermediate inserts at the same location act similarly in terms of their ability to modify somatic *P* dependent phenotypes. The Type I and intermediate elements tested occur at the same location and position effect will not confound the results as it has in past experiments.

2.1.1 Drosophila stocks

Drosophila melanogaster mutations were described previously (LINDSLEY and ZIMM 1992), unless otherwise cited. The *P{lacW}ci*^{Dplac} allele was a *P{lacZ*$^{P\backslash T.W}$*w*$^{+mC}$ *amp*^{R} *ori = lacW}* construct inserted between an 8 base pair duplication starting at 79894 (GTCTGTAC) in AE03854.3, which is about 3 kbp upstream from the *ci* locus on chromosome 4 (LOCKE *et al.* Submitted). The Harwich wild type strain was previously described (ANXOLABEHERE *et al.* 1988; KIDWELL and KIDWELL 1976) and was an inbred *P* strain containing many *P* elements and a *P* cytotype. The Oregon-R strain lacked *P* elements (M strain) as tested by Southern transfers and cannot mobilize *P{w*$^{+}$*}* marked transposons. *P{ry*$^{+}$ *SalI}89D* was a Type I *P* repressor element that had a frame shift mutation in the *Sal* I site at the beginning of exon 3 (KARESS and RUBIN 1984; RIO *et al.* 1986). The *Su(var)3-7*$^{+t6.5}$ transgene was provided by P. Spierer and originally described by G. Reuter *et al.* (1990) . *T1* and *BX2* were successively repeated *P{lacW}* arrays originally generated by D. Dorer and S. Henikoff (1994) and provided by S. Ronsseray. *KP-D(Cy)* and *KP-U(TM6B)* stocks were also provided by S. Ronsseray. Our PCR and Southern blot analysis found that these stocks had 4-6 *KP* elements segregating either with chromosome carrying a *Cy Bl vg*$^{?}$ for *KP-D* or a *TM6B* for *KP-D*. The *P{hsp26-pt-T}ci*$^{2\text{-}M1021.R}$ and *P{hsp26-pt-T}2-M010.R* insertions were originally isolated by Sun *et al.* (2000). The *vg*$^{21\text{-}3}$ allele was described in Williams *et al.* 1988 and supplied by R. Hodgetts. All crosses testing expression from the *w*$^{+}$ transgenes were completed in a *white*$^{-}$ (*w*$^{67c23(2)}$) background to permit the assessment of *w*$^{+}$ transgene expression.

Culture vials were grown at 21° (unless otherwise stated) on standard yeast/cornmeal medium (each liter contains 8 g agar, 18 g Torula yeast, 72 g cornmeal , 96 mL fancy molasses, 2.8 mL propionic acid, and 2.8 g methyl paraben dissolved in 10 mL of 95% ethanol).

2.1.2 Mutagenesis

Three different screens used gamma irradiation, ethyl methanesulfonate (EMS), or *P* element dysgenesis to generate the *Su(PDS)* mutants. In the first screen, *y w; P{lacW}ci*^{Dplac} males were exposed to 40 GRAYS and crossed to *y w; P{ry*$^{+}$ *SalI}89D Sb/ TM6B; ci ey*^{R} females. We selected for suppressor mutants that caused increased numbers of colored ommatidia in *P{ry*$^{+}$ *SalI}89D Sb* progeny, which had predominately white colored eye with a few colored ommatidia. Flies with a putative suppressor mutation that allowed *w*$^{+}$ expression when *P{ry*$^{+}$ *SalI}89D* was present were back crossed to the original *y w; P{ry*$^{+}$ *SalI}89D Sb/ TM6B; ci ey*^{R} stock to confirm that the mutant phenotype transmitted. We also screened for enhancer mutations in the progeny that inherited *TM6B* that normally had uniformly colored eyes. Flies with a putative enhancer mutation were backcrossed to *y w; P{lacW}ci*^{Dplac} to confirm the mutant phenotype transmitted. Males

displaying a mutant phenotype were crossed successively to a *w; CyO/ Xa* and *w; TM6B/ Ly* to map the mutation to chromosome 2 or 3 by testing for segregation from the balancer and to stock the mutant with the appropriate balancer.

In the second screen, *y w; dp; e; P{lacW}ci^{Dplac}* males were starved for 8 hours, then placed overnight in a fly vial containing a piece of Whatmann filter paper soaked with 600 μL of a solution containing 25 mM EMS, 1% sucrose, and 0.1% bromophenol blue (ASHBURNER 1989a). Males with blue abdomens from bromophenol blue ingestion were preferentially mated to *w; Sb P{ry^{+} SalI}89D /TM3, Ser; ci ey^{R}* females. We screened for a suppressed eye phenotype in the *Sb* progeny and an enhanced eye phenotype in progeny with wild type bristles. The mutants were stocked using a *w; CyO/ Bl; Sb P{ry^{+} SalI}89D/ TM2* stock.

In the last screen, *P{PZ}* inserts, *P{PZ}05137^{05137}* and *P{PZ}CtBP^{03463}*, which flank *Su(var)3-7*, were mobilized to generate mutants within 87E. To generate mutants, *y w; Sb e Δ2-3(99B)/ P{PZ} ry^{506}* (where *P{PZ}* was either *P{PZ}05137^{05137}*or *P{PZ}CtBP^{03463}*) males were crossed to *y w; P{ry^{+} SalI}89D; P{lacW}ci^{Dplac}* females. Progeny with wild type bristles and an increased number of red ommatidia were back crossed to a *y w; P{ry^{+} SalI}89D; P{lacW}ci^{Dplac}* stock to confirm the mutant phenotype transmitted and then stocked as in the second screen.

2.1.3 Genetic mapping

The Su(PDS) phenotype of *Su(var)3-7^{P9}* was mapped by crossing *w; Su(var)3-7^{P9}/ Ki^{1} rs^{1} Ubx^{1} H^{2} Dr^{1}* heterozygous females to *y w; P{ry^{+} SalI}89D; P{lac W}ci^{Dplac}* males. Although *Dr^{1}* flies had reduced eye size, the relative amount of red pigment in the eye tissue could still be assessed.

The Su(PDS) phenotype of *Su(PDS)P80* and *Su(PDS)P86* were mapped by crossing *w; Su(PDS) /Coa Ki H* heterozygous females to *y w; P{ry^{+} SalI}89D; P{lacW}ci^{Dplac}* males. The Su(var) phenotype was mapped by crossing *In(1)w^{m4}, w^{m4}; Su(PDS)P86/ Coa Ki H* heterozygous females to *In(1)w^{m4}, w^{m4}* males.

Su(PDS) mutations on chromosome 2 were mapped by crossing *w; mutant/ wg^{Sp-1} Bl^{1} L^{rm} Bc^{1} Pu^{2} Pin^{B}* females to *w; P{ry^{+} SalI}89D; P{lacW}ci^{Dplac}*. Recombination events between *wg^{Sp-1} Bl^{1} and L^{rm}* were used to determine the genetic location for the *Su(PDS)* mutations.

2.1.4 Staining imaginal discs for β-galactosidase activity from *P{lacW}ci^{Dplac}*

Following the method described by Ashburner (1989b) (ASHBURNER 1989b), imaginal discs were dissected and washed in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 pH 7.4). The discs were fixed in 0.75% gluteraldehyde 1X PBS. Then the discs were washed in 1XPBT (PBS+0.05% Triton-X). All discs were stained in X-gal staining buffer (10 mM Na_2HPO_4, 150 mM NaCl, 1 mM $MgCl_2$, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.2% 5-bromo-4-chloro-3-indolyl b-D-galactopyranoside pH 7.2) over the same 8 hour time

period at 37°C. Then the discs were washed in PBT and mounted in disc mountant (90% glycerol, 0.1 M Tris-HCl pH 8) solution. We used the *Tubby* (*Tb*) marker to distinguish *Su(var)3-7* mutant larvae from progeny that inherited *TM6B-Tb, Antp^{Hu} e^{1} Tb*. Photographs were taken with a Zeiss Axiophot photomicroscope.

2.1.5 Eye pigment determination

The extent of w^{+} gene activity was determined by measuring the amount of brown eye pigment. Measurements were made based on the method of Ephrussi and Herold (1944) as modified by Locke *et al.* (1988) . The heads recovered from 5-9 day old adult flies were stored at -70°C. Three replicate samples containing either 10 or 20 heads, depending on the assay, of each genotype were extracted in 500 μL of acidified methyl alcohol (AMA: 1% v/v HCl in methanol). After 36-48 hours of continuous agitation to extract the pigments, 2.5 μL of 1% H_2O_2 was add to each tube and then incubated at room temperature for 90-120 minutes. The absorbance at 470 nm was measured in a LKB Ultraspec II spectrophotometer or Genova Life Science Analyser.

To measure the amount of pigment produced by *In(1)w^{m4}, w^{m4}* progeny, mutant male flies were crossed to *In(1)w^{m4}, w^{m4}; Xa/CyO; Xa/TM2* females. Then males with *In(1)w^{m4}, w^{m4}; CyO/?; TM2/?* were crossed to *In(1)w^{m4}, w^{m4}* females. If the mutation was on chromosome 2 then Cy^{-} and Cy^{+} flies of a single sex were separated and compared; if on chromosome 3 Ubx^{-} and Ubx^{+} flies were separated and compared.

2.1.6 Complementation of lethal and maternal effect phenotypes

Mutant alleles were tested to determine if they complemented recessive lethal or maternal effect phenotypes. In each complementation test, at least 50 flies were counted in a balancer class that was expected to occur with equal frequency to the class that had the mutant combination. After counting 50 flies in a balancer class, if the heterozygous mutant combination did not occur, the mutant allele combination was considered lethal (-). When mutant combination occurred at a ratio of 1:19 compared to the balancer class, the mutant combination was labeled semi-lethal (s). If the combination occurred more frequently than 5%, the combination was considered viable (+).

Mutant/balancer flies were crossed to homozygous *Su(var)3-7^{P9}* females to test whether the mutant chromosome could zygotically rescue *Su(var)3-7^{P9}*'s maternal effect. If the mutant chromosome zygotically rescued the maternal effect, there should be a 1:1 ratio between balancer/*Su(var)3-7^{P9}* progeny and mutant/*Su(var)3-7^{P9}* progeny.

*Su(var)3-7** mutant alleles were tested for a maternal effect by crossing *Su(var)3-7*/ Su(var)3-7^{P13}* heterozygous females to *Su(var)3-7^{P13}/ TM6B* males. *Su(var)3-7^{P13}* is a deficiency including *Su(var)3-7* (See Results). If there was no maternal effect, then the progeny that inherited *Su(var)3-7^{P13}* from their father should occur at the same frequency as those that inherited *TM6B, Antp^{Hu} e^{1}*.

2.1.7 Mutation detection and DNA sequencing

Base pair changes in *Su(var)2-5* and *Su(var)3-7* mutant alleles were detected using the Transgenomic WAVE® DNA fragment analysis system. Template was isolated from mutant/+ heterozygous flies and PCR was used to amplify overlapping sections of each gene. Heteroduplexes forming between mutant and wild type sequences could be detected using this system.

Su(var)2-5 allele sequences were determined from hemizygous flies and larvae that did not express green fluorescence protein derived by crossing a *Df(2L)TEAa-ll /CyO-pAct-GFP* stock to a *Su(var)2-5*^{P4} mutant/ CyO-pAct-GFP stock (REICHHART and FERRANDON 1998). Homozygous *Su(var)2-5*^{P5} mutants were isolated from a *Su(var)2-5*^{P5}*/CyO-pAct-GFP* stock by selecting embryos that lacked the *GFP* gene. Since GFP fluorescence was not expressed in the embryos, we used a PCR test with primers within the *GFP* gene to select against embryos that inherited the *CyO-pAct-GFP* chromosome allowing selection for *Su(var)2-5*^{P5} homozygous embryos.

Su(var)3-7 mutant alleles were sequenced as hemizygotes over *Df(3R)126c* (B-3009). All mutations were confirmed by reading from both forward and reverse primers (both strands).

2.1.8 Single fly extraction protocol

This procedure has been modified from a protocol published by Gloor and Engels 1992. Single flies were squished in 50 µL "squishing buffer" (10 mM TRIS-HCl pH 8; 1 mM EDTA pH 8, 25 mM NaCl and freshly added Proteinase K (200 µg/ml)) to a 0.6 ml PCR tube containing a single fly. The Proteinase K was kept as a 20 mg/mL stock solution in the freezer and 1 µL can be mixed into 99 µL of squishing buffer.

Individual flies were mashed with a pipette tip. The best way to do this was to add only a small amount of the 50 µl volume in the tip, and squish the fly with the tip still attached to the pipetman. When the fly seems to be well mashed (separate pieces, some color usually seen in the buffer), the remaining volume in the tip can be added.

The tubes are then placed in the thermocycler and incubated in a PCR machine using the program "FLY1"(Lid at 100°C, 1. 37°C for 45 minutes, 2. Link to Fly 2). This program automatically links to "FLY2" (Lid at 100°C, 1. 99°C for 10 minutes, 2. 4°C).

The DNA preparation was now ready for use as a PCR template. Between 0.5-1.0 µl of the fly mini-preparation usually had sufficient template for a single PCR reaction of 30 µl to 50 µl total volume.

2.1.9 Large-scale genomic DNA extraction protocol

Between 50-100 flies were ground in a glass homogenizer with 500 µl of homogenization buffer (200 mM EDTA pH8, 100mM TRIS pH7.5, 1% SDS), preheated to 60 C. Then the flies and buffer were transferred to a 2 ml screw cap tube, rinse homogenizer with an additional 100 µl of buffer, and add to the tube contents. The homogenizers were

thoroughly cleaned, rinsed with distilled water and returned to their container after use. The 2 mL tubes (flies and buffer) were incubated at 60°C for 30 minutes. After incubating at 60°C, 600 µl of phenol was immediately added. The tubes were mixed for 1 minute by inverting the tube. The tubes were centrifuged down for 3 minutes and the $CHCl_3$ layer removed with a fine tip transfer pipette. The removal of fly bits was avoided. After removing the $CHCl_3$, 600 µl of phenol was added and the tubes mixed by inversion. Since inversion did not always break up the fly tips, a transfer pipette was often used to break the fly bits. After centrifuging for 3 minutes, the phenol layer was removed. This phenol step was repeated again. Then 600 µl of $CHCl_3$ was added and the tubes centrifuged for 3 minutes. Only the aqueous layer was then transferred to a 1.5 mL tube. Fly bits were carefully avoided. The total volume in the 1.5 mL tube (aqueous layer) was increased to 500 µL by adding TE (10 mM TrisHCl pH8 1 mM EDTA). Then to precipitate down the DNA 50 µL 3M NaCl and 1000 µL of cold 95% ethanol were added to tubes. The tubes were mixed by inversion and allowed to precipitate for 1 minute. The tubes were centrifuged for 1 minute and the ethanol removed. The pellet was dried for 10-30 minutes but not allowed to completely dry because a completely dried pellet would not dissolve. The pellets were re-suspended in 200 µL TE and allowed to dissolve overnight with 20 µL RNAseA (0.5 mg/mL). Then 200 µL of phenol were added and the tubes centrifuged for 3 minutes. After discarding the phenol, 200 µL of $CHCl_3$ was added and the tubes mixed by inversion. After centrifuging for 3 minutes, the $CHCl_3$ was removed and the chloroform extraction step was repeated. On the second extraction the aqueous layer was transferred to a new 1.5 mL tube. Then 20 µL of 3 M NaCl and 450 µL of 95% ethanol are added . The tubes were mixed by inversion and left for 1 minute. Then the tubes were centrifuged down at top speed (13 200 rpm) for 1 min and briefly washed with 80% EtOH. After decanting the ethanol the tubes were dried until the pellet looked slightly wet and there were no droplets on the side of the centrifuge tube. The samples were dissolved in 50 µL of TE. Then 5 µL was used to test the quality of the genomic preparation in an agarose gel.

2.1.10 DNA extraction from flies using Qiagen™ kit

In the following procedure, buffers ATL, AL, AW1, AW2, and AE were provided with DNeasy™ tissue kit.

Between 25-50 flies were ground in a 1.5 ml microtube using 180 µl of ATL and a blue disposable pestle. Then 30 µl of proteinase K solution was added and the tube vortexed. While periodically being vortexed, the tubes were incubated at 60 C (incubator) for 60 minutes. Then 200 µl of buffer AL was added, the tubes mixed by vortexing, and incubated at 70°C for 15 minutes. Then 200 µl of room temperature 95% EtOH was added and the tubes mixed by vortexing. Then the entire mixture, including the fly-bits were pipetted into the spin column. After centrifuging at 8000 rpm for 1 minute, the flow-through was discarded.

Then 500 µl of AW1 was added and the columns centrifuged at 8000 rpm for 1 minute. After discarding the flow-through, 500 µl of AW2 was added and the columns centrifuged at 14 000 rpm/ 3 minutes. The flow-through was discarded and a new catch microfuge tube was placed underneath the column. Then 200 µL of AE were added to the column and allowed to equilibrate for 1 minute. Then the column was centrifuged at 8000 rpm for 1 minute. This elution step was repeated with another 200 µL AE. A 1/10 dilution from the 400 µL of genomic DNA provided enough template for PCR. For wave analysis, the DNA had to be salt precipitated before use in the PCR to avoid chaotropic salt contamination.

2.1.11 Plasmid minipreps by alkaline lysis

This protocol has been modified from Sambrook et al. 1989. A test tube containing 5 ml of Lauria Broth (LB; 10 gm tryptone, 5 gm yeast extract, 10 gm sodium chloride, 1 gm glucose add to 1 L of water and autoclaved for 20 minutes) with the appropriate antibiotic was inoculated from a single bacterial colony. Then culture was grown overnight while shaking, at 37°C. In the morning, the cells were centrifuge down in a Sorvall centrifuge at 5000 rpm for 2 minutes. The broth was carefully drained off so that no broth remained. Then the cells were re-suspend in 100 µl glucose solution 1 (50 mM glucose, 25mM TrisHCl pH8, 10 mM EDTA pH 8), by vortexing until no pellet remains. Then the contents were transferred to a labeled 1.5 ml microfuge tube. To the 1.5 mL tube, 10 µl of RNase A solution (0.5 mg/ml) was added. Then 200 µl of lysis solution 2 (0.2 M NaOH, 1% SDS) was added. Tubes were inverted several times to mix thoroughly while being allowed to lyse at room temperature for exactly 5 minutes. Then the lysis solution was neutralized by adding 150 µl of high salt solution 3 (5 M K-acetate 60 mL, glacial acetic acid 11.5 mL H_2O 28.5 mL). Again, the tubes were inverted to mix the contents until a white precipitate formed and the solution was well mixed. The precipitate was centrifuge down for 10 minutes and the supernatant moved to a new microfuge tube. Then 500 µL of phenol was added and the tube was vortexed twice over a two minute period. The tube was centrifuged for 4 minutes and the phenol layer removed. Then 500 µL of chloroform was added, the tube centrifuged for 4 minutes, and the aqueous layer moved to a new tube. Then 1000 µL of cold 95% ethanol was added to the aqueous layer. Precipitation was allowed to proceed at –20°C for 30 minutes. The precipitate was centrifuged down for 10 minutes and the ethanol removed. The tubes were washed with 80% ethanol and centrifuged for 3 minutes. After removing all the ethanol the pellets were allowed to completely dry. The pellets were then dissolved in 50 µL of TE. About 2 µl of high copy and 5 µL of low copy plasmid is enough for a restriction digest.

2.1.12 Rapid cracking procedure

A sterile toothpick transferred approximately 1/3 of a gridded bacterial colony square to a centrifuge tube containing 50 µl of rapid

cracking buffer (Table 2.1.1) (LAW and CRICKMORE 1997). The cells were rubbed off on the side of the tube, and then pushed into the buffer, moving the toothpick up and down. If the cells were left on the toothpick, the DNA had a tendency to stick to the wood when the cells lysed in the buffer. The tubes were not vortexed.

Once all the (1/2 cm^2) gridded squares were transferred to the tubes, the centrifuge tubes were placed in a 37 C incubator for 5 minutes. After the 5 minute incubation, the tubes were placed on ice for another 5 minutes. The tubes were the centrifuged down for 10 minutes. Then 15-20 μL was loaded into each lane of an agarose gel. There was enough sample so that pipetting the viscous pellet was avoided. The samples were compared to a control, which was usually the original plasmid. This procedure worked well to detect inserts that were more than 700 bp.

Table 2.1.1: Rapid cracking buffer

EDTA pH 8	5 mM
Sucrose	10 %
SDS	0.25 %
NaOH	100 mM
KCl	60 mM
Bromophenol blue	0.05 %

2.1.13 Sequencing of PCR products

To sequence a PCR fragment, I ran the product out on an agarose gel. Then the band was excised and placed into a clean microfuge tube. The tube was weighed to determine the volume of the agarose gel slice (*ie.* compare to an empty tube). The DNA was extracted from the gel slice using the Qiagen gel extraction kit. I followed the directions supplied with the kit. The DNA was eluted in 30 - 40 μl of elution buffer supplied with the kit. The reaction mix was described in Table 2.1.2.

Table 2.1.2: Sequencing Reaction

Big Dye premix	2 μl
Reaction buffer	6 μl
DNA	10 μl
Primer (2.5 μM)	2 μl
Total volume	20 μl

The reaction was run in the thermocycler using program SEQ (1. 95°C 30s, 2. 50°C 15s, 3. 60°C 60s, 4. Goto 1 Rep 29, end).

After the cycling was complete, I added 2 μl of EDTA/Na-acetate + 80 μl of 95% EtOH was addede. After being vortexed briefly to mix the solution, tubes were placed in the freezer for about an hour. After precipitation, the tubes were centrifuged for 15 minutes and the ethanol decanted. The pellet was usually not visible. Then the pellet was washed with 200 μl of 80% EtOH and vortexed briefly. The tubes were centrifuged down again for 15 minutes before discarding the supernatant. After carefully removing the ethanol, the tubes were completely dried in a vacuum.

2.1.14 Southern transfer

The genomic DNA was run on a 0.8% agarose gel overnight at 35V. Images were taken alongside a ruler and saved to disk. As well, distance between the well and each fragment in the lane loaded with the 1 kb^+ ladder (Invitrogen) was recorded before starting the transfer. The gel was then soaked and gently shaken in 0.25 M HCl for 10 minutes. After rinsing off the HCl with water, the gel was soaked in 0.5 M NaOH/ 1.5 NaCl for 30 minutes. Special care was taken during this step because the gel was slippery after this treatment. The gel was rinsed in water and gently shaken in 1.5 M NaCl/ 0.5 M TRIS-HCl pH7.5 for another 30 minutes. During the previous steps, I would cut a Genescreen plus membrane and 3 pieces of Whatman paper to exactly the size of the gel and equilibrate them in 10XSSC for 10 minutes. As well, three long pieces of Whatman paper the width of the gel were cut. On a glass plate resting over a container, the 3 long strips of Whatman were placed to wick up 10XSSC that fills half the container. Excess 10XSSC was placed on the Whitman strips and the gel placed on top. A pipette was rolled across the top of the gel to remove any air bubbles. Then 10XSSC was placed on top of the gel. Then the Genescreen plus membrane was placed gently on top. The pipette again was used to remove any air bubbles between membrane and gel. Then 3 pieces of Whatman (gel size) were placed on top and again a pipette was used to remove any air bubbles. On top of the Whatman, 5 –7 cm of paper towel were laid across the top and then a glass plate with a weight was placed on the very top. The next day after removing the paper towel, I used a pencil to

mark the lane positions. Then the membrane was soaked in 0.5 M NaOH for 1 minute and rinsed in 0.2 M TRIS-HCl pH7.5. The membrane was dried at 60°C on saran wrap for an hour.

2.1.15 Random primer labeling and hybridization

The DNA and dH_20 were placed in a labeled screw-cap tube (Table 2.1.3). Then the tube was boiled for 3-5 minutes. After boiling, the tube was placed immediately on ice. To the tube on ice, the agents for random primer labeling were added (Table 2.1.3). The contents in the tube were mixed and centrifuged down. Then reaction was incubated at 37 C for ~3 hours.

Table 2.1.3: Random primer labeling reaction

DNA (50-100ng) + dH_2O - Boil for 3-5 min	33 µl
5X OLB	10 µl
^{32}P-dCTP (10 mCi/ mL at 3000 Ci/mmol)	5 µl
Klenow Gibco (18012-021 1-9 U/ µL)	2µl
Total volume	50 µl

When making 5XOLB (Table 2.1.4), aliquot 100 µL into separate tubes. Add 2 µL β mercaptoethanol to each tube before use and keep in freezer.

Table 2.1.4: 5XOLB

Tris pH 7.5	1 M	100 µL
$MgCl_2$	1 M	12.5 µL
dATP, GTP, TTP	100 mM each	1.25 µL
HEPES pH 6.6	2 M	250 µL
pd(N)6 hexamers (Pharmacia 27-2166-01)	90 A260	150 µL
H_20		250 µL
	Total	520 µL

2.1.15.1 Prehybridization

I placed the membrane in a bottle or a Tupperware dish with enough hybridization solution to cover the membrane (Table 2.1.5). When using bottles, I used a nylon mesh to prevent overlapping membranes from trapping probe. I pre-hybridized the membrane at 65 C for 30 minutes for DNA blots. If using 50% formamide buffer (50% formamide, 5XSSC, 1% SDS), I reduced the hybridization temperature to 42 C.

Table 2.1.5: Hybridization buffer

$NaPO_4$ pH7.2	1 M	50 mL

SSC	20X	300 mL
10 % SDS	(w/v)	100 mL
H_2O		550 mL
	Total	1000mL

2.1.15.2 Hybridization

I boiled the labeled probe in the kettle for 3-5 minutes. Then I diluted the probe in ~500 µl of hybridization solution, and added the entire amount immediately to the container or tube, being careful not to add the probe directly onto the membrane, as this may cause a region of high background. The hybridization reaction proceeded overnight at 65 C in regular hybridization solution, or 42 C in 50% formamide solutions.

2.1.15.3 Stringency washes

I placed the membrane into a covered dish containing pre-warmed Wash I (2XSSC, 0.1%) and then incubated at 65 C while shaking for 30 minutes. The radioactive hybridization solution was poured carefully into the waste bottle.

I transferred the membrane into a new dish containing pre-warmed Wash II (0.2XSSC, 0.1%) and Incubated at 65 C while shaking for 30 minutes. The radioactive wash buffer I should be poured carefully into the waste bottle.

In the final wash, the membrane was transferred into a new dish containing pre-warmed Wash III (0.1XSSC, 0.1%SDS) and incubated at 65 C while shaking for 30 minutes. The wash buffer can be diluted and poured down the drain. If a lower level stringency wash was desired, I only used Wash 1, and monitored with a Geiger counter to check the radioactivity level. The recipe for 20XSSC was indicated in Table 2.1.6.

Table 2.1.6: Making 20XSSC

NaCl	175.3 g/L
Nacitrate	88.2 g/L

2.1.15.4 Autoradiography

I placed the wet membrane onto a piece of wet filter paper, and wrapped well with Saran wrap, so that the membrane did not dry out. Then I checked for signal using the Geiger monitor. If the signal was strong, a lower exposure time (~4 hours) was sufficient. The X-ray film was placed on top of the blot in the cassette with orientation dots. Then the cassette was incubated at -80 C.

2.1.16 Stripping

The Southern blots were denatured in excess Strip I (0.4 M NaOH) at 42°C for 30 minutes. Then the filter was neutralized in excess Strip II (0.2 M Tris pH 7.8), 0.1XSSC, 0.1% SDS) at 42°C for 30 minutes. Special care was taken to drain off all excess NaOH.

2.1.17 Polymerase chain reaction (PCR)

A basic 1X master mix was described in Table 2.1.7. This master mix was used at 50 μL for making probes and template DNA for sequencing reactions. Otherwise, the master mix was halved and a 25 μL reaction produced enough product to detect on an agarose gel.

Table 2.1.7: PCR master mix for 50 μL

Buffer (supplied)	10X	5 μL
dNTP	10 mM	1 μL
$MgCl_2$	50 mM	1.5 μL
Primer F	10 μM	2.5 μL
Primer B	10 μM	2.5 μL
$dH_2O^{sterile}$		36.25 μL
Taq (Gibco)		0.250 μL
Template		1 μL
	Total	50 μL

2.1.17.1 Primers

The sequence for each primer used to characterize the *Su(var)2-5* and *Su(var)3-7* alleles is listed in Table 2.1.8.

Table 2.1.8: Primers used to characterize the *Su(var)2-5* and *Su(var)3-7* mutations

Primer	5′ Sequence 3′
Su(var)2-5$^+$	
2FHP1	GTAGTCTTAGCAGTCGCACGC
394BHP1	CGCACCCGCCTGTCGATGAT
328FHP1	AACCCTGAGAGCTCGGCAAAG
804BHP1	GTAGGTACACTCATTCCATAGCT
779FHP1	CGAAAGTCCGAAGAACCAACAG
1220BHP1	CCACTGAGGAGGGCACCATTT
1154FHP1	TGGGTGCCTCCGACAATAAT
1542BHP1	TGAGCAACAAGTCGCAAATAA
227FHP1	TTTGCGTGTGTGTGCGGTTG
Su(var)3-7$^+$	
7551F3-7	GGCGAAAAACCTC
8092B3-7	CGGGTTCACGTTCCTGGTCTT

Primer	5′ Sequence 3′
7919F3-7	GGATGATGACGATGGGGATGT
8354B3-7	AACGCTAAGGTGCCATCGGAG
8211F3-7	CCGGGAGACAGCCAGGATGAC
8710B3-7	TGGAAAATTAATGCGGACTCGGA
8505F3-7	CGAGGAGCGTAAGTGTCACAG
8897B3-7	TTCGCAAACTTTCGGTGATCTCA
8716F3-7	TCCGCATTAATTTTCCAACGTGT
9261B3-7	ATCCCCGCCGCTATTGGTCA
9141F3-7	ACGTGGAGTTTGTCTATTTGCGC
9595B3-7	CCGCGAATATCTGAAGAGCTGAG
9569F3-7	CCGCTGAAAAGGGGTGAGTTGAC
9998B3-7	CTAGAGGACTTATGAGCAAAACT
9933F3-7	ACTCGGAAAAGGGTATGTTGCT
10442B3-7	TTTGTAGGAGACCACGGGAA
10401F3-7	CGGCAACCATGAAGGGCAAGG
10903B3-7	CGCATCAACGGTGGAAGTGGGA
10871F3-7	GCCGCCGCTGCCGCCAATG
11327B3-7	TCGGCGAAATGCTGTCAAAGAAG
11294F3-7	AAGCCACCGGAGCCACGACAT
11703B3-7	CGGCGTATATTCTCTGGCAGGTC
11652F3-7	CCAGTTACCGGGGCTTCCTCT
12089B3-7	GGAGCTCTATGGCCTGCGGTTA
12013F3-7	CCTCGACTCCAATAATGCGTG
13032B3-7	GGCCCTTGCAGAGTCCAGAGC
12973F3-7	CAACTTGCTTCGCGCTATCTT
13991B3-7	ATCCCACTGCACATATTCGGC
13958F3-7	CCATTTTCATTGCCACTCACAT
16530B3-7	CCGCAGACATTCGACATCAGTC
Other	
GFP1493	AGTGCCATGCCCGAAGGTTA
GFP1181	GGTAAAAGGACAGGGCCATCG

Primer	5′ Sequence 3′
JS1	ATACATAGTTTCATTACGG
JS2	GCGTTTGTATGTATATTGGS

2.1.17.2 PCR conditions for primer pairs spanning *Su(var)2-5*

Table 2.1.9 listed the primer pairs used to characterize the *Su(var)2-5* alleles. Table 2.1.10 indicated the thermocycler programs used to amplify each fragment. Wave and sequencing analysis detected mutations in these amplified products.

Table 2.1.9: Primer pairs used to characterize the *Su(var)2-5* alleles

Primer Pair	Expected size (bp)	Optimum Annealing Temperature (°C)
2F-394B	392	61.1
328F-804B	476	66
779F-1220B	441	67.4
1154-1542	388	59.6

Table 2.1.10: Thermocycler program used to amplify fragments from *Su(var)2-5*

1. 95°C 3:00′
2. 65°C 1:00′
3. 73°C 1:00′
4. 95°C 1:00′
5. Optimum annealing temperature 0:45
6. 73°C 0:30
7. Goto 4 Rep 29

2.1.17.3 Primer pairs used to analyze the *Su(var)3-7* alleles

Table 2.1.11 described the primer pairs used to amplify sections from *Su(var)3-7* alleles for sequencing and WAVE analysis. Table 2.1.12 indicated the thermocycler programs used to amplify these fragments.

Table 2.1.11: Primer pairs used to analyze the *Su(var)3-7* alleles

Primer Pair	Size (bp)	Temp F (°C)	Temp. B (°C)	Optimum Annealing Temp. (°C)	Program
7551F-8092B	541	60.99	64.22	65.2	Dan1
7919F-8354B	435	62.57	64.52	61.8-67.1	Dan1
8211F-8710B	499	68.42	60.99	60.6	Dan2
8505F-8897B	392	64.52	60.99		
8716F-9216B	500	59.20	64.50	59-64.5	Dan2
9141F-9595B	454	62.77	64.55	63.1-65.9	Dan1
9569F-9998B	429	66.33	59.20	59	Dan3
9933F-10442B	509	60.81	62.57	60.1-63.1	Dan2
10401F-10903B	502	66.47	66.40	64.5-67.9	Dan1
10871F-11327B	456	70.95	62.77	61.8-67.1	Dan1
11294F-11703B		66.47	66.33	64.5-7.1	Dan1
11652F-12089B	437	66.47	66.40	65.2-66.5	Dan1

Table 2.1.12: Thermocycler programs to amplify fragments from *Su(var)3-7*

Thermocycler Programs		
Dan1	Dan2	Dan3
1. 95°C 3:00′	1. 95°C 3:00′	1. 95°C 3:00
2. 67°C 1:00′	2. 63°C 1:00′	2. 62°C 1:00
3. 73°C 1:00′	3. 73°C 1:00′	3. 73°C 1:00
4. 95°C 1:00′	4. 95°C 1:00′	4. 95°C 1:00
5. 65°C 0:45′	5. 60.6°C 0:45′	5. 59°C 0:45
6. 73C° 0:30′	6. 73°C 0:30′	6. 73°C 0:30
7. Goto 4 Rep 29	7. Goto 4 Rep 29	7. Goto 4 Rep 29

2.2 Constructing the P{I SalI} construct

To place the FRT sequences between exons 1-2 (intron1-2) and exons 2-3 (intron2-3), fragments spanning 1-2 and 2-3 were PCR amplified using Vent$_R$®DNA polymerase (New England Biolabs) from pπ25.1. These fragments had an *Nco* I site added where the FRT was placed and were subcloned into pBluescriptSKII(+) (pBSKII(+)).These constructs were sequenced using T7 and T3 primers to confirm the lack of mutations in each intron sequence that contained the FRT sequence. Then an oligonucleotide with the FRT sequence was cloned into each *Nco* I site. Then the fragments containing the FRT sequences replaced the exons1-3 sequence within pP{ry}. The process was detailed below for each intron. Table 2.2.1 lists the primers used in P{I Sal}'s construction.

Table 2.2.1: Primers and FRT sequences used to construct P{I Sal}

Primer Name	Sequence5′-3′
2152B (*Nco*I)	TTTCCATGGTACGAAACTTACATTCTGATATAC
1483R	GGGTCCGCCGGTATCATAC
2700F	TGGTCACGACATTTACTCTT
2358F	CAATTCAGGAGGCACTTCATC
2173F (*Nco*I)	GTACCATGGAAAAATCAGATAATCCTTGAAATTC
3017B (*Nco*I)	TACCATGGTTCTCAACTGAACTTTCCTGAAACA
3039F (*Nco*I)	GAACCATGGTAGTTATGTGCTGTCTATTGTGTTTTG
2033F	CCGAATGGACCCGGATACTC
2376R	TGAAGTGCCTCCTGAATTGT
2888F	TTCGACCATCCCACTCCACT
3269F	CGATGAGATGTTAAGCAATAT
3195B	GCCAGCTTTCAGAGTTGTCC
2712B	TTGAAATGGGAGCCTTTTGGG
2632F	GCTCGCAACCTTATGGCAAG
3413B	TAAGTCCGCCGTGAGACACC
T7	TAATACGACTCACTATAGGG
T3	TAACCCTCACTAAAGGGA
FRT1	CATGGGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGG AATAGGACTTCC
FRT2	CATGGGAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCC
3269F	CGATGAGATGTTAAGCAATAT
PLW-1	TCCACTTAACGTATGCTGC
PLW-2	GAAGTATACACTTAAATTCAG
11RPT-5	CGTAAGGGTTAATGTTTTCAAA
12672F	AGTTCGATCGACGTCTAATGTA
24037B	GGCCCTCAATCAGGCGTTGGCA
RY1	TGGCATAACTAATTCCCTACGA

The intron1-2 was amplified as two separate fragments using the following primer sets: 2033F-2152B and 2173F-2712B (Table 2.2.1-2). Both primers 2152B and 2173F had a *Nco* I restriction site added to their

5′ ends, which did not occur in the *P* element sequence and allows insertion of the FRT sequence in later steps. The amplified fragments were gel isolated, digested with *Nco* I, and then ligated together. Following the ligation the fragments were double digested with *Kpn* I and *EcoR* I and ligated into pBSKII(+). After *E. coli* DH5α transformation and plating on X-gal ampicillin plates, both blue and white colonies were sequenced. Surprisingly, after DNA sequencing, all the white colonies showed mutations within the primer 2142B sequence while blue colonies had an insert with correct intron2-3 and *Nco* I restriction site. After digesting the correct pBSKII(+)intron1-2 with the *Nco* I site, the FRT oligonucleotide was cloned into the construct. Digestion with *Xba* I, which had a restriction site within the FRT sequence, and sequencing using T7 and T3 primers determined the orientation of the FRT sequence.

The intron 2-3 was amplified as two separate fragments using the following primer sets: 2632F-3017B and 3039F-3413B (Table 2.2.1-2). Both primers 3017F and 3039B have an *Nco* I restriction site added to their 5′ ends, which does not occur in the P element sequence and allows insertion of the FRT sequence in later steps. The amplified fragments were gel isolated, digested with *Nco* I, and then ligated together. Following the ligation, the fragments were double digested with *Sal* I and *EcoR* I and ligated into pBSKII(+). After *E. coli* DH5α transformation, white colonies were selected on X-gal ampicillin LB plates. After digesting the pBSKII(+)intron2-3 with *Nco* I, the FRT oligonucleotide was cloned into the construct. Digestion with *Xba* I, which had a restriction site within the FRT sequence, and sequencing using T7 and T3 primers determined the FRT orientation.

Table 2.2.2: Primer pairs used to construct P{I Sal}

PrimerF	PrimerB	Optimum Temp. (°C)	Thermocycler Program
2033	2152	56-57	DanOpt2
2173	2712	56-62	DanOpt2
2632	3017	57-67	DanOpt2
3039	3413	57-67	DanOpt2

Table 2.2.3: Reaction conditions to construct P{I Sal}

Reaction mix		Thermocycler - DanOpt2
1XThermo Pol Buffer	5 μL	1 T=95°C, 3:00
DNA template	2μL	2 T=70°C 1:00
100X BSA	5 μL	3 T=73°C 1:30
10mM dNTP	1 μL	4 T=95°C 1:00
10 μM primerF	2.5 μL	5 T=62 0:45
10 μM primerB	2.5 μL	+0 +0
Vent	0.25 μL	R=3°/S +0.01
H_20	31.75 μL	G= 6°C
		6 T=73°C 0:45
		7 Goto 4 rep 29

The introns with the FRT sequences in the same direction were isolated from their respective constructs to replace the original P sequence in pP{ry}. In a single reaction, the gel isolated *Kpn* I-*EcoR* I intron1-2 (642 bp), *EcoR* I-*Sal* I intron2-3 (753 bp) was ligated into the gel isolated pP{ry} ~12 kbp band that had the *Kpn* I-*Sal* I (~1.2 kbp) fragment from the P element removed. The P{FRT ry} construct was transformed into *E. coli* DH5α.

The *Sal* I site in P{FRT ry} was filled in using Vent (PCR mix – primers and 0.5 μL Vent). After ligation, *Sal* I was used to re-digest any constructs that ligated without filling in the end the 5' *Sal* I overhang. Then the construct was transformed into *E.coli* DH5α cells. Sequencing confirmed that the new P{I Sal} construct matched the expected sequence and that the FRT sequences had the same orientation.

2.2.1 Injecting Fly Embryos

2.2.1.1 Preparing DNA

The EndoFree Plasmid Midi Kit (Qiagen) isolated DNA from bacteria transformed with pP{ry}, pP{FRT ry}, and pP{I Sal} for fly injections. The extracts were checked for appropriate *Nco* I and *Sal* I restriction digests and quantified using the absorbance at 260 nm. Helper plasmid DNA (Δ2-3 plasmid) was provided by Lynn Podemski and isolated using the same kit.

The day before injections, 12.5 μg of P element construct DNA and 2.5 μg of Δ2-3 DNA were ethanol precipitated (0.3 M sodium acetate and two volume 95% ethanol) and re-suspended in 25 μL dH_2O. The next morning the sample was centrifuged down, the supernatant removed, and the pellet washed in 80% ethanol. After centrifugation, the wash was removed and the sample was dried. Then 25 μL of distilled sterile water was added and the sample was centrifuged for 30 min at 15 000 rpm to remove particulates that clog the injection needle. After

centrifugation, 5 μL was pipetted from the top of the water layer and then three 5 μL aliquots were stored for the daily injections.

2.2.1.2 Preparing cover slips

Glue was prepared by adding 3M™ double-sided scotch tape (Cat no 136 NA) to a scintillation vial filled with heptane. After shaking the mixture overnight, the scotch tape was removed. Then the glue was placed as two small stripes running the width on both sides of cover slip. The cover slip was placed on a microscope slide and was held in place by a small drop of 80% glycerol.

Some injections were done using just the double-sided scotch tape placed on the slide. This procedure worked just as well as the one previously described to hold the embryos in place.

2.2.1.3 Egg laying

Egg laying chambers consisted of 10" long, 4" diameter black plastic sewer pipes covered on one end with mosquito netting. The pipe diameter was the same diameter as the petri dish holding the agar. The petri dish contained 4% agar and fit inside the sewer cap. The sewer cap, with the petri dish inside, fit over the opposite end of the pipe to the mosquito netting. A thick yeast paste was made with 2% ethanol and applied to the plate. About 8-12 bottles filled with ry^{506} flies were added to each egg laying chamber. Flies were used for a week and then discarded by freezing the chambers at –20°C.

2.2.1.4 Loading injection needles

Borosilicate glass injection needles (.90 mmX10 cm Cat#120-90-10) were formed using a Sutter P-87 needle puller. I loaded 5 μL of the injection mix into the injection needle with a 100 μL capillary tube pulled into a needle-like shape using Bunsen burner. The injection mix was forced into the injection needle using a pipetteman with a narrow-ended P200 gel loading pipette tip. After the injection needle was loaded, the needle tip was broken against the side of a coverslip to form a sharp point for easy entry into the egg.

2.2.1.5 Collecting, removing the chorion, and injecting embryos

I used distilled water to wash the eggs off the petri dish through mosquito netting into a cell strainer (100 μm Falcon 35-2360). The mosquito netting caught the dead flies. The embryos in the filter were immersed and agitated in fresh 50% bleach solution for 50-60 seconds to remove the chorion. Then the bleach was immediately washed with distilled water.

I used a dissecting needle to place embryos 1-2 egg widths apart on the edge of agar blocks that were the same width as the coverslip. The large posterior end of the embryos pointed out. On older embryos the opaque germ cells were seen at this end.

After the eggs were lined up, they were pressed gently on to the glue line on the coverslip slide. The embryos would stick and not move when the needle pierces the posterior end. If they moved, more scotch tape

was used to make the glue and the glue was reapplied to the coverslips already made.

Then the microscope slide and embryos were placed in a bottle of very old drierite ($CaSO_4$) for 7 minutes 45 seconds. After drying the embryos, most appeared firm and were not shrunken. Then the embryos were injected using a LEITZ micromanipulator one after another at 100X magnification. Vaseline was used to surround the coverslip and hold in the hydrocarbon oil. The embryos were covered with 200 Halocarbon oil (CAS# 9002-83-9) and incubated at 18-19°C for the next three days. Over the next three days, the slides were checked twice a day for larvae that hatched. Special care was taken because the larvae blend in with the vaseline.

2.2.1.6 Isolating transformants

Hatched larvae from a single day (up to 12 larvae) were added to normal fly food and kept at 21°C. Before adding the larvae, 500 µL dH_2Os was added to the top of the food and worked with a dissecting needle. Larvae were added to the food by spearing the food with one quick motion.

After the flies eclosed, they were individually mated to ry^{506} flies. Their offspring were checked for a ry^+ phenotype. If found, up to 4 ry^+ male offspring were mated to virgins from a *Gl Sb H/ TM2, Ubx ry* stock. If only ry^+ female were produced, then the insert was considered to occur on chromosome 1 and stocked by backcrosses until only ry^+ occurred. If ry^+ *TM2* male progeny occurred then they were consecutively crossed to virgin *Df(2R)C321/ CyO*; ry^{506} and *Gl Sb H/ TM2, Ubx* females. If all Ubx^+ progeny were ry^+ this indicated there was an insert on chromosome 3 and this insert was stocked over *TM2, Ubx*. If there was *Ubx* ry^+ progeny a second insert on chromosome 2 or 4 was sought. The *TM2, Ubx; CyO* progeny were crossed to virgin ry^{506} female to determine if ry^+ segregates from *CyO*. Inserts on chromosome 2 were stocked over *CyO* in stocks homozygous for ry^{506}.

The transformants were numbered1-6. Multiple lines from a single transformant were tested and labeled with different letters because these derivatives could be a different insertion. For example, from the first G_0, 2 different ry^+ males were isolated and the designations were 1a and 1b. After I determine which chromosome had the insert a dash and chromosome number was placed beside the designation. For example, the ry^+ marker in transformants *1a* and *1b* segregated with chromosome 3 and therefore the full name of these inserts was *P{I SalI}1a-3* and *P{I SalI}1b-3*.

2.2.2 Testing whether the inserts cause PDS

After being stocked, males with the inserts were crossed to a virgin *w; dp; e; P{lacW}ci*^{Dplac} females. Male progeny inherited the *w* allele from their mother and the w^+ activity resulted from *P{lacW}ci*^{Dplac}. Both *BX2* and *T1* were tested similarly by crossing to *w; BX2/ Cy* and *w; T1/ Cy* stocks respectively.

The vg^{21-3} phenotype was tested by crossing a male with the insert/ balancer to a *w; Bl/ CyO; Sb* P{ry$^+$ SalI}89D/ *TM2* and collecting *w; CyO/+; TM2/* insert males. These males were crossed to a *w;* vg^{21-3}*; TM6B/ Sb P{ry$^+$ SalI}89D* females and *w: CyO/* vg^{21-3}*; TM6B/* insert males were collected. In the last cross, these males were crossed to a vg^{21-3} stock and flies with either a strong vestigial phenotype or wild wing phenotype (Cy+) were scored.

2.2.3 Generating the Type II derivatives from the *P{I SalI}* inserts using *hsFLP*

With chromosome 2 inserts, the *P{I SalI}/ CyO* males were crossed to a *P{ry hsFLP}1* w^{118}*; Adv^1/ CyO* (Bloomington – 6). For chromosome 3 inserts, *P{I SalI} / TM2 or TM6B* males were crossed to a *P{ry hsFLP}1* y^1 w^{118}*; Dr^{Mio}/ TM3, ry* (Bloomington-7) stock. The cross was incubated for 2-3 days then the parents were removed. The progeny in the vial were heat-shocked at 37°C for two hours in a hot water bath every 2-3 days until the flies started to eclose. Males flies were collected that had the insert/ balancer and crossed to *w; Bl/ CyO; Sb P{ry$^+$ SalI}89D/ TM2* females.

I used a PCR test to detect exon 2 loss using primers 2033F-3195R and 2632F-3413B. The PCR program used was 1. 95°C 2:00, 2. 95°C 1:00, 3. T=66°C 0:45, 4 73°C 1:20, 5. Goto 2 Rep 29, and 5. Hold 5°C enter. For details on this PCR see results.

2.2.4 Inverse PCR to detect insertion site

Genomic DNA was extracted using the Qiagen DNeasy tissue kit (Cat#69506). Then 25 µL of the genomic DNA was digested with either *Hha* I or *Sau3A* I in a total digest volume of 25 µL. The digests were heat inactivated at 70°C for 15 minutes. Then 20 µL from the restriction digests was ligated at a total volume of 180 µL, using 5 µL T4 DNA Ligase (1 U/ µL Gibco), overnight at 4°C. The DNA was precipitated from the ligation mixture using 20 µL 3M sodium acetate and 400 µL 95% ethanol at –20°C for 90 minutes. After spinning down and washing the DNA, it was dissolved in 50 µL dH2O.

Inverse PCR for the *Hha* I digested genomic DNA used primers 3269F and 11RPT-5. Basic PCR reaction mix using 10 µL from the ligation reactions was used and the program was 1. 95°C 3:00, 2. 65°C 1:00, 3. 73°C 2:00, 4. 95°C 1:00, 5. 57°C 0:45, 6. 73°C 3:00, 7. Go to 4 Rep 29, and 8. Hold 5°C enter. The inverse PCR from the *Sau3A* I digests used primers 11RPT-5 and 2173F in a PCR with the same conditions as the PCR used for *Hha* I digests.

2.2.5 Generating new *P{I SalI}* inserts using *Δ2-3*

To generate new *P{I SalI}* inserts that caused w^+ variegation from *P{lacW}ci^{Dplac}*, males with the insert were crossed to a *w; TM3, Ser/ Sb Δ2-3(99B);* ci^1 ey^R stock. The inserts chosen did not cause variable silencing before being mobilized by *Δ2-3(99B)*. The males that had the *Sb Δ2-3* chromosome and the *P{I SalI}* insert were crossed to a *w; dp; e;*

P{lacW}ci^{Dplac} females and the progeny was screened for flies that had a variegated color pattern in their eyes. A similar screen used *P{II SalI}*, rather than *P{I SalI}*, in the exact same crossing scheme to search for new inserts that caused variegation.

2.2.6 Testing whether *P{I SalI}* and *P{II SalI}* inserts modify P dependent phenotypes

To test *P{lacW}ci*^{Dplac}, inserts/balancer males were crossed to a *w; dp; e; P{lacW}ci*^{Dplac} females. Both males and females could be studied because the inserts were stocked in a w^- background over a *CyO*, *TM2*, or *TM6B* balancer chromosome. The one insert on chromosome 4 was stocked over M^{578}. In each case, the insert was compared to the balancer chromosome to determine if it caused eye pigment variegation.

To test *BX2*, the inserts/balancer males were crossed to *w; BX2/ Cy* females. In this case, inserts were compared to the original stock. This is because *CyO / BX2* was semi-lethal and this genotype occurred at reduced frequency. Furthermore, the PDS on *BX2* had a parental effect and this will be described in the Results section.

To test M^{F2}, the inserts/ balancer males were crossed to M^{F2}/ ey^D females. The ey^D progeny had wild type bristles. The progeny that inherited the balancer and M^{F2} had Minute bristles, which were compared to the progeny that inherited the insert and M^{F2}. If the bristles more closely resembled the narrow scutellar short bristles found in the balancer/M^{F2} progeny than the insert/M^{F2} flies, then the phenotype was considered Minute. However, if the scutellar bristles more closely resembled the wild type bristle phenotype in the ey^D flies, then the bristle phenotype was scored wild type.

To test the $vg^{21\text{-}3}$ phenotype the insert was first crossed to a *w; Bc/ CyO; Sb P{ry*$^+$ *SalI}89D/ TM2* females. Then *w; CyO/+; TM2/* insert males from this cross were crossed to *w;* $vg^{21\text{-}3}$*; TM6B/ Sb P{ry*$^+$ *SalI}89D* . The *w; CyO/* $vg^{21\text{-}3}$*; TM6B/* insert males produced by this cross were crossed to *w;* $vg^{21\text{-}3}$ females. All the Cy progeny were removed and the progeny that inherited *TM6B* were compared to the progeny that had the insert. The progeny that inherited the *TM6B* chromosome had a strong vestigial phenotype.

3 Results

3.1 Testing *KP* elements for the ability to cause PDS at *P{lacW}ci^{Dplac}*

Josse *et al.* (2002) demonstrated that two different chromosomes that had *KP* elements could enhance silencing at *P{lacW}* repeats. Since PDS at *P{lacW}* arrays and at *P{lacW}ci^{Dplac}* could involve a similar mechanism, I tested whether these *KP* elements would cause variable silencing at *P{lacW}ci^{Dplac}*. Two different *KP* chromosomes were tested: *KP-U* and *KP-D*. The *KP-D* chromosome was marked with dominant *Cy* and *Bl* alleles. The *KP-U* chromosome was balanced in a *Cy; TM6B / Xa* stock. Ronsseray indicated (personal communication) that the *KP-U* elements segregated with the Cy chromosome. Crossing males with these *KP* chromosomes to *w; dp; e; P{lacW}ci^{Dplac}* females produced male progeny where the *w^{+mC}* transgene was silenced in most of the ommatidia (Figure 3.1.1). This silencing was stronger than silencing associated with *P{ry^{+} SalI}89D*.

Males produced from the original cross to *w; dp; e; P{lacW}ci^{Dplac}* females were backcrossed to the same stock to determine which chromosome was causing PDS. The crosses using the *KP-D* confirmed that the silencing was inherited with only the *Cy Bl* chromosome. However, in the crosses with *KP-U*, the silencing did not segregate with the *Cy* but with the *TM6B* chromosome. This genetic evidence, as well as molecular evidence described later, found that the *KP-U* elements segregated with the *TM6B* chromosome rather than the Cy chromosome.

3.1.1 *KP* elements also suppress *M^{F2}*, *vg$^{21-3}$*, and the ci phenotype caused by *P{hsp26-pt-T}ci$^{2-M1021.R}$*

I wanted to determine whether these *KP* elements acted like *P{ry^{+} SalI}89D* in terms of their effect on the *P* dependent phenotypes caused by *vg$^{21-3}$*, *M^{F2}* and *P{hsp26-pt-T}ci$^{2-M1021.R}$*. *P{ry^{+} SalI}89D* suppressed the mutant phenotypes caused by these alleles. In the following sections, the *KP* element tested acted similarly to *P{ry^{+} SalI}89D* and suppressed the mutant phenotypes caused by these *P* dependent alleles.

3.1.1.1 Testing *M^{F2}*

I found that *KP-U* chromosomes suppressed the *M^{F2}* phenotype (Figure 3.1.2 and Figure3.1.3). When *M^{F2}* was present the bristles appeared short and slender, but when combined with *TM6B, KP-U* the bristles were wild type in length and width. In comparison, *P{ry^{+} SalI}89D* also suppressed *M^{F2}* and produced the same phenotypic change. Testing *KP-D*'s ability to suppress *M^{F2}* was complicated by the presence of *Bl*, and I could not determine whether the *KP* elements on this chromosome suppressed the *M^{F2}* phenotype. *Bl* produced a bristle phenotype that is epistatic to the Minute phenotype.

3.1.1.2 Testing $vg^{21\text{-}3}$ and vg^{2a33}

Both *KP-U* and *KP-D* suppressed the $vg^{21\text{-}3}$ phenotype (Figure 3.1.4). Normally, a homozygous $vg^{21\text{-}3}$ produced a small malformed wing. When *KP-U* was present, a wild type wing formed when the flies were homozygous for $vg^{21\text{-}3}$.

I received the *KP-D* element on a chromosome labeled as *Cy Bl vg*, but the *vg* allele was not labeled. I confirmed that this chromosome had a vg^{-} allele by crossing it to vg^{BG}, which was not a *P* dependent allele. When *Cy Bl vg* was heterozygous with vg^{BG}, a strong vestigial phenotype was produced indicating this chromosome had a strong vg^{-} allele (Figure 3.1.4 [B]). Next, I crossed the *Cy Bl vg* chromosome to $vg^{21\text{-}3}$ (a *P* sensitive allele) and the heterozygote produced only a weak vestigial phenotype. Since $vg^{21\text{-}3}$/ vg^{BG} and vg^{BG}/ *Cy Bl vg* produced a strong vestigial phenotype, the *Cy Bl vg*/ $vg^{21\text{-}3}$ should also have produced a strong vestigial phenotype. The fact that it did not indicated the *KP-D* chromosome suppressed the $vg^{21\text{-}3}$ allele. Presumable, this suppression resulted from the many *KP* elements inserted in this chromosome.

Both $vg^{21\text{-}3}$ and vg^{2a33} had *P* elements inserted at the same site in the 5′ end of the *vg* gene. They had different internal deletions within the *P* elements (See Discussion) which appears to be the reason why $vg^{21\text{-}3}$ was suppressed when *P{ry⁺ SalI}89D* was present while vg^{2a33} was not (HODGETTS and O'KEEFE 2001). I crossed the *KP-U* and *KP-D* chromosomes to vg^{2a33} to determine if they suppressed this allele. These *KP* elements acted similarly to *P{ry⁺ SalI}89D* and suppressed the $vg^{21\text{-}3}$ allele but did not suppress the vg^{2a33} allele (Figure 3.1.5). This indicated the mechanism through which the Type I elements (*P{ry⁺ SalI}89D*) and Type II (*KP*) act through may be very similar. In both cases, the slight change between vg^{2a33} and $vg^{21\text{-}3}$ disabled the ability for *P* elements to suppress the mutant phenotype.

3.1.1.3 Testing *P{hsp26-pt-T}ci*$^{2\text{-}M1021.R}$

In *P{hsp26-pt-T}ci*$^{2\text{-}M1021.R}$ / ci^{1} heterozygotes, the wing showed a ci phenotype, suggesting there was ectopic *ci* expression in the posterior wing compartment (HANNA S. PERSONAL COMMUNICATION). The ci phenotype was characterized by a gap in the L4 wing vein. I discovered that the presence of either *P{ry⁺ SalI}89D* or *TM6B, KP-U* suppressed this ci phenotype (Figure 3.1.6). Penetrance for the ci phenotype was not complete and half the *P{hsp26-pt-T}ci*$^{2\text{-}M1021.R}$ / ci^{1} ey^{R} heterozygotes had the ci phenotype. When either *P{ry⁺ SalI}89D* or *KP-U* elements were present, all the wings were wild type and the ci phenotype did not occur. The presence of *P{ry⁺ SalI}89D* or the *KP* elements did not suppress the ci phenotype that occurred in ci^{1} homozygotes. Therefore, the *P* element suppression was limited to the *P{hsp26-pt-T}ci*$^{2\text{-}M1021.R}$ / *ci* combination.

3.1.2 Do *KP* elements act similarly to *P{ry⁺ SalI}89D* and suppress variegating *w⁺* transgenes on chromosome 4?

A series of w^+ transgenes on chromosome 4 had a variegated w^+ expression pattern (SUN *et al.* 2000). In contrast to the gene silencing at the *P{lacW}ci*^{Dplac} locus, the presence of *P* elements, such as *P{ry⁺ SalI}89D,* suppressed these variegating w^+ transgenes to produce an eye with more colored facets (HANNA S. PERSONAL COMMUNICATION). I wanted to test the *KP* elements and determine if they suppressed variegation from these w^+ transgenes. Males with the *KP* chromosome were crossed to females that had the w^+ insert and *Sb P{ry⁺ SalI}89D* chromosome (Figure 3.1.7 [A]). The w^+ transgene bearing progeny that inherited *Sb P{ry⁺ SalI}89D* could be compared to progeny that inherit the *KP* chromosome. The w^+ transgenes studied include *P{hsp26-pt-T}39C-12* (102B) and *P{hsp26-pt-T}39c-34* (102A) (Figure 1.1.1). The *KP-D* chromosome strongly suppressed variegation from *P{hsp26-pt-T}39C-12* and weakly suppressed variegation from *P{hsp26-pt-T}39c-34* (Figure 3.1.7 [B]). The *P{ry⁺ SalI}89D* chromosome had the same effect as *KP-D* with each insert. The *KP-U* chromosome also suppressed variegation from *P{hsp26-pt-T}39c-12* (Figure 3.1.8). However, *KP-U* weakly suppressed *P{hsp26-pt-T}39c-34,* compared to the *P{ry⁺ SalI}*89D class. Pigment analysis of *P{hsp26-pt-T}39c-12* bearing progeny found that *P{ry⁺ SalI}89D* strongly suppressed variegation compared to *KP-U* in both males and females (Figure 3.1.9). In summary, the *KP* chromosomes suppressed variegation similarly to *P{ry⁺ SalI}89D* but there was a quantitative difference. Based on visual analysis and pigment results the *P{ry⁺ SalI}89D* chromosome was a stronger suppressor than either *KP* chromosomes tested.

Finding that *P{ry⁺ SalI}89D* was a stronger suppressor than *KP-U* was surprising because this did not correlate with their PDS effects at *P{lacW}ci*^{Dplac}. I had expected that *P* elements that caused strong silencing at *P{lacW}ci*^{Dplac} would cause strong suppression at the w^+ inserts at other locations on chromosome 4. The *KP* chromosomes weakly suppressed variegation from the *P{hsp26-pt-T}* inserts but strongly caused PDS at *P{lacW}ci*^{Dplac}. However, the variegating inserts tested contained a *P{hsp26-pt-T}* construct, which may show different sensitivities than *P{lacW}ci*^{Dplac}.

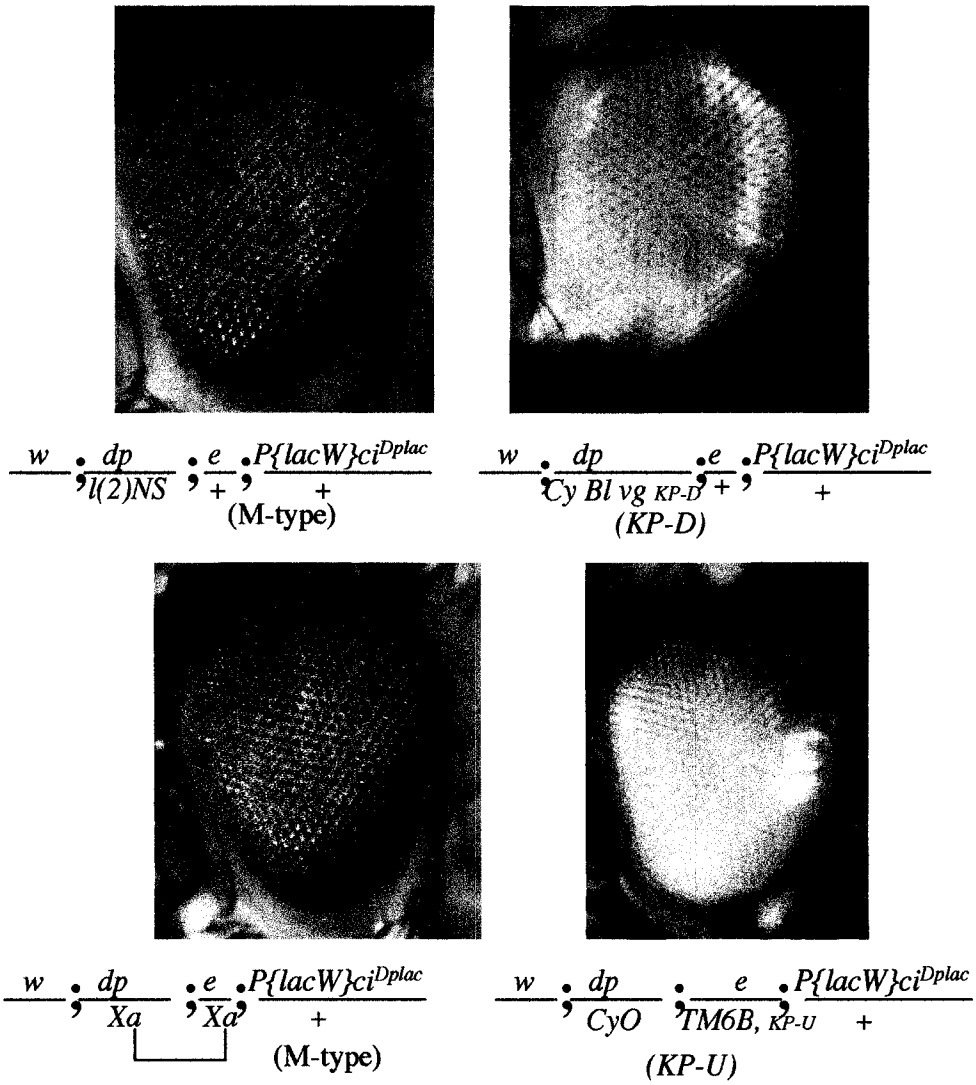


Figure 3.1.1 - *KP* elements can silence w^{+mC} expression from $P\{lacW\}ci^{Dplac}$. The genotype of each fly is written below each picture. The *Cy Bl vg* and *TM6B* chromosomes have multiple *KP* elements inserted in them.

A P1: $\frac{w}{}; \frac{CyO}{dp}; \frac{TM6B}{e}$ ♂ X ♀ $\frac{w}{w}; \frac{M^{F2}}{ci^D}$

B

Pictures of F1 Males

Wild type bristles $w; \frac{ci^D}{+}$ Minute bristles $w; \frac{M^{F2}}{+}$

Wild type bristles $w; \frac{TM6B}{+}; \frac{ci^D}{+}$ Wild type bristles $w; \frac{TM6B}{+}; \frac{M^{F2}}{+}$

Wild type bristles $w; \frac{CyO}{+}; \frac{ci^D}{+}$ Minute bristles $w; \frac{CyO}{+}; \frac{M^{F2}}{+}$

Figure 3.1.2 - *KP-U* suppresses the M^{F2} phenotype as seen with the scutellar bristles. *KP-U* elements are on the *TM6B* chromosome. **A.** Crossing scheme to generate the flies depicted in B. **B.** Photographs of scutellar bristles depicting the Minute M^{F2} phenotype and its suppression by *KP-U*. The Minute phenotype is characterized by short narrow bristles which can be seen in the top right picture. This phenotype is suppressed when *TM6B, KP-U* present (middle row right column).

A

P1: $\frac{w}{}; \frac{CyO}{dp}; \frac{TM6B}{e}$ X $\frac{w}{w}; \frac{M^{F2}}{ci^{D}}$

Pictures of F1 Males

B

Wild type bristles $w; \frac{ci^{D}}{+}$ Minute bristles $w; \frac{M^{F2}}{+}$

Wild type bristles $w; \frac{TM6B}{+}; \frac{ci^{D}}{+}$ Wild type bristles $w; \frac{TM6B}{+}; \frac{M^{F2}}{+}$

Wild type bristles $w; \frac{CyO}{+}; \frac{ci^{D}}{+}$ Minute bristles $w; \frac{CyO}{+}; \frac{M^{F2}}{+}$

Figure 3.1.3 - *KP-U* suppresses the M^{F2} phenotype as seen with the sternopleural bristles. *KP-U* elements are on the *TM6B* chromosome. **A.** Crossing scheme to generate the flies depicted in B. **B.** Photographs of sternopleural bristles depicting the M^{F2} phenotype and its suppression by *KP-U*. The Minute phenotype is characterized by short narrow bristles which can be seen in the top right picture. This phenotype is suppressed when *TM6B, KP-U* present (middle row right column).

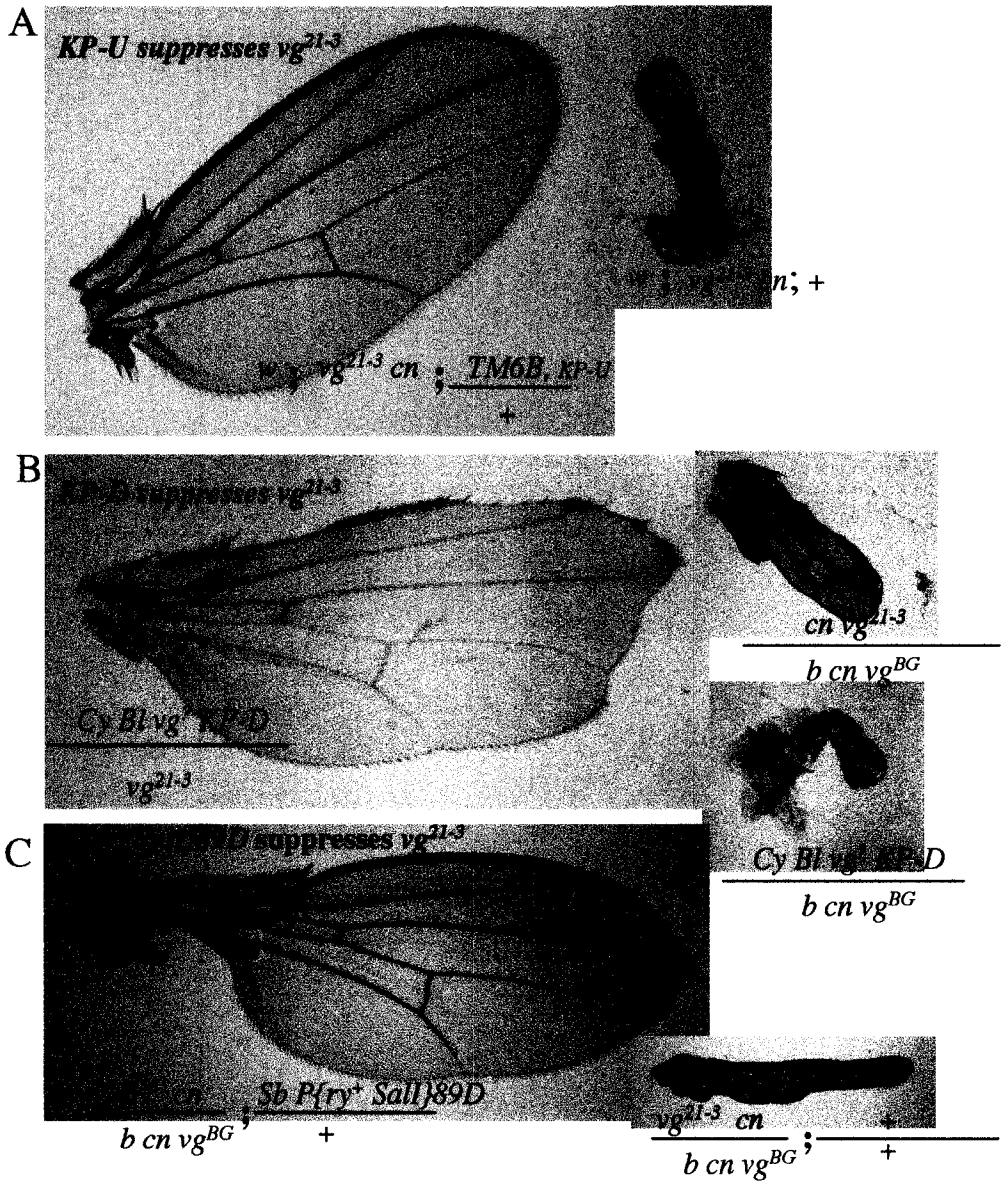


Figure 3.1.4 - *TM6B, KP-U* and *Cy Bl vg? KP-D* suppress vg^{21-3}. The genotype for each wing picture is below each wing. Pictures are not to scale. **A.** *KP-U* suppresses vg^{21-3}. **B.** *KP-D* suppresses vg^{21-3}. The *vg* allele on the *Cy Bl vg?* is not indicated in this stock. The chromosome does have a vg^{-} allele because it produces a vestigial phenotype over vg^{BG}. The vg^{BG} is not a *P* elements dependent allele and expresses the vestigial phenotype when *P* elements are present. **C.** *P{ry+ SalI}89D* suppresses the vg^{21-3} phenotype.

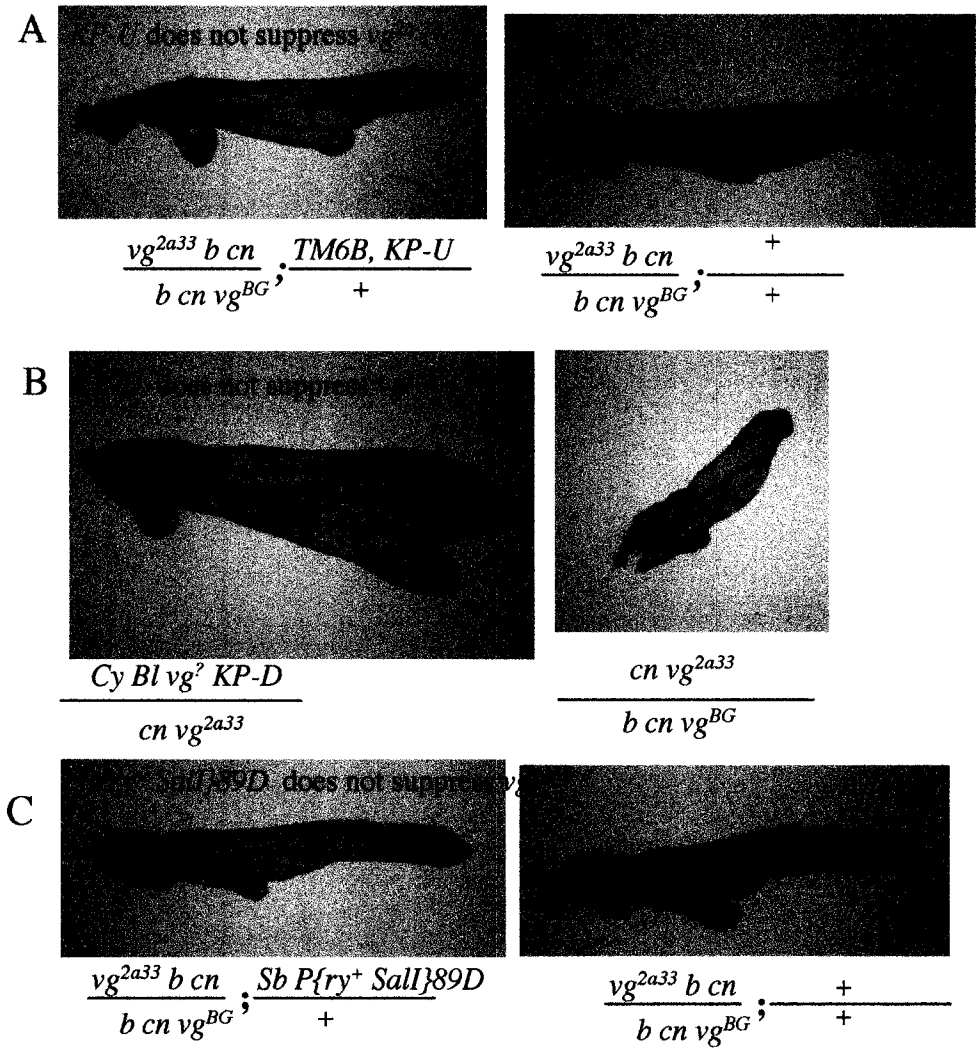
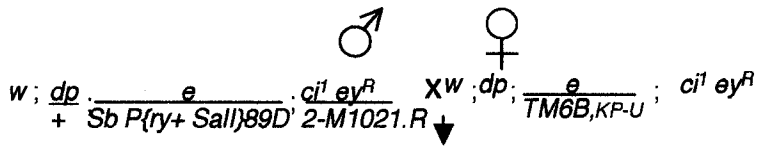
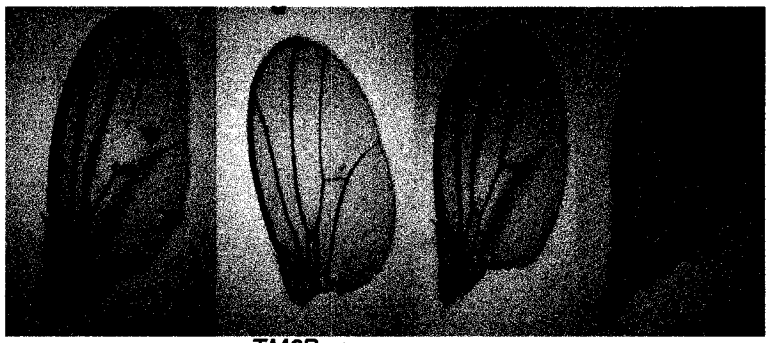


Figure 3.1.5 - *KP-D* and *KP-U* do not suppress vg^{2a33}. The genotype is below each picture. **A.** *KP-U* does not suppress vg^{2a33}. **B.** *KP-D* does not suppress vg^{2a33}. **C.** *P{ry⁺ SalI}89D* does not suppress vg^{2a33}.

A



B



C

Genotype	e/e		KP-U/Sb P{ry+ SalI}89D		KP-U/e		Sb P{ry+ SalI}89D / e	
ci phenotype	-	+	-	+	-	+	-	+
2-M1021.R/ ci1 eyR	39	34	0	26	0	28	0	41
ci1 eyR/ ci1 eyR	7	16	12	10	13	5	10	26

Figure 3.1.6 - *P{ry+ SalI}89D* and *TM6B, KP-U* suppress the ci phenotype that occurs in the *P{hsp26-pt-T}ci2-M1021.R / ci1 eyR* heterozygotes. **A**. Crossing scheme to generate different classes. The *P{hsp26-pt-T}ci2-M1021.R* insertion is abbreviated *2-M1021.R*. **B**. Picture of wing phenotypes. The alleles on chromosome 3 are indicated below each picture; otherwise the genotype is w; dp/+; #3/#3 *P{hsp26-pt-T}ci2-M1021.R/ ci1 eyR* fly. The arrow points to a gap in the L4 wing vein which is the ci phenotype. **C**. Table describing the frequency with which the ci phenotype occurs in each genotypic class. The top row indicates the chromosome 3 combination inherited. The second row indicates whether the phenotype is wild type (+) or has a ci phenotype (-). The *TM6B, KP-U* chromosome is abbreviated *KP-U*. The first column indicates the chromosome combination inherited on chromosome 4. The *2-M1021.R/ ci1 eyR and ci1 eyR/ ci1 eyR* classes are counted separately and there are fewer *ci1 eyR/ ci1 eyR* flies counted.

Therefore I tested *P{hsp26-pt-T}ci*$^{2\text{-}M1021.R}$, which was inserted in the *ci* distal regulatory region next to *P{lacW}ci*^{Dplac}. Since this was the same construct as the variegating w^+ transgenes inserts tested elsewhere on chromosome 4, it had the same sensitivity. Like *P{lacW}ci*^{Dplac}, this w^+ insert variegated when *P{ry*$^+$ *SalI}89D* was present. I tested for silencing at this construct when either a *KP* or *P{ry*$^+$ *SalI}89D* was present. *P{ry*$^+$ *SalI}89D, KP-U,* and *KP-D* all produced a similar variegated phenotype with *P{hsp26-pt-T}ci*$^{2\text{-}M1021.R}$ (Figure 3.1.7-8). Pigment analysis comparing *KP-U* and *P{ry*$^+$ *SalI}89D,*confirmed that *KP-U* was the stronger silencer (Figure 3.1.10). Therefore, there was no correlation between the ability of the *P* elements to silence w^+ expression within the *ci* distal regulatory region and the *P* elements ability to suppress w^+ variegation elsewhere on chromosome 4.

3.1.2.1 The *KP* and *P{ry*$^+$ *SalI}89D* combination did not have a combined effect on silencing or suppression.

The crosses described above also produced a genotype class that had both *P{ry*$^+$ *SalI}89D* and *KP* chromosomes (Figure 3.1.8-10). The two P inserts in this class had no combined effect on expression from the w^+ transgenes tested. Phenotypically this class did not look consistently more suppressed or silenced compared to classes that inherited only one of these *P* elements.

Pigment analysis for *P{hsp26-pt-T}39C-12* produced contradictory results between males and females. In *males,* the *KP-U / Sb P{ry*$^+$ *SalI}89D* class was as strongly suppressed as the *Sb P{ry*$^+$ *SalI}89D/ e class* (Figure 3.1.9). In females, the *KP-U* / Sb *P{ry*$^+$ *SalI}89D* was more suppressed compared to either the *KP-U* / e or *P{ry*$^+$ *SalI}89D* / e class. This stronger effect could be produced by the combined effects from both *P* inserts.

With *P* element silencing of *P{hsp26-pt-T}ci*$^{2\text{-}M1021.R}$, the *KP-U/ P{ry*$^+$ *SalI}89D* class was slightly more silenced compared to the other two classes in both males and females, but the difference was not significant (Figure 3.1.10). These results indicated the combined dose of both *KP-U* and *P{ry*$^+$ *SalI}89D* had no consistent additive effect on w^+ variegation from the various inserts tested.

In the pigment analysis described above, some values were derived from less than three samples. In the rest of this thesis, pigment analysis always used the average of 3 samples. In this case, collecting flies was difficult because there were 8 genotypic classes and classes with *KP-U* occurred at a reduced frequency.

3.1.3 Confirming that the *KP-U and KP-D* chromosomes contained *KP* elements

I wanted to confirm that these chromosomes contained only *KP* elements and that there were no autonomous P elements. I designed a series of PCR tests using different primers to detect autonomous P elements that contained exon 2. The first reaction was designed to detect the presence of *P* elements by using the primer PRPT, which anneals

within the 31 bp repeat so that the 3′ end points into the *P* element. Using PRPT, the full length *P* element would produce a 2.9 kbp band and from the *KP* element a 1.1 kbp band was expected. Using genomic DNA from the *KP-U* and *KP-D* stocks as template produced a ~1.1 kbp band consistent with them having a *KP* element (Figure 3.1.11-12). In reactions with the *KP-U* stock DNA, *P* elements segregated with the *TM6B* chromosome rather than the *Cy* chromosome (Figure 3.1.12). This corresponds with the fact that the PDS effect also segregates with this *TM6B* balancer. *P* strains, such as π2 and Harwich, produced bands smaller than 2.9 kbp. These smaller bands were probably produced because *KP* elements and other Type II elements occurred naturally in these stocks and were preferentially amplified in the PCR using PRPT.

To confirm that the *KP* chromosomes did not have exon 2, primers that anneal within exon 2 were used in PCR reactions. Two different reactions using 411F-2376B (0.9 kbp) and 411-2712B (1.3 kbp) amplified products from the *P* strains tested. The primers 2376B and 2712B anneal within exon 2 and reactions that used these primers to amplify template from *P* strains produced the appropriate size band as well as smaller size bands. The smaller product probably resulted from the many *P* derivatives that occur in these *P* strains and these derivatives had deletions in this region. However, the production of the appropriate size band indicated that this test successfully detected exon 2 in *P* strains that had autonomous *P* elements. These PCR reactions specific to exon 2 did not produce any product from the *KP* chromosomes tested. Therefore, both *KP* chromosomes had no autonomous P elements.

Southern analysis by Lynn Podemski (Biological Sciences, U. of Alberta) revealed that the *KP-D* chromosome had 4 inserts and the *KP-U* chromosome had between 4-6 different *KP* inserts. Therefore, the effects seen with these *KP* elements could be the result of expression from more than one *KP* element. *KP-U* is reported as being a double *KP* element with one being inserted within the other (JOSSE *et al.* 2002).

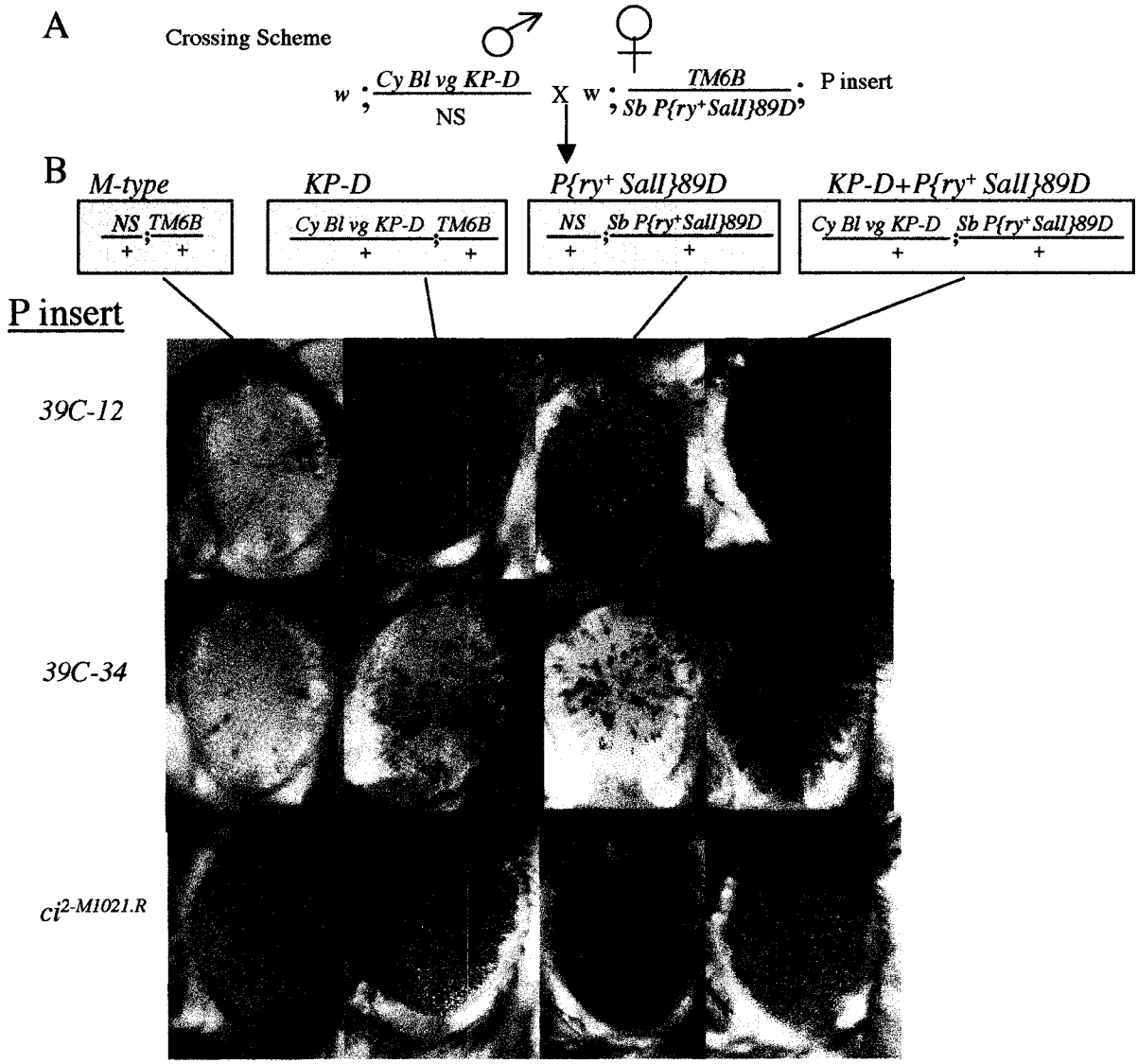


Figure 3.1.7 - *KP-D* acts similarly to *P{ry+ SalI}89D* and modifies w^+ transgene expression on chromosome 4. **A.** Crossing scheme to generate the flies in B. **B.** Pictures of eyes that have *KP-D* and *P{ry+ SalI}89D* with a w^+ transgene on chromosome 4. The abbreviation for each *P{hsp26-pt-T}* insert is displayed to left of each row of eyes. The chromosome or combination of chromosomes tested is indicated above each column of eye pictures.

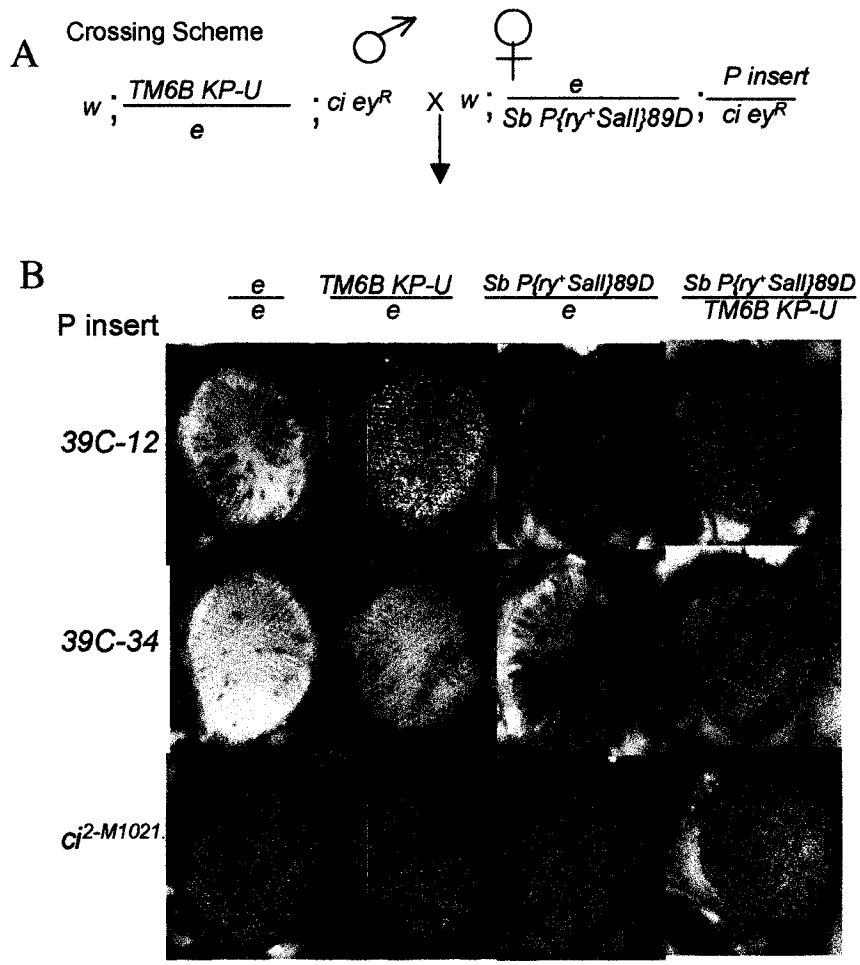


Figure 3.1.8 - *KP-U* acts similarly to *P{ry⁺ Sall}89D* and modifies expression from *P w⁺* inserts on chromosome 4. **A.** Crossing scheme to generate the flies in B. **B.** Pictures from male eyes that inherit *KP-U* and *P{ry⁺ Sal}89D*. The abbreviation for each *P{hsp26-pt-T}* insert is displayed to left of each row of eyes. At the top of each column of eyes is the *KP-U*, *e*, or *P{ry⁺ Sall}89D* combination tested.

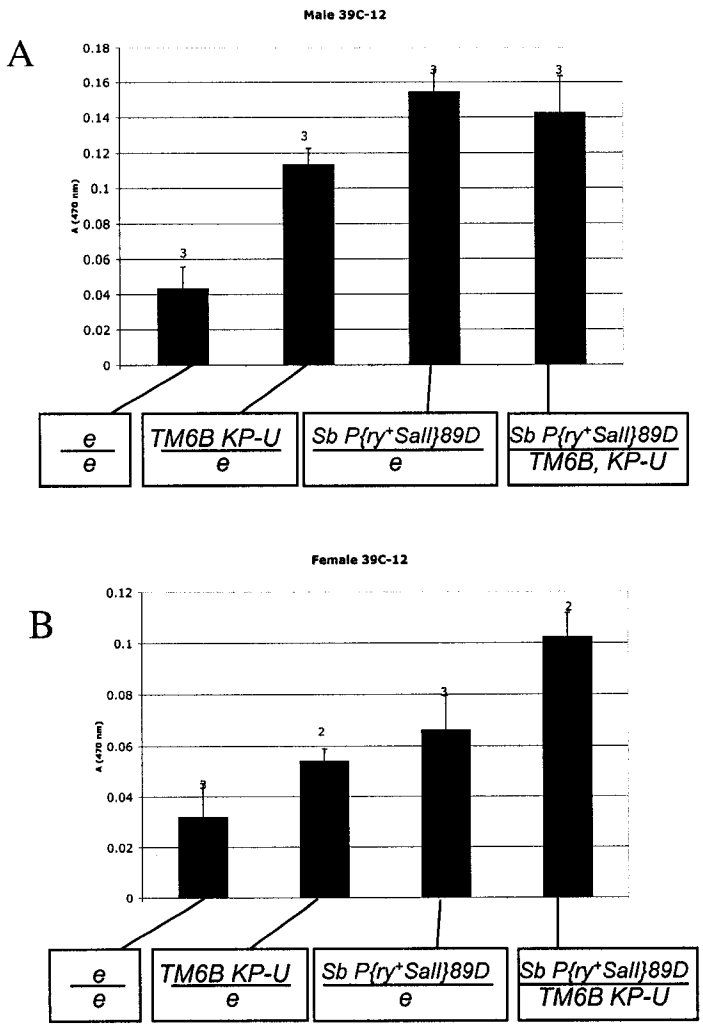


Figure 3.1.9 - Both *KP-U* and *P{ry⁺ Sall}89D* suppress w^+ variegation from *P{hsp26-pt-T}39C-12.* This insert occurs at cytological site 102B. The number of samples taken is indicated above each bar. Each sample contains 10 *Drosophila* heads. The genotype is indicated below each bar. **A.**Pigment is analysis using male heads. **B.** Pigment analysis using female heads.

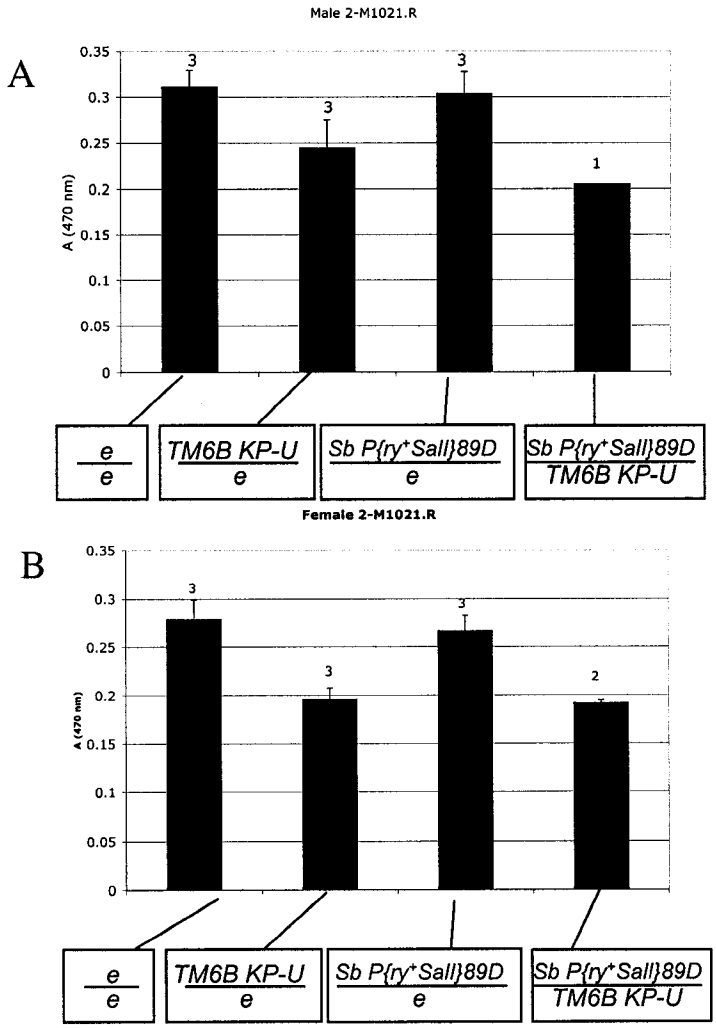
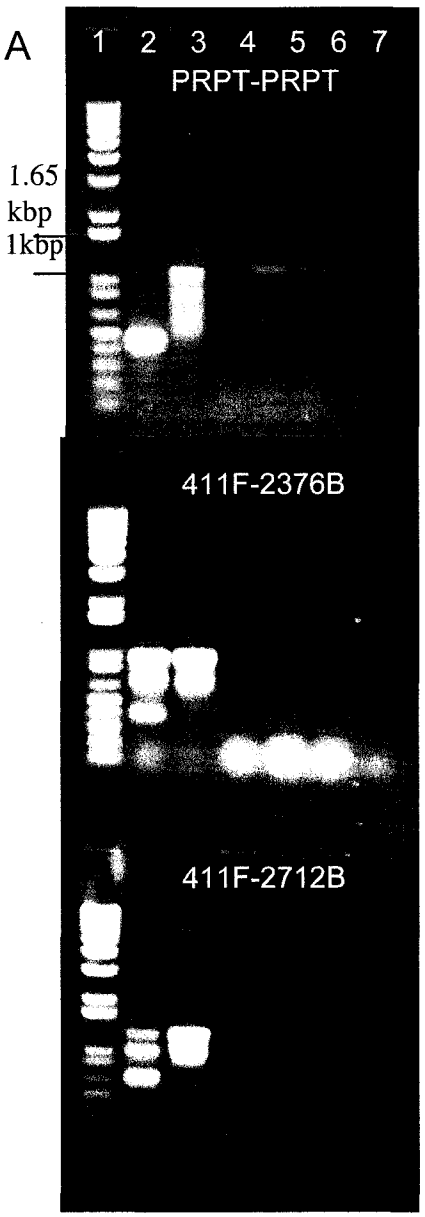


Figure 3.1.10 - Both *KP-U* and *P{ry⁺ Sall}89D* reduce w^+ expression from *P{hsp26-pt-T}ci*$^{2\text{-}M1021.R}$. This insert occurs in the *ci* distal regulatory region and is subject to *P* element silencing. The number of samples taken is indicated above each bar. Each sample contains 10 *Drosophila* heads. The genotype is indicated below each bar. **A.**Pigment is analysis using male heads. **B.** Pigment analysis using female heads.



B

Lane#	Template
Lane	Description
1	1 kb+ Ladder (Gibco)
2	Harwich
3	PI2
4	Oregon-R (M Stock)
5	KP-D (male)
6	KP-D (female)
7	No template

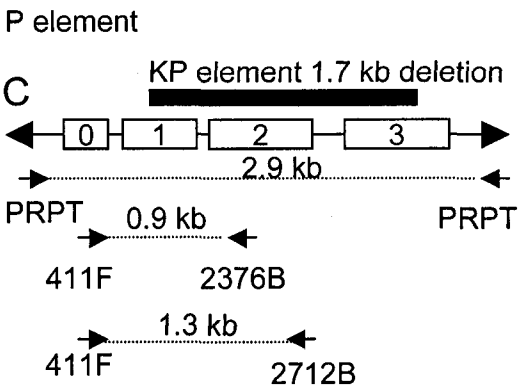


Figure 3.1.11 - PCR tests to determine if there are any autonomous *P* elements on the chromosome containing the *KP*-D elements. **A.** 1% Agarose gels stained with ethidium bromide. Each gel is loaded with the PCR using the primers indicated below the wells. The template added to the PCR loaded in each lane is indicated in Table B. **B.** Table describing the template added to each PCR reaction. **C.** A diagram depicting the location of each primer used in the PCR.

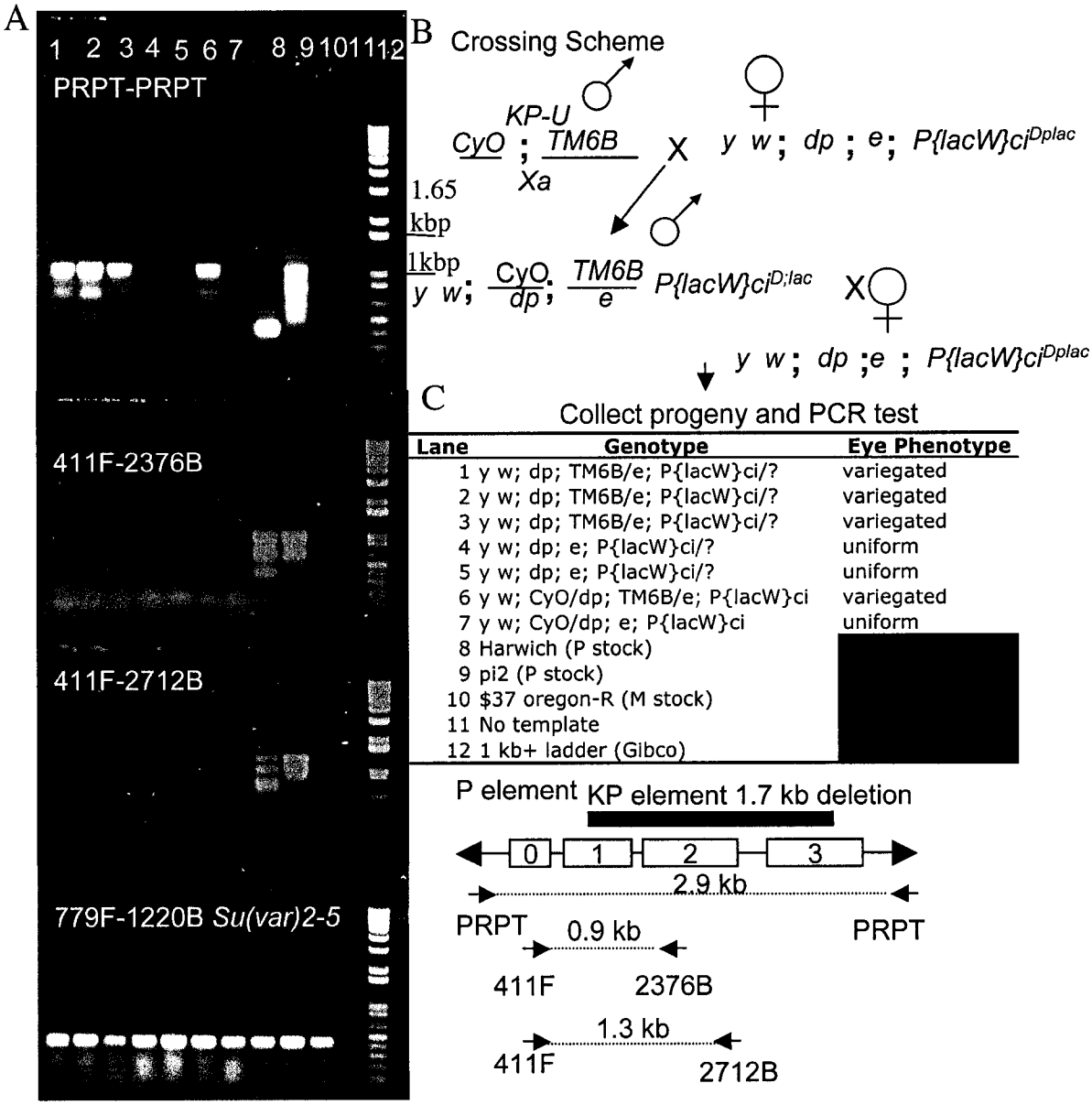


Figure 3.1.12 - PCR tests to confirm that the *TM6B* chromosome has only *KP* elements. **A.** 1% Agarose gels stained with ethidium bromide. Each gel is loaded with reactions using the primers indicated below the wells. The template added to each reaction is depicted in Table C. The 779F-1220B primers anneal within the *Su(var)2-5*$^{+}$ gene and are included as a positive control. **B.** Crossing scheme to test for effect on *P{lacW}ci*^{Dplac} expression. **C.** Table describing what template is added to the PCR loaded into each lane. **D.** Diagram depicting primers used within the P element and the expected size product.

3.2 Screens for dominant mutations that suppress *P* element dependent silencing

In the presence of *P{ry⁺ SalI}89D*, a non-autonomous P element construct that mimicked P cytotype (ROBERTSON and ENGELS 1989), the w^{+mC} transgene in $P\{lacW\}ci^{Dplac}$ was variably silenced and the eye was mostly white with a few patches of red ommatidia (Figure 3.2.1). I conducted several screens for dominant mutations that suppressed the P element dependent silencing (Su(PDS)) of the w^{+mC} transgene in $P\{lacW\}ci^{Dplac}$.

In the first screen, I scored 4,742 progeny from γ-irradiated males and recovered two *Su(PDS)* mutants on chromosome 3 as well as nine translocations (Table 3.2.1-2). The translocations presumably suppressed PDS by a position effect. I also screened 29,601 progeny from EMS treated males and isolated two mutations on chromosome 2 and seven mutations on chromosome 3 that suppressed PDS (Table 3.2.1).

In a final mutagenesis, I isolated four *Su(PDS)* mutants using the Δ2-3 transposase source to mobilize two P constructs inserted near *Su(var)3-7⁺* (Table 3.2.1). The total progeny screened from males producing transposase and containing either $P(PZ)05137^{05137}$ or $P\{PZ\}CtBP^{03463}$ was 19,407 and 18,835 flies respectively.

The variegated eye phenotype in our screens also enabled us to search for dominant mutations that silenced w^{+mC} expression from $P\{lacW\}ci^{Dplac}$ in the absence of *P* elements, but none was recovered. I isolated two mutants with completely white eyes from the EMS screen. Since they mapped to the $P\{lacW\}ci^{Dplac}$ chromosome, I assumed they were in the w^{+mC} gene in the *P{lacW}* construct and did not consider them further.

In total, there were two mutants on chromosome 2 and thirteen mutants on chromosome 3 isolated that suppressed the PDS at $P\{lacW\}ci^{Dplac}$ (Table 3.2.1).

Table 3.2.1: *Su(PDS)* mutants

Mutant	Mutagen	Mutation	Amino Acid Change	Zygotic Rescue ✂	Maternal Effect✂	Associated Phenotypes	Source
Complementation Group 1: *Su(var)2-5*[a]							
Su(var)2-5^{P4}	EMS	C363T[a]	Q64nonsense	ND	ND	RL, Su(var)	Screen
Su(var)2-5^{P5}	EMS	!		ND	ND	RL, Su(var)	Screen
Complementation Group 2: *Su(var)3-7*[b]							
Su(var)3-7^{P1}	P(PZ)05137^{05137}	!		-(54)	ND	RL	Screen
Su(var)3-7^{P2}	P(PZ)05137^{05137}	!		-(40)	ND	RL	Screen
Su(var)3-7^{P9}	gamma	! 2610-2622	D742frameshift	-(563)	-(53)	Su(var)	Screen
Su(var)3-7^{P12}	P{PZ}CtBP^{0346}	! 2990-3445	! 896D-1020P	-(111)	ND	Su(var)	Screen
Su(var)3-7^{P13}	gamma	!		-(62)	ND	RL	Screen
Su(var)3-7^{P25}	EMS	G1971A	C529Y	-(269)	s 4%(431)	RL	Screen
Su(var)3-7^{P32}	P(PZ)05137^{05137}	!		-(61)	ND	RL	Screen
Su(var)3-7^{P47}	EMS	! 87AB-88F;89A		-(206)	ND	RL	Screen
Su(var)3-7^{P49}	EMS	C2045T	Q554nonsense	-(99)	-(407)	RL	Screen
Su(var)3-7^{P71}	EMS	G1596A	G404E	-(135)	s 1% (190)	RL	Screen
Su(var)3-7^{P43}	EMS	G1632A[1]	G416E	-(60)	s 1% (578)	RL	Screen
Df(3R)ry615				-(68)	ND	RL	Bloomington
Df(3R)126c				-(65)	ND	RL	Bloomington
Su(var)3-7$^{7.1A}$				-(46)	ND		Carol Seum
Su(var)3-7^{9}				-(54)	ND		Carol Seum
Su(var)3-7^{14}				-(58)	ND		Carol Seum
Complementation Group3							
Su(PEIS)P80	EMS	unknown		+	+	RL	Screen
Complementation Group 4							
Su(PEIS)P86	EMS	unknown		+	+	RL, Su(var)	Screen

[a]The PubMed accession number for *Su(var)2-5* is M57574.1 or GI 157640; [b] For *Su(var)3-7* the pubmed accession number is X25187.1 for *Su(var)3-7*; !=deletion; [b]RMEL=recessive maternal effect larval lethal; RL Recessive Lethal; [1]has a Juan element inserted 3' after the transcript termination; ✂Testing whether mutants could zygotically rescue the maternal effect of *Su(var)3-7*^{P9}. See results for details. A "-" sign indicated the mutation cannot zygotically rescue while a "+" indicated the mutation can rescued the maternal effect phenotype. ✂Testing whether these mutations had a maternal effect caused by *Su(var)3-7*. See results for details. A "-" sign indicated that mutant has a maternal effect, an "s" indicated a weak maternal effect, and a "+" sign indicated there was no maternal effect. Numbers in brackets indicated total progeny scored and the percent indicated how many mutant flies survived out of the expected number of flies.

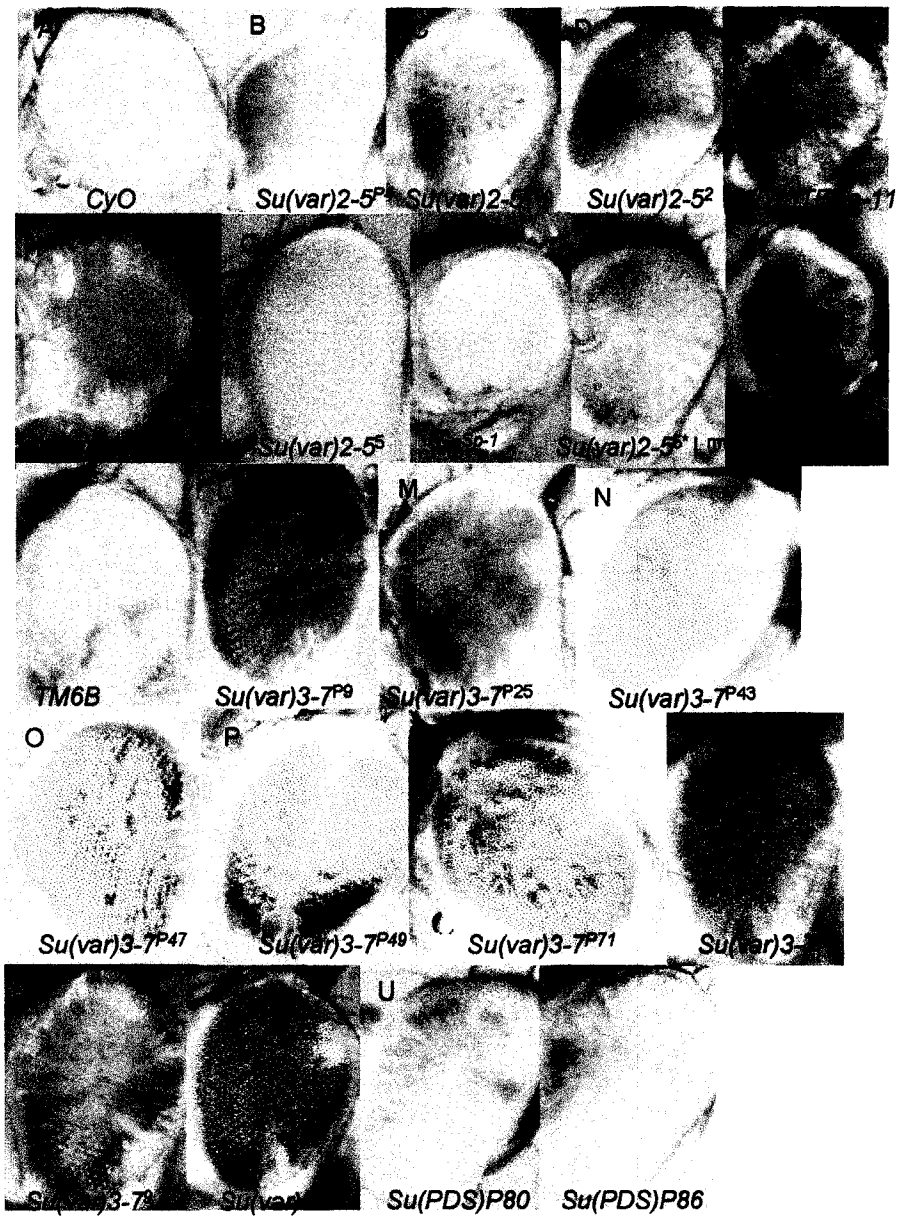


Figure 3.2.1 - Mutations in *Su(var)2-5* and *Su(var)3-7* produce a Su(PDS) phenotype. Pictures are of male progeny from a cross between *y w; P{ry⁺ SALl}(89D); P{lacW}ci^{Dplac}* females and a male with the indicated mutation. All red pigment was produced from the *P{lacW}ci^{Dplac}* chromosome. At the bottom of each picture is the chromosome tested for a Su(PDS) phenotype: A. *CyO*, B. *Su(var)2-5^{P4}*, C. *Su(var)2-5^{P5}*, D.*Su(var)2-5^{2}* , E. *Df(2L)TEAa-11*, F. *Su(var)2-5^{4}*, G. *Su(var)2-5^{5}*, H. *wg^{Sp-1} Bl^{1} L^{rm} Bc^{1} Pu^{2} Pin^{B}*, I. *Su(var)2-5^{5*} L^{rm} Bl^{1}*, *J. Df(3R)ry615*, K.*TM6B,* L. *Su(var)3-7^{P9}*, M. *Su(var)3-7^{P25}*, N.*Su(var)3-7^{P43}*, O. *Su(var)3-7^{P47}*, P. *Su(var)3-7^{P49,}* Q. *Su(var)3-7^{P71}*, R. *Su(var)3-7^{14}*, S. *Su(var)3-7^{9}* T. *Su(var)3-7$^{7.1}$*,U. *Su(PDS)P80*, and V. *Su(PDS)P86*. The *CyO* and *TM6B* balancers produce a wild type phenotype and the eye is predominantly white.

Table 3.2.2: Translocations of *P{lacW}ci*^{Dplac} that suppress PDS

Translocation	Chromosomes	Cytological	Source
12-15a	complex*		This screen
12-1	3-4	66C7-10	This screen
12-3	3-4	92B-C	This screen
$404	3-4	71A	Scott Hanna
RE13-27a	2-4	57A-B	This screen
RE10-10a	3-4	63E	This screen
RE10-17a	3-4	62BC	This screen
12-23a	complex		This screen
RE10-27b	1-4	6AD	This screen
RE10-54b	2-4	47F-48A	This screen
$388	3-4	68D-E	Scott Hanna

*complex rearrangements involved more than two chromosomes.

3.2.1 Position effect can suppress PDS at *P{lacW}ci*^{Dplac}

The γ-irradiation screen produced nine translocations that move *P{lacW}ci*^{Dplac} away from its original location on chromosome 4 (Table 3.2.2). These translocations suppressed the silencing caused by *P{ry*$^{+}$ *SalI}89D* and in most of the translocations w^{+mC} was expressed uniformly throughout the eye (Figure 3.2.2). However, variegation still occurred in some of the translocations when *P{ry*$^{+}$ *SalI}89D* was present.

I compared pigment values from the translocations including *P{lacW}ci*^{Dplac} in M-type and *P*-type (*P{ry*$^{+}$ *Sal}89D*) flies. In M-type flies, the amount of pigment produced from each translocation remained relatively constant no matter the distance from the centromere. In contrast, pigment levels increased when *P{ry*$^{+}$ *SalI}89D* was present as distance from the centromere increased (Figure 3.2.3 blue bar). This increase in pigment occurred in one translocation that was variegated (Figure 3.2.3 *T(2;4)RE13-27a*). Presumably, increased expression in the ommatidia that still had w^{+} expression caused the increased pigment levels.

These translocations behave similarly to other w^{+} transgenes inserted at positions other than the *ci* distal regulatory region. As mentioned in section 3.1, the presence of *P{ry*$^{+}$ *SalI}89D* increased expression from other w^{+} transgenes on chromosome 4. These translocations emphasized the position specific effect *P* elements had on w^{+} transgene expression in *P* constructs. Silencing of a single w^{+} transgene was only described for w^{+} inserts within the *ci* distal regulatory region on chromosome 4.

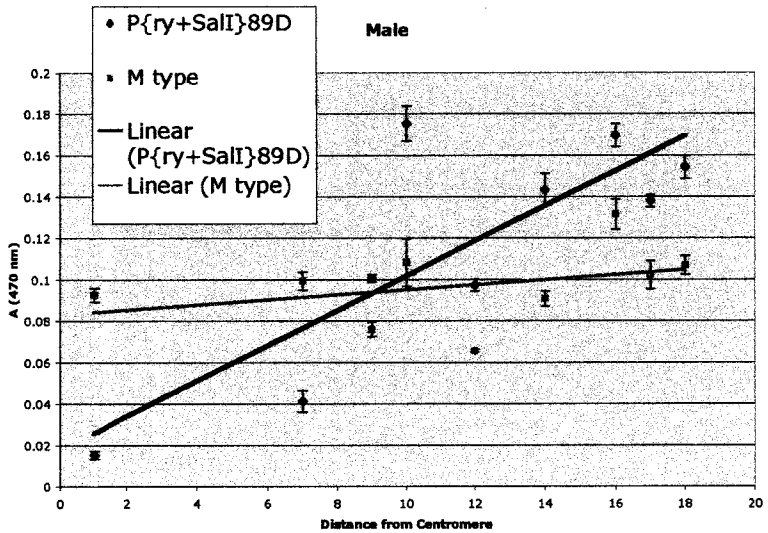
T(2;4)RE10-54b T(3;4)404 T(3;4)12-3

T(3;4)12-1 T(1;4)RE10-27b T(2;4)RE13-27a

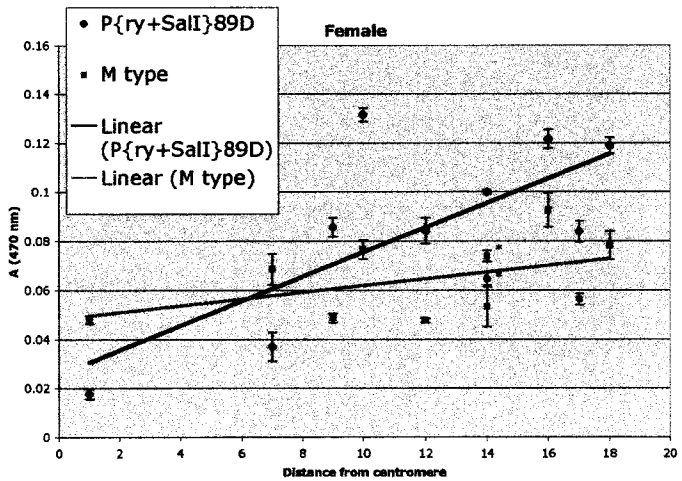
T(3;4)RE10-17a

Figure 3.2.2 - Pictures of eyes where the sole source of w^+ expression is from a translocation of *P{lacW}ci*^{Dplac}. Each frame has 4 flies from each translocation either as *Sb P{ry*$^+$*Sall}89D* or with no *P* element present (M-type). Each frame has a male and a female from each genotypic class. Purple arrows point to variegation seen with some of these translocations when *P{ry*$^+$ *Sall}89D* is present.The crossing scheme to generate these flies involved crossing a male fly that has the translocation to females from a *w; Sb P{ry+ Sall}89D/ TM6B* stock.

A



B



C

Mutation	*P{lacW}cl^{Dplac}*	*T(2;4)RE10-54b*	*T(3;4)404*	*T(3;4)12-3*	*T(3;4)388*	*T(3;4)12-1*	*T(1;4)RE10-27b*	*T(2;4)RE13-27a*	*T(3;4)RE10-10a*	*T(3;4)RE10-17a*
Distance from Centromere	1	7	9	10	12	14	14*	16	17	18
Cytological Location	101	47F-47A	71A	92B-C	68D-E	66C	6A-D	57A-B	63E	62BC

Figure 3.2.3 – Translocations including *P{lacW}cl^{Dplac}* suppress PDS. Pigment values were obtained as described in Materials and Methods from 3 samples consisting of 10 heads. The Y-axis is the cytological distance from the centromere and the X-axis is the Absorbance (470 nm). **A**. Data from male heads. **B**. Data from female heads. **C**. Cytological location and distance from centromere for each translocation tested. The flies used to measure the pigment levels were generated from a cross between males carrying the translocations and *y w; TM6B/ Sb P{ry^{+} SalI}89D* females.

3.2.2 *Su(PDS)* mutations on chromosome 2 are in *Su(var)2-5*

Recessive lethal complementation analysis found that our chromosome 2 *Su(PDS)* mutations were allelic. Both *Su(PDS)* mutations failed to complement the recessive lethal phenotype of *Su(var)2-5*^{2}, *Su(var)2-5*^{5}, and *Df(2L)TEAa-ll*, a deficiency that included the *Su(var)2-5* locus. The Su(PDS) phenotype in one of our *Su(PDS)* mutations (*Su(var)2-5*^{P4}) mapped to ~2-30.5 cM (N=464), which was the approximate location of *Su(var)2-5* (2-31.1). Therefore, I named our new *Su(PDS)* mutations *Su(var)2-5*^{P4} and *Su(var)2-5*^{P5} since they were alleles of *Su(var)2-5*.

Denaturing HPLC (Wave™) analysis indicated that there was a mutation within one of the first three exons of *Su(var)2-5*^{P4} (Data not shown). Sequencing of the *Su(var)2-5*^{P4} allele in hemizygous larvae (*Su(var)2-5*^{P4}/ *Df(2L)TEAa-ll*) revealed a nonsense mutation at position C562T (M57574.1 GI:157640), and only 63 amino acids (aa) of the original 209 aa were predicted to be encoded by this mutant. No mutation was noted for *Su(var)2-5*^{P5}. However, PCR primers that amplified sequences within *Su(var)2-5*$^{+}$, did not produce any product from homozygous *Su(var)2-5*^{P5} mutants. Control primers, could amplify product from other regions indicating the template DNA was intact (Figure 3.2.4). Cytological analysis of + / *Su(var)2-5*^{P5} polytene chromosomes could not find a visible deletion. Taken together, these results indicated this allele had a small deletion that included the *Su(var)2-5* locus.

My recovery of *Su(var)2-5* mutant alleles suggested I test existing alleles for a Su(PDS) phenotype. Both *Df(2L)TEA-11* and *Su(var)2-5*^{2} produced a Su(PDS) phenotype (Figure 3.2.1 [DE]). I mapped the Su(PDS) phenotype in *Su(var)2-5*^{2} to ~2-31.2 (~9.3 cM to the right of *wg*$^{Sp-1}$; N=450) and the published distance for the *Su(var)2-5* locus was 2-31.1 cM (THE FLYBASE CONSORTIUM 2003). While *Su(var)2-5*^{2} did show a PDS phenotype, *Su(var)2-5*^{4} and *Su(var)2-5*^{5} did not (Figure 3.2.1 [FG]). I confirmed by sequencing that the *Su(var)2-5*^{5} allele had the expected mutation.

We hypothesized and confirmed that a second locus enhanced the Su(PDS) phenotype and this locus masked the Su(PDS) phenotype that should have been produced by the chromosomes that had *Su(var)2-5*$^{-}$ mutation. To remove the second locus that was masking the Su(PDS) phenotype that *Su(var)2-5*^{5} produced, I recombined the chromosome that had the *Su(var)2-5*^{5} allele with a chromosome marked with *wg*$^{Sp-1}$ *Bl*^{1} *L*^{RM} *Bc*^{1} *Pu*^{1} *Pin*^{B} (Figure 3.2.1 [H]). I isolated two recombinant chromosomes that produced a Su(PDS) phenotype and retained the *Su(var)2-5*^{5} mutation. Sequencing confirmed these recombinants had the *Su(var)2-5*^{5} allele (Figure 3.2.1 [I]) and gained the Su(PDS) phenotype, thereby supporting our contention that there was a second modifier present on the chromosome. The recombination data was consistent with a single enhancer locus near *L* (2-98; N=183) that neutralized the Su(PDS) effect. This enhancer was not pursued further.

3.2.3 Chromosome 3 *Su(PDS)* mutations affect *Su(var)3-7* and two other loci

Of the 13 *Su(PDS)* mutants I mapped to chromosome 3, 11 belong to one complementation group, while the two remaining mutants affected unidentified genes (see below). The group of 11 mutants was associated with mutations in *Su(var)3-7*, and consequently I have adopted the *Su(var)3-7* name for this group of *Su(PDS)* mutants (Table 3.2.1).

I genetically mapped the Su(PDS) phenotype of one of our initial γ-ray induced mutations (*Su(var)3-7*^{P9} (Figure 3.2.1 [L])) to 3-50.9 (N=1205). This genetic location corresponded with the cytological location 87E and deficiencies including this region, such as *Df(3R)126c*, *Df(3R)ry615* (Figure 3.2.1 [J]), and *Df(3R)ry27* strongly suppressed PDS at *P{lacW}ci*^{Dplac}. No other set of overlapping chromosomes 3 deficiencies consistently produced a strong Su(PDS) phenotype (Figure 3.2.5). This genetic and cytological location corresponds with the location of *Su(var)3-7*. In the following sections, complementation analysis with known *Su(var)3-7* mutants, rescue by a *Su(var)3-7*$^{+}$ transgene, as well as molecular characterization all confirmed that *Su(var)3-7*^{P9} had a mutation interrupting *Su(var)3-7*$^{+}$ function.

3.2.3.1 Complementation Analysis

In addition to the Su(PDS) eye color phenotype, the *Su(var)3-7* mutants had a recessive maternal effect larval (RMEL) lethal phenotype. I used this characteristic to assemble these alleles into a *Su(var)3-7* complementation group (Table 3.2.1). In the RMEL phenotype, homozygous *Su(var)3-7*$^{-}$ mutant progeny from homozygous mothers of either *Su(var)3-7*^{P9} or *Su(var)3-7*^{P12} alleles stop development as first instar larvae. The mouth hooks from these first instar larvae had none of the teeth seen at later larval stages (Figure 3.2.6) (ASHBURNER 1989a). A single functional *Su(var)3-7*$^{+}$ allele, in mother or progeny, allowed development to proceed to the adult stage. These *Su(var)3-7* alleles were tested for the ability to produce maternal product that is deposited in the egg and zygotic product produced in the offspring. By crossing the *Su(PDS)* mutants on chromosome 3 to homozygous *Su(var)3-7*^{P9} females, I tested whether these mutants produced zygotic product. *Su(var)3-7*$^{+}$ alleles, which produced zygotic product, allowed development to proceed and rescued the *Su(var)3-7*^{P9}'s RMEL phenotype. All eleven *Su(PDS)* mutations and *Df(3R)126c*, were unable to complement *Su(var)3-7*^{P9}*s* zygotic RMEL phenotype (Table 3.2.1).

A. Table describing PCR loaded into each lane

Lane#	Primers	Template	Description	Result
1.	2F-394B	G43-3	*Su(var)2-5*^{P5}	-
2.	779F-9998B	G43-3		-
3.	9569F-9998B	G43-3		+
4.	1181F-1493B	G43-3		-
5.	2F-394B	G43-5	*Su(var)2-5*^{P5}	-
6.	779F-9998B	G43-5		-
7.	9569F-9998B	G43-5		+
8.	1181F-1493B	G43-5		-
9.	2F-394B	G43-27	*Df(2L)TE29Aa-11*	-
10.	779F-9998B	G43-27		-
11.	9569F-9998B	G43-27		+
12.	1181F-1493B	G43-27		-
13.	2F-394B	G43-28	*Df(2L)TE29Aa-11*	-
14.	779F-9998B	G43-28		-
15.	9569F-9998B	G43-28		+
16.	1181F-1493B	G43-28		-
17.	2F-394B	G42.2-3	*CyO-GFP/Su(var)2-5*^{P4}	+
18.	779F-9998B	G42.2-3		+
19.	9569F-9998B	G42.2-3		+
20.	1181F-1493B	G42.2-3		+
21.	2F-394B	H3-7	*w; dp; e; P{lacW}ci*^{Dplac}	+
22.	779F-9998B	H3-7		+
23.	9569F-9998B	H3-7		+
24.	1181F-1493B	H3-7		-
25.	2F-394B	water	No Template	-
26.	779F-9998B	water		-
27.	9569F-9998B	water		-
28.	1181F-1493B	water		-
29.	1 kb+ Ladder			

B. 0.8% Agarose Gel stained with Ethidium Bromide

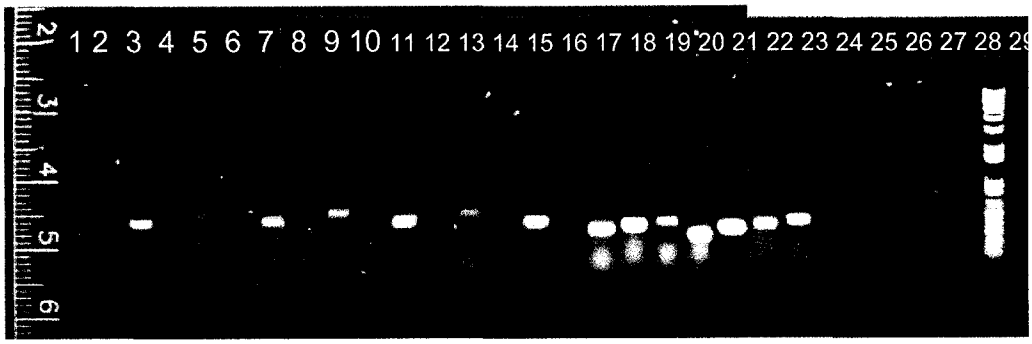


Figure 3.2.4 – *Su(var)2-5*^{P5} is a deletion that includes *Su(var)2-5.* Primers 2F-394B (392 bp distant) and 779F-1220B (441 bp distant) anneal within the *Su(var)2-5*$^{+}$ sequence. Primers 1181F-1493B (312 bp apart) anneal within the *GFP* gene on the *CyO* chromosome. Embryos homozygous for *Su(var)3-7*^{P5} do not produce a band while those that produce a band are *Su(var)3-7*^{P5}*/Cyo-GFP*. Primers 9569F-9998B (429 bp apart) anneal within the *Su(var)3-7*$^{+}$ and are included as a positive control. **A.** Table describing the PCR reactions loaded in each lane of the agarose gel B. A (+) sign indicates the expected size fragment occurs; a (-) sign indicates the expected size band does not occur. **B.** 0.8% agarose gel loaded with PCR reaction described in Table A.

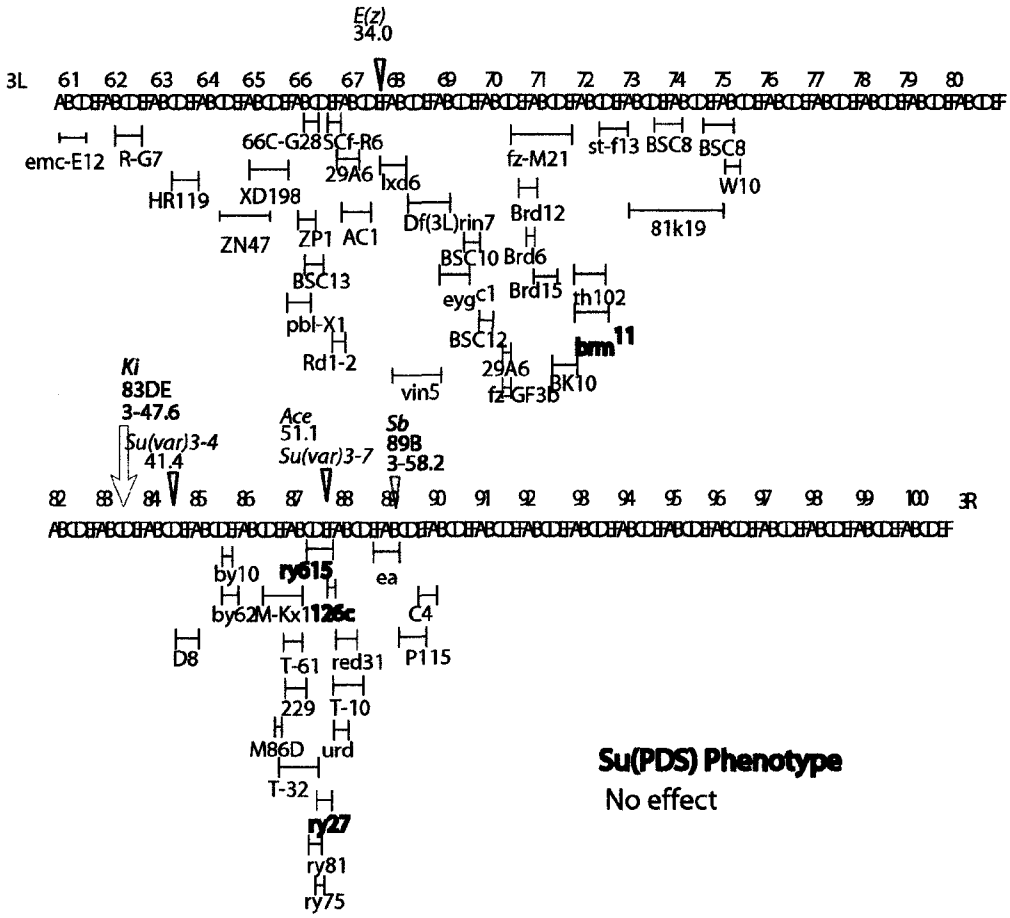


Figure 3.2.5 - Diagram describing the deficiencies on chromosome 3 tested for a Su(PDS) phenotype. Stocks with the deficiency were crossed with a *w; P{ry[+] Sall}89D; P{lacW}ci[Dplac]*. Flies that inherited the deficiency were compared with flies that inherited the balancer. If there was a significant increase in the number of red ommatidia, the deficiency was considered to contain a *Su(PDS)* mutation.

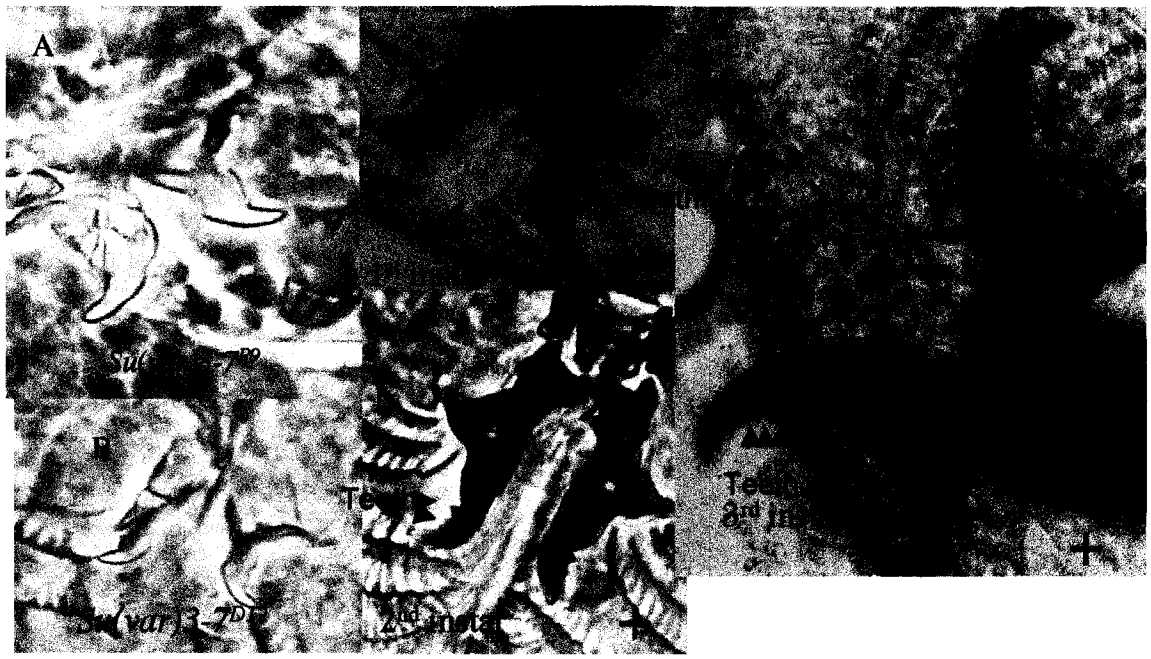


Figure 3.2.6 - Pictures of mouth hooks taken from *Su(var)3-7*$^{-}$ larvae and wild type larvae. The *Su(var)3-7*$^{-}$ mouth hooks are taken from homozygous *Su(var)3-7*$^{-}$ larvae produced from a cross between homozygous Su(var)3-7^{-} mothers and fathers. None of the progeny from such a cross reach pupation and all larvae resemble 1st instar larvae. Vials with mutant larvae and wild type larvae were started at the same time. After larvae started to pupate in the vials with wild type larvae, the larvae in the mutant vials were harvested and the pictures taken. The mouth hooks from the homozygous mutants have no teeth indicating they are from 1st instar larvae. The *Su(var)3-7* allele is indicated at the bottom of each picture. **A**. Picture of mouth hooks taken from a 1st instar larva that is homozygous for *Su(var)3-7*^{P9} and produced from a homozygous mother. **B**. Picture taken of mouth hooks dissected from a 1st instar larvae homozygous for *Su(var)3-7*^{D12} and produced by a homozygous mother. **C.** Picture from a wild type 1st instar larva. The average 1st instar larval has the beginning of one mouth hook (Ashburner 1989). and **D**. Pictures of a 2nd instar larva from wild type (oregon-R) crosses. The 2nd instar larvae have about 3 teeth (Ashburner 1989). **E**. Picture of 3rd instar mouth hooks. The average number of teeth on a mouth from a 3rd instar larva is 12 (Ashburner 1989). Pictures are taken at 100X magnification with a phase contrast microscope.

As well, I tested three previously isolated *Su(var)3-7* mutants produced by site directed mutagenesis (SEUM *et al.* 2002) and all failed to complement *Su(var)3-7*^{P9}'s RMEL phenotype. As well as failing to complement the RMEL phenotype, *Su(var)3-7*$^{7.1}$, *Su(var)3-7*^{9} and *Su(var)3-7*^{14} also produced a Su(PDS) phenotype (Table 3.2.1). *Su(var)3-7*^{9} was a hypomorphic allele (SEUM *et al.* 2002) and had a weaker Su(PDS) phenotype than *Su(var)3-7*$^{7.1}$ and *Su(var)3-7*^{14}, which were reported to be amorphic alleles (Figure 3.2.1 [RST]).

I tested each of the *Su(var)3-7* mutants, which interrupted zygotic RMEL function, for the ability to produce maternal *Su(var)3-7*$^{+}$ product. To test for maternal product, females with a particular *Su(var)3-7* mutant were crossed to *Su(var)3-7*^{P13}/ *TM6B* to generate mutant / *Su(var)3-7*^{P13} heteroallelic mothers. These heteroallelic mothers were crossed back to Su(var)3-7^{P13}/ TM6B males. If the *Su(var)3-7* mutant allele being tested had a maternal effect, then the heteroallelic (*Su(var)3-7* mutant / *Su(var)3-7*^{P13}) mothers should not produce heteroallelic (*Su(var)3-7* mutant / *Su(var)3-7*^{P13}) adult progeny because no maternal *Su(var)3-7* product is contributed to their offspring. In this assay, the progeny inherited the *Su(var)3-7* mutant allele being tested, which could produce *Su(var)3-7* zygotically, but according to the previous tests none of *Su(var)3-7* mutants produced enough zygotic product to allow development to proceed. All the *Su(var)3-7* alleles that failed to act zygotically had a maternal effect. In the case of *Su(var)3-7*^{P25}, *Su(var)3-7*^{P43}, or *Su(var)3-7*^{P71} the effect was weak and heterozygous mothers (*Su(var)3-7* mutant / *Su(var)3-7*^{P13}) rarely produced *Su(var)3-7* mutant progeny (*Su(var)3-7* mutant / *Su(var)3-7*^{P13}) that developed into adults (Table 3.2.1).

Since mutations in *Su(var)3-7* were not recessive lethal, heteroallelic lethal combinations must result from a mutation affecting another common locus (Table 3.2.3). Complementation assays testing for recessive lethality subdivided the eleven *Su(var)3-7* mutations into two groups. The first group consisted of five alleles that had mutations that included both *Su(var)3-7* and adjacent genes in 87E (Table 3.2.3 grey boxes). Although not cytologically visible, *Su(var)3-7*^{P13} probably had a deletion in the 87E region since it was lethal over *Df(3R)126c* and *Df(3R)ry615*, which were deficiencies that include 87E. *Su(var)3-7*^{P47} had a visible cytological deletion that included 87E. Three other mutants, *Su(var)3-7*^{P1}, *Su(var)3-7*^{P2}, and *Su(var)3-7*^{P32}, were lethal in combination with *Su(var)3-7*^{P13}. The second groups included six alleles that had lesions within the 87E region that only affected *Su(var)3-7*. Of the remaining six mutants, only *Su(var)3-7*^{P9} and *Su(var)3-7*^{P12} were homozygous viable. All the EMS induced mutants were homozygous lethal and second site mutations, outside the 87E region, maybe responsible for the recessive lethality associated with these chromosomes. Also, common second site mutations would explain why such allele combinations, as *Su(var)3-7*^{P71}/ *Su(var)3-7*^{P80}, *Su(var)3-7*^{P43}/ *Su(var)3-7*^{P86}, and *Su(var)3-7*^{P71}/ *Su(var)3-7*^{P49} were semi-lethal or lethal.

Table 3.2.3: Complementation assay for recessive lethality between *Su(PDS)* mutations on chromosome 3

Mutant	P86	P80	P71	P49	P47	P43	P32	P25	P13	P12	P9	P2	P1
P1	ND	ND	ND	ND	ND	ND	ND	ND	-	ND	+	ND	-
P2	ND	ND	ND	ND	ND	ND	ND	ND	-	ND	+	-	
P9	+	+	+	+	+	+	+	+	+	+	+		
P12	ND	ND	ND	ND	ND	ND	ND	ND	+	+			
P13	+	+	+	+	-	+	-	+	-				
P25	+	+	+	+	+	+	ND	-					
P32	ND	ND	ND	ND	ND	ND	ND						
P43	s	+	+	+	+	-							
P47	+	+	+	+	-								
P49	+	+	-	-									
P71	+	s	-										
P80	+	-											
P86	-												

Combinations of mutations isolated on chromosome 3 were tested for viability (See Material and Methods). A "+" sign indicated the combination was viable. A "s" sign indicated that combination occurs less than 5% of the time than expected. A "-" sign indicated the combination was lethal. The "ND" indicates cross was not done. Grey boxes represent *Su(var)3-7* alleles that have a deficiency or more than one recessive lethal mutation in the 87E region.

3.2.3.2 Molecular Analysis

Molecular analysis of the *Su(var)3-7* mutants identified using the genetic evidence described above confirmed that these alleles had a mutation interrupting *Su(var)3-7*. This molecular analysis started with Southern blots made using *Hind* III, *Sac* I, and *EcoR* I digested genomic DNA from stocks with the *Su(var)3-7* mutations heterozygous with a balancer. *Hind* III digestion splits the *Su(var)3-7*$^{+}$ into two restriction fragments that were 5 and 11 kbp long (Figure 3.2.7). Probing with Probe 1:9569F-11703B found that *Su(var)3-7*^{P12} and *Su(var)3-7*^{P43} had changes in fragment length (Figure 3.2.8 Lanes 7 and 9). The same Probe 1 hybridized to a 2.7 kbp fragment in the *Sac* I Southern blot (Figure 3.2.9). In lane 14 of the *Sac* I Southern blot, there was an extra band indicating a change that occurred in 3' end of the *Su(var)3-7*^{P12}. Probing an *EcoR* I Southern blot with Probe 1 found that *Su(var)3-7*^{P12} and *Su(var)3-7*^{P43} had extra bands that were not due to incomplete digestion (data not shown). These Southern blots were probed again with Probe 2: 13958-16530. Probe 2 hybridized only to the expected bands for *Sac* I and *EcoR* I Southern blots (Figure 3.2.10 and data not shown for the *EcoR* I blot) and this indicated the changes noted occurred within the 3' end of the *Su(var)3-7*. Re-probing the *Hind* III Southern blot produced extra bands for *Su(var)3-7*^{P12} and *Su(var)3-7*^{P43} confirming that there was a change in the *Hind* III restriction fragment at the 3' end of the gene

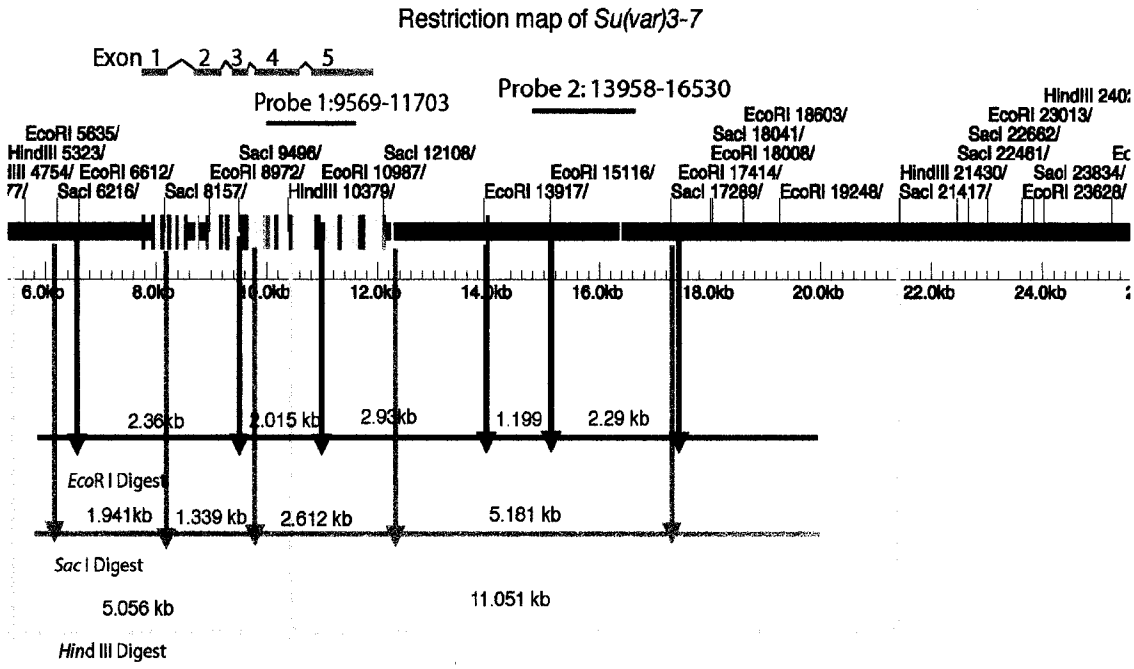
(Figure 3.2.11). PCR did not amplify sequence from *Su(var)3-7*^{P12} hemizygotes (*Su(var)3-7*^{P12}*/ Df(3R)126c*) using primer pairs 10401F-10903B and 10871F-11327B but primers 10871F-11703B produced a 400 bp fragment where a 900 bp product was expected. The 10871F-11703B product from *Su(var)3-7*^{P12} template was sequenced and I found a 455 bp deletion (Table 3.2.1).

Using PCR and overlapping primer pairs that span the Su(var)3-7 coding sequence, I did not find any gross molecular changes in the *Su(var)3-7*^{P43} hemizygote (*Su(var)3-7/ Df(3R)126c*). Together, the PCR and Southern blot analysis indicated that a molecular change had occurred at the 3′ end of the last exon but not within the coding sequence. The primer pairs 12013F-13032B and 12973F-13991B amplified product after the last exon in *Su(var)3-7*$^{+}$ from wild type alleles but primer pair 12973F-13991B did not produce any product using template from a *Su(var)3-7*^{P43} hemizygote with the same PCR conditions. Extending the annealing time to 6 minutes (from 1^{20} minutes) produced a ~5 kp band where the wild type allele only produced a ~1 kbp band. This 5 kbp band was sequenced and a *Juan* element insert was found more than 319 bp down stream from the longest identified transcript for *Su(var)3-7*$^{+}$ (CLEARD *et al.* 1995). The exact position of this insert was not determined because it was not expected to interrupt *Su(var)3-7*$^{+}$ function.

Mutations in *Su(var)3-7*^{P49}, *Su(var)3-7*^{P43}, and *Su(var)3-7*^{P9} were detected in ~500 bp fragments spanning *Su(var)3-7* using denaturing HPLC. DNA sequencing of amplified fragments from hemizygotes identified the mutations within these alleles (Table 3.2.1). Mutations in *Su(var)3-7*^{P25} and *Su(var)3-7*^{P71} were not detected in denaturing HPLC screens but by sequencing the entire coding region in hemizygotes (Table 3.2.1).

In summary all 11 *Su(PDS)* mutations had mutations in the *Su(var)3-7* gene (Table 3.2.1).

A



B

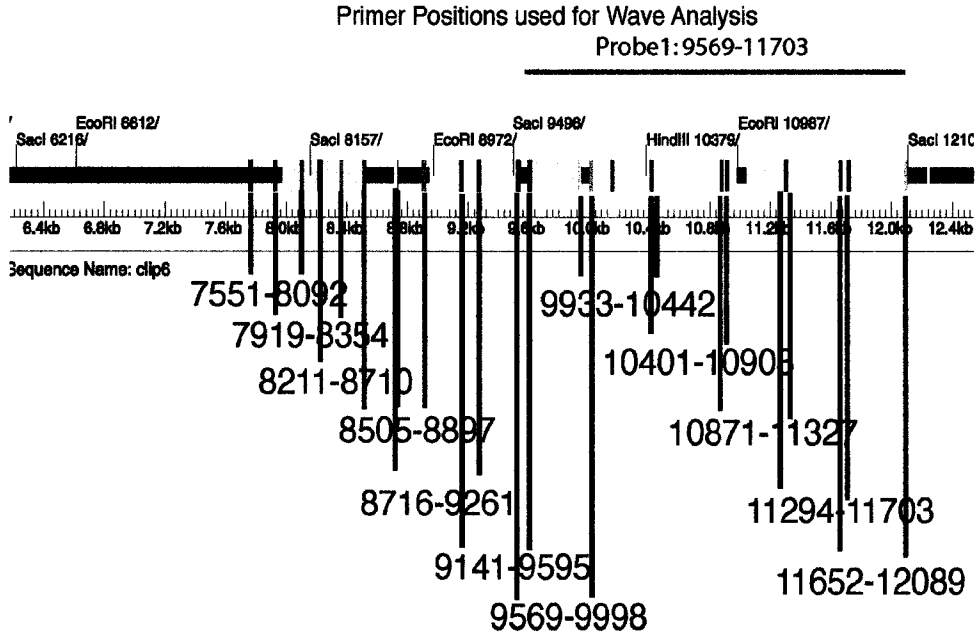
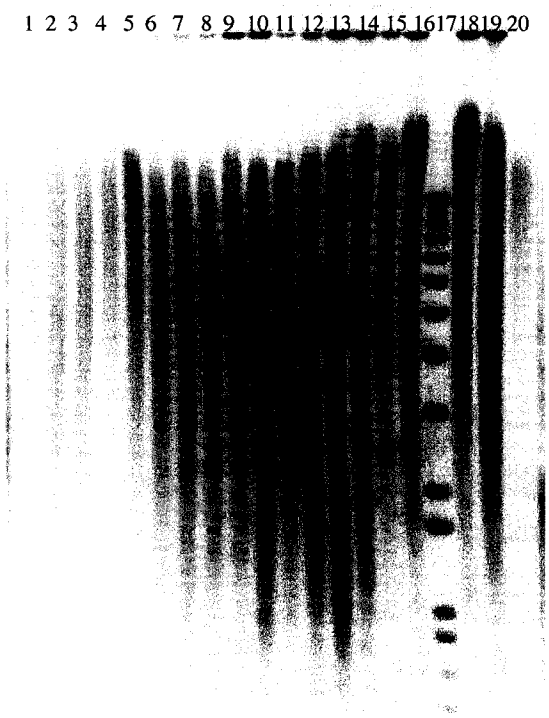
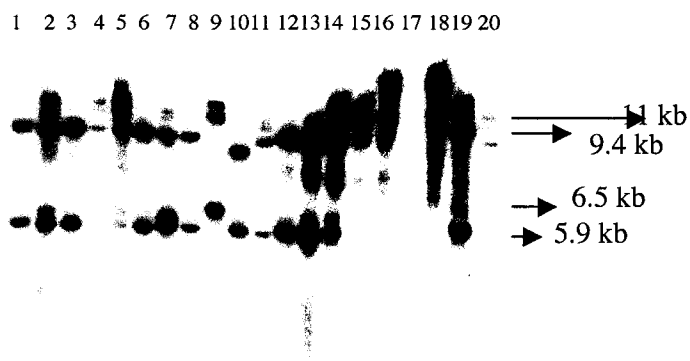


Figure 3.2.7 Restriction map and primers used to find mutations in *Su(var)3-7*. **A**. Restriction map of *Su(var)3-7*. The colors are specific to different restriction enzymes and the numbers represent the expected sizes. Black is specific to *Eco*R I, Orange is *Sac* I, *Hind* III is yellow. **B.** Primers pairs used in wave analysis and sequencing of *Su(var)3-7* mutants.

A. Agarose Gel



B. Autoradiograph 5 days exposure



C

Lane	Description (*Hind* III digested)
1.	*w; Su(var)3-7^{P13}/ TM6B*
2.	*w; Su(var)3-7^{P25}/ TM2*
3.	*w;Su(var)3-7^{P25}/ TM2*
4.	*w;Su(var)3-7^{P47}/ TM2*
5.	*w;Su(var)3-7^{P49}/ TM2*
6.	*w;Su(var)3-7^{P71}/ TM2*
7.	*w;Su(var)3-7^{P43} / TM2*
8.	*w;Su(var)3-7^{P2} / TM2*
9.	*w;Su(var)3-7^{P12}/ TM2*
10	*w;Su(var)3-7^{P1}/ TM2*
11	*w;Su(var)3-7^{P32}/ TM2*
12	*w; Sb P{ry^{+} Sall}89D/ TM3, ser; ci^{1} ey^{R}*
13	*w; CyO/ Bc; TM2/ Sb P{ry^{+} Sal}89D*
14	*w; TM6B/ Ly*
15	P{PZ}CtBP^{03463} ry^{506}/ TM3, ry^{RK} Sb Ser
16	ry^{506} P{PZ}05137^{05137}/TM3, ry^{506} Sb Ser
17	1 kb^{+} Ladder
18	*w; e; dp; P{lacW}ci^{Dplac}*
19	w;Su(var)3-7^{P9}/ *TM6B*
20	*w; P{lacW}ci^{Dplac}*

Figure 3.2.8 - *Hind* III digestion and Southern hybridization blot using Probe1: 9569F-11703B. **A.** 0.8% Agarose gel stained with ethidium bromide. **B.** Autoradiograph of Southern hybridization blot using 9569F-11703B. **C.** Table indicating which *Hind* III digested genomic DNA is added to each lane. The expected size band is 11 kbp. The genomic DNA in lane 9 electrophoresed slower possibly because of high salt concentration. Some of the lanes were loaded with partially digested genomic DNA: 13, 14, 15 16, 18, and 19. Lane 20 did not have enough DNA loaded.

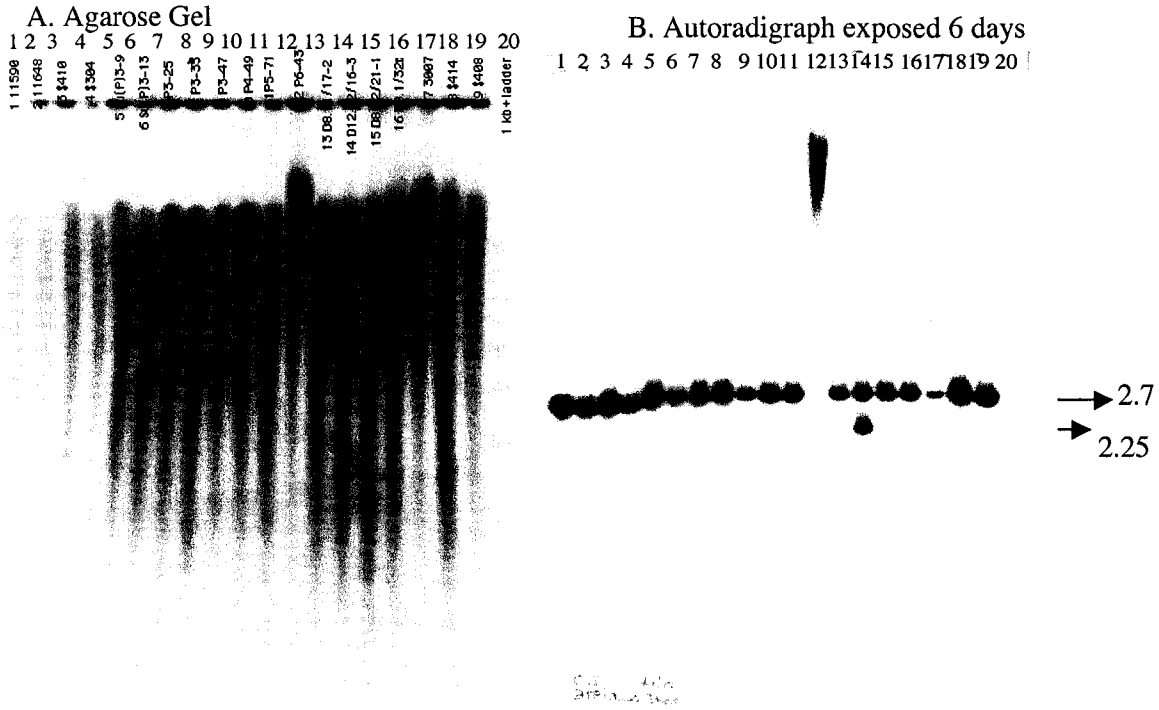
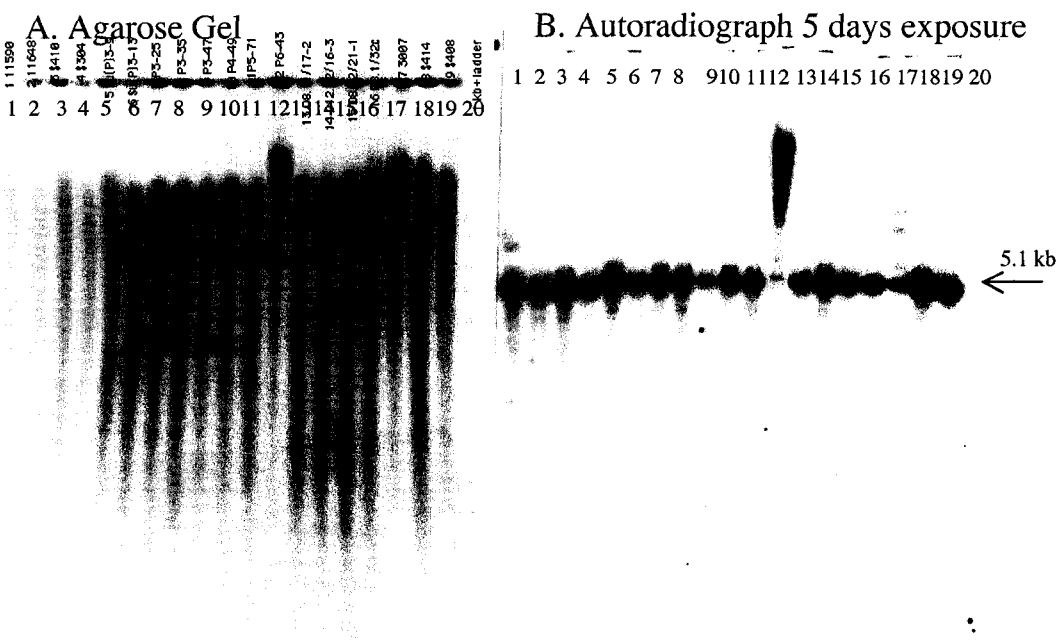


Figure 3.2.9 - *Sac* I digestion and Southern hybridization blot using Probe 1: 9569F-11703B. **A.** 0.8% Agarose gel used to make the Southern blot. **B.** Autoradiograph of Southern blot probed with radiolabeled PCR product from primers 9569F-11703B. **C.** Table describing which *Sac* I digested genomic DNA is loaded into each lane. Lanes 12 and 17 are partially digested.

C

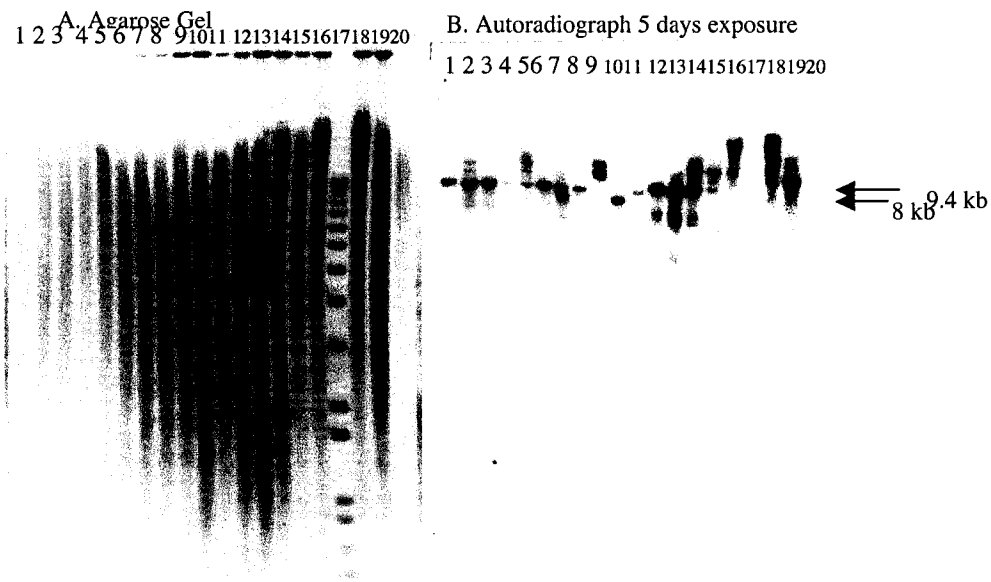
Lane	Description
1.	P{PZ}CtBP^{03463} ry^{506}/ TM3, ry^{RK} Sb Ser
2.	ry^{506} P{PZ}05137^{05137}/TM3, ry^{506} Sb Ser
3.	*w; e; dp; P{lacW}ci^{Dplac}*
4.	w; *P{lacW}ci^{Dplac}*
5.	*w; Su(var)3-7^{P9}*/ TM6B
6.	*w; Su(var)3-7^{P13}*/ TM6B
7.	*w;Su(var)3-7^{P25}/ TM2*
8.	*w;Su(var)3-7^{P25} / TM2*
9.	*w;Su(var)3-7^{P47} / TM2*
10	*w;Su(var)3-7^{P49}/ TM2*
11	*w;Su(var)3-7^{P71} / TM2*
12	*w;Su(var)3-7^{P43} / TM2*
13	*w;Su(var)3-7^{P2} / TM2*
14	*w;Su(var)3-7^{P12}/ TM2*
15	*w;Su(var)3-7^{P1}/ TM2*
16	*w;Su(var)3-7^{P32}/ TM2*
17	*Df(3R)ry615/TM3, Sb^{1}Ser^{1}*
18	*w; CyO/ Bc; TM2/ Sb P{ry^{+} Sal}89D*
19	*w; TM6B/ Ly*
20	1 kb^{+} Ladder



C

Lane	Description
1.	P{PZ}CtBP^{03463} ry^{506}/ TM3, ry^{RK} Sb Ser
2.	ry^{506} P{PZ}05137^{05137}/TM3, ry^{506} Sb Ser
3.	*w; e; dp; P{lacW}cl^{Dplac}*
4.	w; *P{lacW}cl^{Dplac}*
5.	*w; Su(var)3-7^{P9}*/ TM6B
6.	*w; Su(var)3-7^{P13}*/ TM6B
7.	*w;Su(var)3-7^{P25}/ TM2*
8.	*w;Su(var)3-7^{P25} / TM2*
9.	*w;Su(var)3-7^{P47} / TM2*
10	*w;Su(var)3-7^{P49}/ TM2*
11	*w;Su(var)3-7^{P71} / TM2*
12	*w;Su(var)3-7^{P43} / TM2*
13	*w;Su(var)3-7^{P2} / TM2*
14	*w;Su(var)3-7^{P12}/ TM2*
15	*w;Su(var)3-7^{P1}/ TM2*
16	*w;Su(var)3-7^{P32}/ TM2*
17	*Df(3R)ry615/TM3, Sb^{1}Ser^{1}*
18	*w; CyO/ Bc; TM2/ Sb P{ry^{+} Sal}89D*
19	*w; TM6B/ Ly*
20	1 kb^{+} Ladder

Figure 3.2.10 - *Sac* I digestion and Southern hybridization blot of *Su(var)3-7* mutants using Probe 2 **A.** 0.8% Agarose gel stained with ethidium bromide used to make the blot. **B.** Autoradiograph of the hybridization blot using Probe 2: 13958-16350. **C.** Table indicating from which stock the *Sac* I digested genomic DNA was isolated.



C

Lane	Description (*Hind* III digested)
1.	*w; Su(var)3-7^{P13}/ TM6B*
2.	*w; Su(var)3-7^{P25}/ TM2*
3.	*w;Su(var)3-7^{P25}/ TM2*
4.	*w;Su(var)3-7^{P47}/ TM2*
5.	*w;Su(var)3-7^{P49}/ TM2*
6.	*w;Su(var)3-7^{P71}/ TM2*
7.	*w;Su(var)3-7^{P43} / TM2*
8.	*w;Su(var)3-7^{P2} / TM2*
9.	*w;Su(var)3-7^{P12}/ TM2*
10	*w;Su(var)3-7^{P1}/ TM2*
11	*w;Su(var)3-7^{P32}/ TM2*
12	*w; Sb P{ry^{+} SalI}89D/ TM3, ser, ci^{1} ey^{R}*
13	*w; CyO/ Bc; TM2/ Sb P{ry^{+} SalI}89D*
14	*w; TM6B/ Ly*
15	P{PZ}CtBP^{03463} ry^{506}/ TM3, ry^{RK} Sb Ser
16	ry^{506} P{PZ}05137^{05137}/TM3, ry^{506} Sb Ser
17	1 kb^{+} Ladder
18	*w; e; dp; P{lacW}cl^{Dplac}*
19	*w;*Su(var)3-7^{P9}/ *TM6B*
20	*w; P{lacW}cl^{Dplac}*

Figure 3.2.11 - *Hind* III digestion and Southern hybridization blot probed using Probe 2: 13958-16530. **A.** 0.8% Agarose gel used to make the Southern blot. **B.** Autoradiograph after hybridizing with probe 2. **C.** Table describing what genomic DNA was loaded into each lane of the agarose gel.

3.2.4 Transgene *Su(var)3-7$^{+t6.5}$* rescues the Su(PDS) and RMEL phenotypes of *Su(var)3-7^{P9}*

Since the *Su(PDS)* mutations were dominant and most likely dose dependent, I wanted to test the effect *Su(var)3-7^{+}* dosage had on *w^{+mC}* silencing at *P{lacW}ci^{Dplac}* using a previously reported *Su(var)3-7^{+}* transgene. The *Su(var)3-7^{+}* locus was originally identified by transforming *Drosophila* with a series of truncated genomic fragments from 87E and selecting fragments that enhanced *In(1)w^{m4}*, *w^{m4}* (REUTER *et al.* 1990). The smallest fragment that enhanced variegation was called *Su(var)3-7$^{+t6.5}$*. In addition to the *Su(var)3-7* gene, this 6.5 kbp transgenic construct contained another gene which shared sequence similarity with *Su(var)3-7* and was called *Ravus* (DELATTRE *et al.* 2002). I attribute the effects of the *Su(var)3-7$^{+t6.5}$* transgene, described below, to the *Su(var)3-7^{+}* gene within this construct rather than to *Ravus*, because *Ravus* did not modify hPEV and unlike *Su(var)3-7*, its protein product did not associate with the chromocenter (DELATTRE *et al.* 2002).

Using a stock that had the *Su(var)3-7$^{+t6.5}$* and *Su(var)3-7^{P9}* alleles (*w; Su(var)3-7$^{+t6.5}$/CyO ; Su(var)3-7^{P9}/ TM2, Ubx*) ,in a single cross I generated three different *Su(var)3-7^{+}* dosages (1, 2, and 3) and observed their effect on *P{ry^{+} SalI}89D* silencing of *P{lacW}ci^{Dplac}* (Figure 3.2.12). When two functional copies of *Su(var)3-7^{+}* were combined with *P{lacW}ci^{Dplac}* and *P{ry^{+} SalI}89D*, the eyes were predominately white indicating *w^{+mC}* was consistently silenced in most of the ommatidia. A mutation affecting one of the two *Su(var)3-7* copies suppressed this silencing and the eye was predominately red colored. When the number of functional *Su(var)3-7^{+}* copies was returned to two in a *Su(var)3-7^{P9}* / + heterozygote by adding the *Su(var)3-7$^{+t6.5}$* transgene, the *w^{+mC}* transgene was silenced and produced a mostly white eye. This dose dependent effect on silencing was best seen when flies were reared at 29°C because at lower temperatures *w^{+mC}* expression was predominately silenced at both 2 and 3 doses (Figure 3.2.12). Thus, gene silencing at *P{lacW}ci^{Dplac}* acted similarly to hPEV at *w^{m4}* where an increased *Su(var)3-7* dosage enhanced silencing.

The *Su(var)3-7$^{+t6.5}$* transgene also rescued the RMEL phenotype of *Su(var)3-7^{P9}* both paternally and maternally. Homozygous *Su(var)3-7^{P9}* progeny, produced by homozygous mothers develop into adults when they inherit *Su(var)3-7$^{+t6.5}$* paternally. Also, *homozygous Su(var)3-7^{P9}* mothers that had one copy of *Su(var)3-7$^{+t6.5}$* can produce *Su(var)3-7^{P9}* homozygous offspring that develop into adults. Therefore *Su(var)3-7$^{+t6.5}$* rescued both the maternal and zygotic phenotypes in the *Su(var)3-7^{P9}* homozygous mutant.

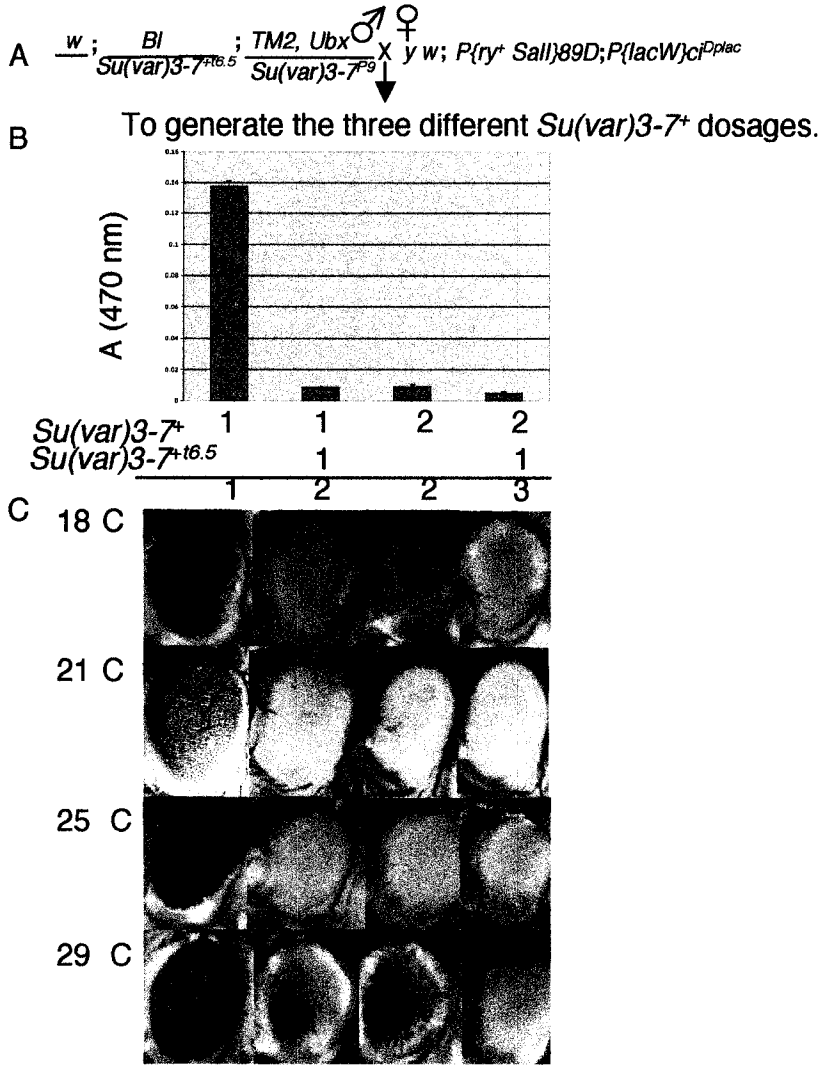


Figure 3.2.12 - *Su(var)3-7*$^{+}$ acts dose-dependently to silence w^{+mc} expression from *P{lacW}ci*^{Dplac}. **A**. Parents crossed to produce the observed progeny. **B**. Pigment accumulation from the w^{+mc} transgene at 21°C in males. As indicated at the bottom of the graph *Su(var)3-7*$^{+}$ dosage increases from 1, 2, 2, to 3 doses. Measurements were taken from 3 samples with 10 heads each. **C**. Eyes from male flies with increasing *Su(var)3-7*$^{+}$ dosage as in B. Each row of eyes is collected from flies raised at the rearing temperature indicated to the left of each row.

3.2.5 *Su(var)3-7* mutations did not interrupt *P* element suppression of the other *P* element dependent phenotypes

I tested whether the *Su(var)3-7* mutants interrupted *P{ry⁺ SalI}89D* activity and if this suppressed w^{+mC} silencing at $P\{lacW\}ci^{Dplac}$. If the *Su(var)3-7* mutants acted directly on *P{ry⁺ SalI}89D*, then other loci that had a *P{ry⁺ SalI}89D* dependent phenotype would be affected by mutations in *Su(var)3-7*. To test this possibility, I used the *vestigial* (*vg*) allele combination $vg^{21\text{-}3}$ / vg^{B}, which produced a strong vestigial wing phenotype (Figure 3.2.13 [A]), which was suppressed by *P{ry⁺ SalI}89D* (Figure 3.2.13 [B]). I observed that this suppression was not changed by the presence of $Su(var)3\text{-}7^{P9}$ mutants (Figure 3.2.13 [C]). Therefore, mutations in *Su(var)3-7* did not interrupt *P* element suppression at *vg*, and presumably other loci, by inhibiting *P{ry⁺ SalI}89D* activity.

In somatic cells, *P{ry⁺ SalI}89D* repressed expression from enhancer traps that have the *P* promoter fused to the *lacZ* gene (*P-lacZ*) (LEMAITRE and COEN 1991). To determine if mutations in *Su(var)3-7* interrupted this repression, I stained imaginal discs for β-galactosidase activity (Figure 3.2.14-15). In the absence of *P{ry⁺ SalI}89D*, the *P-lacZ* expression from $P\{lacW\}ci^{Dplac}$ mimicked *ci* expression and the anterior portion of the wing imaginal disc was stained (SCHWARTZ *et al.* 1995). *P{ry⁺ SalI}89D* completely repressed *P-lacZ* expression from $P\{lacW\}ci^{Dplac}$ and no staining occurred in the wing imaginal disc with or without the $Su(var)3\text{-}7^{P9}$ mutation, using the same staining conditions (Figure 3.2.14). In an assay using a different enhancer trap, the presence of *P{ry⁺ SalI}89D* repressed *P-LacZ* expression from $P\{PZ\}ap^{rk568}$, but did not reduce it to the point were β-galactosidase activity was undetectable as with $P\{lacW\}ci^{Dplac}$ (Figure 3.2.15). With $P\{PZ\}ap^{rK568}$, β-galactosidase staining was also not affected by mutations in *Su(var)3-7* when *P{ry⁺ SalI}89D* was present or absent (Figure 3.2.15). These results indicated that mutations in *Su(var)3-7* did not affect *P{ry⁺ SalI}89D* directly, nor did this mutation prevent *P{ry⁺ SalI}89D* from interacting at $P\{lacW\}ci^{Dplac}$ or $P\{PZ\}ap^{rK568}$. *P{ry⁺ SalI}89D* continued to repress *P-lacZ* expression from *P-LacZ* transgenes during conditions that suppressed w^{+mC} silencing. Therefore silencing at w^{+mC} and repression from *P-lacZ* occurred through different mechanisms.

3.2.6 *Su(var)3-7* can suppress PDS induced by *P* strains

Thus far, *Su(var)3-7* mutations suppressed silencing caused by a single Type I repressor element but wild *P* strains have many *P* elements of both the Type I and II classes, which may act through different mechanisms. To determine if mutations in *Su(var)3-7* continued to suppress silencing caused by a *P* strain, I crossed $Su(var)3\text{-}7^{P9}$ and $Su(var)3\text{-}7^{P13}$ chromosomes, dominantly marked with *Kinked* (*Ki*), into a Harwich background (*P* stock) (Figure 3.2.16). Then *Ki Su(var)3-7⁻* males were crossed to females from a *y w*; $P\{lacW\}ci^{Dplac}$ stock. The *P* elements inherited from Harwich strain consistently silenced expression from w^{+mC} at $P\{lacW\}ci^{Dplac}$ in Ki^{+} progeny, but not in the *Ki* progeny, which inherited the $Su(var)3\text{-}7^{P9}$ mutation.

Su(var)3-7^{P9} also suppressed silencing triggered by *KP* elements present on *KP-D (Cy Bl)* and *KP-U (TM6B)* chromosomes (Figure 3.2.16). These results demonstrated that mutations in *Su(var)3-7* suppressed both Type I (*P{ry*$^{+}$ *Sall}89D*) and Type II *P* elements, as well as multiple types of *P* elements within a single individual. Therefore different Types of P elements appeared to be acting through a common pathway that depended on *Su(var)3-7*$^{+}$ to silence w^{+mC} expression at *P{lacW}ci*^{Dplac}.

3.2.7 Increasing *Su(var)3-7*$^{+}$ dosage silences w^{+mC} from *P{lacW}ci*^{Dplac} in M strains

Given that *Su(var)3-7*$^{+}$ enhances silencing of w^{+mC} expression in the presence of *P* elements, I wanted to determine if *Su(var)3-7*$^{+}$ acted at *P{lacW}ci*^{Dplac} in the absence of *P* elements. I wanted to compare these results to inserts where PDS did not occur, such as *P{lacW}3-76a* and *P{hsp26-pt-T}2-M010.R*. Both of these inserts showed uniform w^{+} expression throughout the eye and did not variegate when *P* elements were present (LOCKE *et al.* Submitted). *P{hsp26-pt-T}2-M010.R* was a different *P* construct inserted in a more distal region on chromosome 4 (102B) (SUN *et al.* 2000) and *P{lacW}3-76a* was a *P{lacW}* insert on chromosome X (THE FLYBASE CONSORTIUM 2003).

I measured the amount of red eye pigment produced by w^{+mC} in the presence of 1, 2, and 3 *Su(var)3-7*$^{+}$ doses (Figure 3.2.17 [A]). In M type flies, as *Su(var)3-7*$^{+}$ dosage increased, pigment levels in *P{lacW}ci*^{Dplac} flies decreased. The largest decrease occurred between 2 and 3 doses. Pigment levels for *P{hsp26-pt-T}2-M010.R* remained relatively constant at 1 and 2 doses but dropped significantly at 3 doses. In contrast to the two loci on chromosome 4, *Su(var)3-7*$^{+}$ dosage had no affect on w^{+} expression from *P{lacW}3-76a* (chromosome X) (Figure 3.2.17). Therefore, *P{lacW}ci*^{Dplac} behaved differently from other w^{+} transgenes in euchromatic regions because it was sensitive to *Su(var)3-7*$^{+}$ dosage. However, this feature was not unique to *P{lacW}ci*^{Dplac} because an increased *Su(var)3-7*$^{+}$ dosage also enhanced w^{+} variegation from the other chromosome 4 insert.

I recovered chromosomal translocations of *P{lacW}ci*^{Dplac} that suppressed PDS. These rearrangements could act by making w^{+mC} expression insensitive to *Su(var)3-7*$^{+}$ dosage, like other euchromatic regions. According to this hypothesis, translocations would behave like inserts in euchromatic regions and be insensitive to *Su(var)3-7*$^{+}$ dosage.

Testing two of these translocations (*T(2;4)13-27a* and *T(2;4)10-54b*) revealed that increasing the *Su(var)3-7*$^{+}$ dosage to three copies continued to cause w^{+mC} repression in M strains (Figure 3.2.17 [A]). The pigment levels produced in flies that had these translocations follow the same trend as *P{lacW}ci*^{Dplac} and as the *Su(var)3-7*$^{+}$ dosage increased pigment values decreased. There was almost no difference in the values between translocation *T(2;4)13-27a* and *P{lacW}ci*^{Dplac}. Therefore the w^{+mC} gene in these translocations continued to behave like other chromosome 4 inserts and retained their sensitivity to *Su(var)3-7*$^{+}$ dosage.

Thus far, the pigment values describe the total pigment that accumulated but ignores variegation. Pigment analysis gave a quantitative measurement of the total amount of pigment produced in the eye. However, the variegated phenotype I am studying resulted from different amounts of pigment accumulating in individual ommatidia producing a variegated phenotype. Continuous gradual repression did not result in a variegated phenotype because variegation occurred even when there were high pigment values. At three *Su(var)3-7*$^{+}$ doses, *P{hsp26-pt-T}2-M010.R* was variegated but produced very high pigment values compared to *P{lacW}ci*^{Dplac}, which also was variegated. In comparison, the translocations did not variegate and had a similar pigment levels to *P{lacW}ci*^{Dplac}. For there to be high pigment values when variegation was present, the w^{+} gene must be expressed at high levels in the colored ommatidia while being completely silenced in the white ommatidia. As the *Su(var)3-7*$^{+}$ dosage increased, pigment values decreased when the original *P{lacW}ci*^{Dplac} allele and translocations of this allele were present. With the translocations, the lower pigment values indicated w^{+} expression was reduced but that lack of variegation indicated it was never completely silenced when the translocations were present. With *P{lacW}ci*^{Dplac}, the lower pigment values could result because w^{+} expression was completely silenced in some ommatidia as well as reduced in others. Thus silencing resulted in variegation that occurred as a separate binary (on/off) switch, which can be switched regardless of expression levels as measured using the pigment assay. With the translocations, increasing the *Su(var)3-7*$^{+}$ dosage resulted in gradual repression but the lack of white ommatidia indicated that w^{+} was not completely turned off. The translocations did not prevent *Su(var)3-7*$^{+}$ dosage from repressing expression but did interrupt the off switch.

3.2.8 *Su(PDS)P80* and *Su(PDS)P86*

In addition to the *Su(PDS)* mutations in *Su(var)2-5* and *Su(var)3-7*, I isolated single alleles at two other loci that also suppressed PDS. The transcription units affected in *Su(PDS)P80* and *Su(PDS)P86* were unidentified. The Su(PDS) phenotype of *Su(PDS)P80* genetically mapped to 3-51.5 (N=1876) while *Su(PDS)P86* maps to 3-31.9 (N=560). Both complement the *Su(var)3-7*^{P9} mutant RMEL phenotype, and in comparison with mutations in *Su(var)3-7*, produced a weak Su(PDS) phenotype (Figure 3.2.1 [F-H]). *Su(PDS)P80* acted as a weak Su(var) in of w^{m4} tests compared to *Su(PDS)86*, which was a strong Su(var).

Further analysis found that the *Su(var)* phenotype in *Su(PDS)86* mapped to 3-44.5 (N=1130), which was a different location than where the *Su(PDS)* mutant mapped. These results indicated the *Su(PDS)P86* had two mutations. Recombination between chromosome with the *Su(PDS)P86* mutation and a chromosome labeled with *Coa*, *Ki*, and *H* produced recombinant chromosomes that produced a Su(var) phenotype but did not have Su(PDS) phenotype (Figure 3.2.1 [H])(Figure 3.2.18). Therefore, by itself the *Su(var)* mutation did not produce a Su(PDS) phenotype. Either the second mutation could solely

produce a Su(PDS) phenotype or in combination with the *Su(var)* mutation produced a Su(PDS) phenotype. While the original *Su(PDS)86* chromosome was homozygous lethal, the recombinant *Su(PDS)86* that had the Su(var) was homozygous viable but homozygotes were female and male sterile.

I tested deficiencies that correspond to regions where these mutations genetically mapped for complementation to the recessive lethality and maternal effects for both *Su(PDS)80* and *Su(PDS)86*. However, all the deficiencies tested thus far complement both phenotypes in both mutations. These *Su(PDS)* mutants have not been studied further.

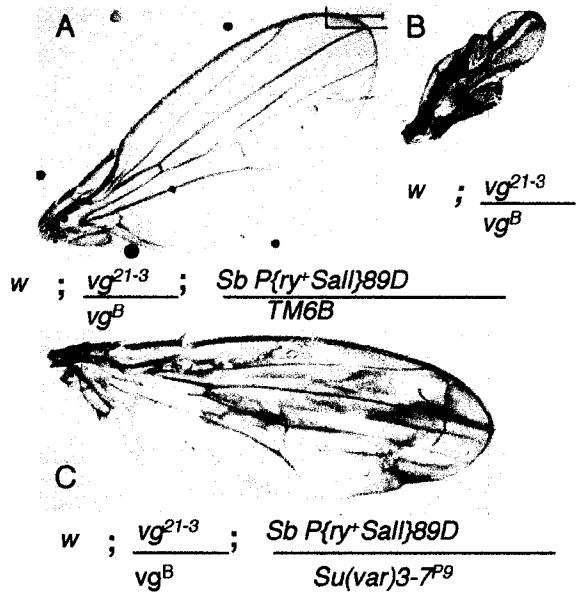


Figure 3.2.13 - *Su(var)3-7^{P9}* does not affect *P{ry^{+} SALl}89D* suppression of *vg$^{21-3}$*. The wings in **A.** and **B.** are generated from a cross between a *y w; CyO/vg$^{21-3}$; TM6B/Sb P{ry+ Sall}89D* male and a *y w; vg^{B}/CyO; Su(var)3-7^{P9}/ TM6B* female. The wings in **C.** is generated from a cross between *vg^{B} cn* male and *w; vg$^{21-3}$* female.

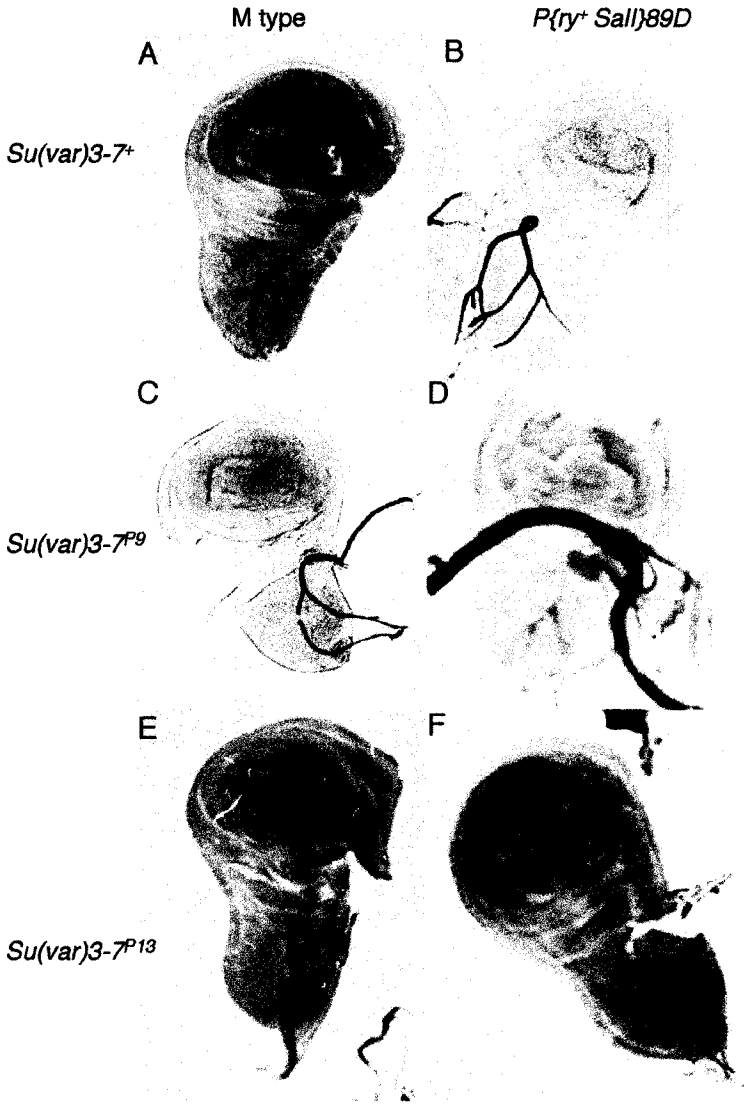


Figure 3.2.14 - Mutations in *Su(var)3-7* do not prevent *P{ry+SalI}89D* from suppressing *P-lacZ* expression from *P{lacW}ci*^{Dplac}. Wing imaginal discs are stained for ß-galactosidase to determine *P-lacZ* activity. The genotypes of the imaginal are as follows: **A.** *w; P{lacW}ci/+*, **B.** *w; P{ry+ SalI}89/ +; P{lacW}ci/+*, **C.** w; *Su(var)3-7*^{P9}*/+; P{lacW}ci*, **D.** *w; Su(var)3-7*^{P9}*/P{ry+ SalI}89D; P{lacW}ci*, **E.** *w; Su(var)3-7*^{P13}*/+; P{lacW}ci/+*, **F.** *w; Su(var)3-7*^{P13}*/P{ry+ SalI}89D; P{lacW}ci/+*

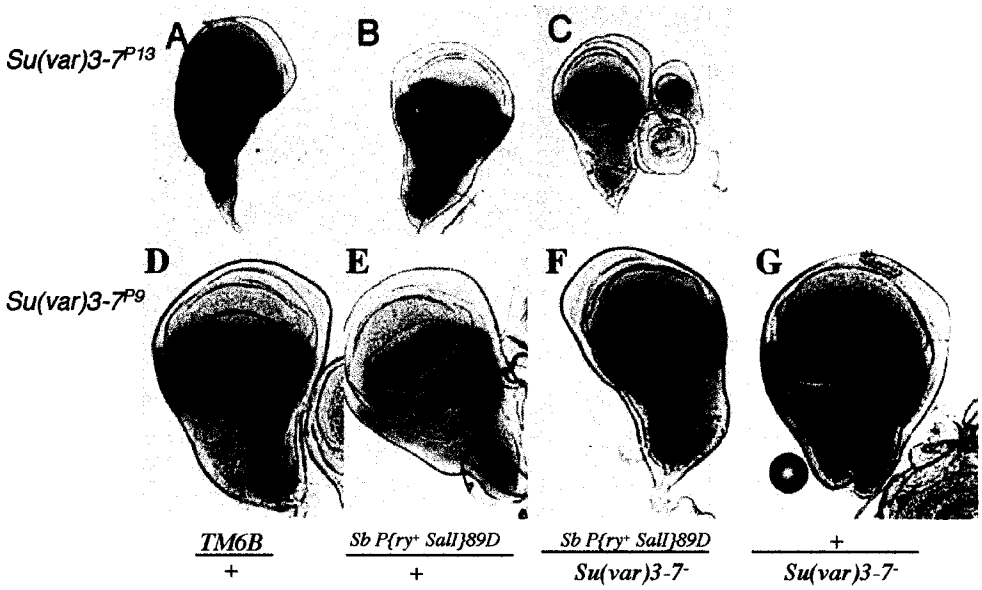


Figure 3.2.15 - Staining imaginal discs for β-galactosidase activity to determine if mutations in *Su(var)3-7* affect *P-lacZ* expression from *P{PZ}ap*^{rk588}. **A.** *w; P{PZ}ap*^{rk588}*; TM6B/+*, **B.** *w; P{PZ}ap*^{rk588}*; Sb P{ry*$^{+}$ *SalI}89D/+*, **C.** *w; P{PZ}ap*^{rk588}*; Sb P{ry*$^{+}$ *SalI}89D/ Su(var)3-7*^{P13}, **D.** *w; P{PZ}ap*^{rk588}*; TM6B/+*, **E.** *w; P{PZ}ap*^{rk588}*; Sb P{ry*$^{+}$ *SalI}89D/+*, **F.** *w; P{PZ}ap*^{rk588}*; Sb P{ry*$^{+}$ *SalI}89D/ Su(var)3-7*^{P9}, and **G.** *w; P{PZ}ap*^{rk588}*; +/ Su(var)3-7*^{P9}. Staining differences occur between *Su(var)3-7*^{P13} and *Su(var)3-7*^{P9} wing discs because discs between the two rows were stained at different times.

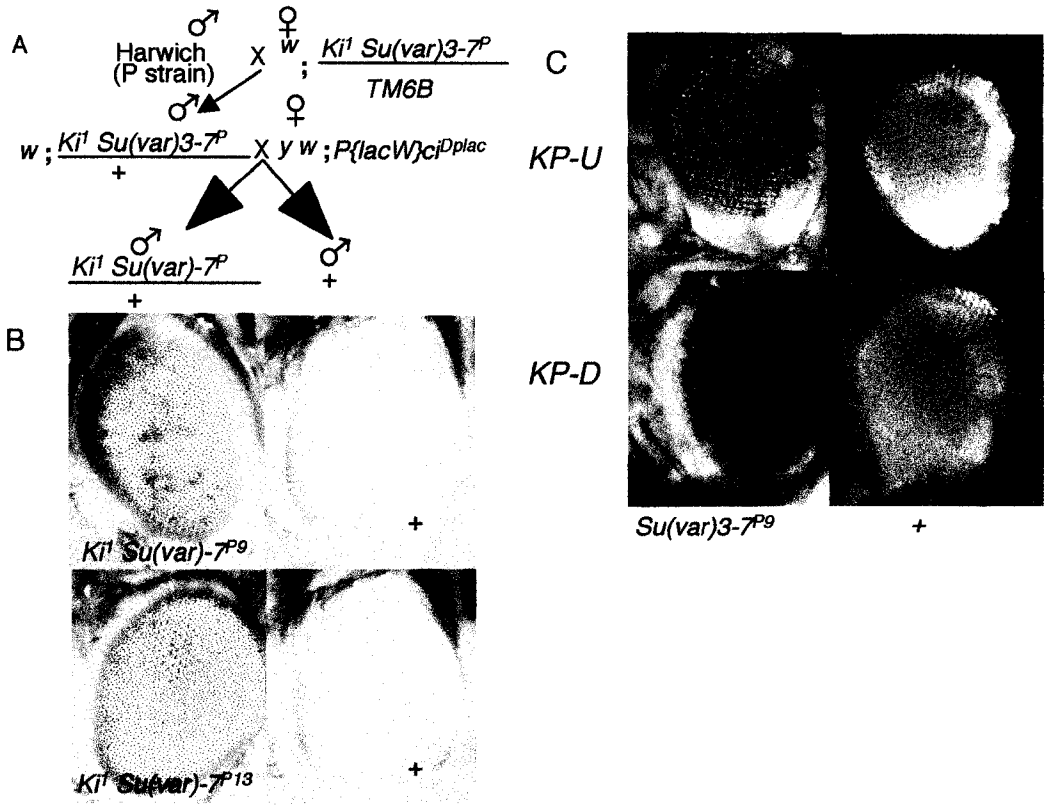


Figure 3.2.16 - Mutations in *Su(var)3-7* suppress PDS induced by a *P* strain and *KP* elements. **A.** Crossing scheme where *Su(var)3-7*^{P9} or *Su(var)3-7*^{P13} was crossed to the Harwich (*P* strain). **B.** Pictures of male eyes where *w*$^{+}$ expression occurs solely from *P{lacW}ci*^{Dplac} after crossing to a *P* strain. The difference in genotype is indicated at the bottom of each picture. As indicated in A, flies on the left inherit *Ki*^{1} and the linked *Su(var)3-7*$^{-}$ allele. Flies on the right inherit a functional *Su(var)3-7*$^{+}$ copy. **C.** *Su(var)3-7*^{P9} suppresses *KP* silencing of *P{lacW}ci*^{Dplac}. Again the sole source of *w*$^{+}$ originates from *P{lacW}ci*^{Dplac}. The left column inherits the *Su(var)3-7*^{P9} mutation and the right column has two functional *Su(var)3-7*$^{+}$ copies. The top row has the *KP-U* chromosome and the bottom row has the *KP-D* chromosome present.

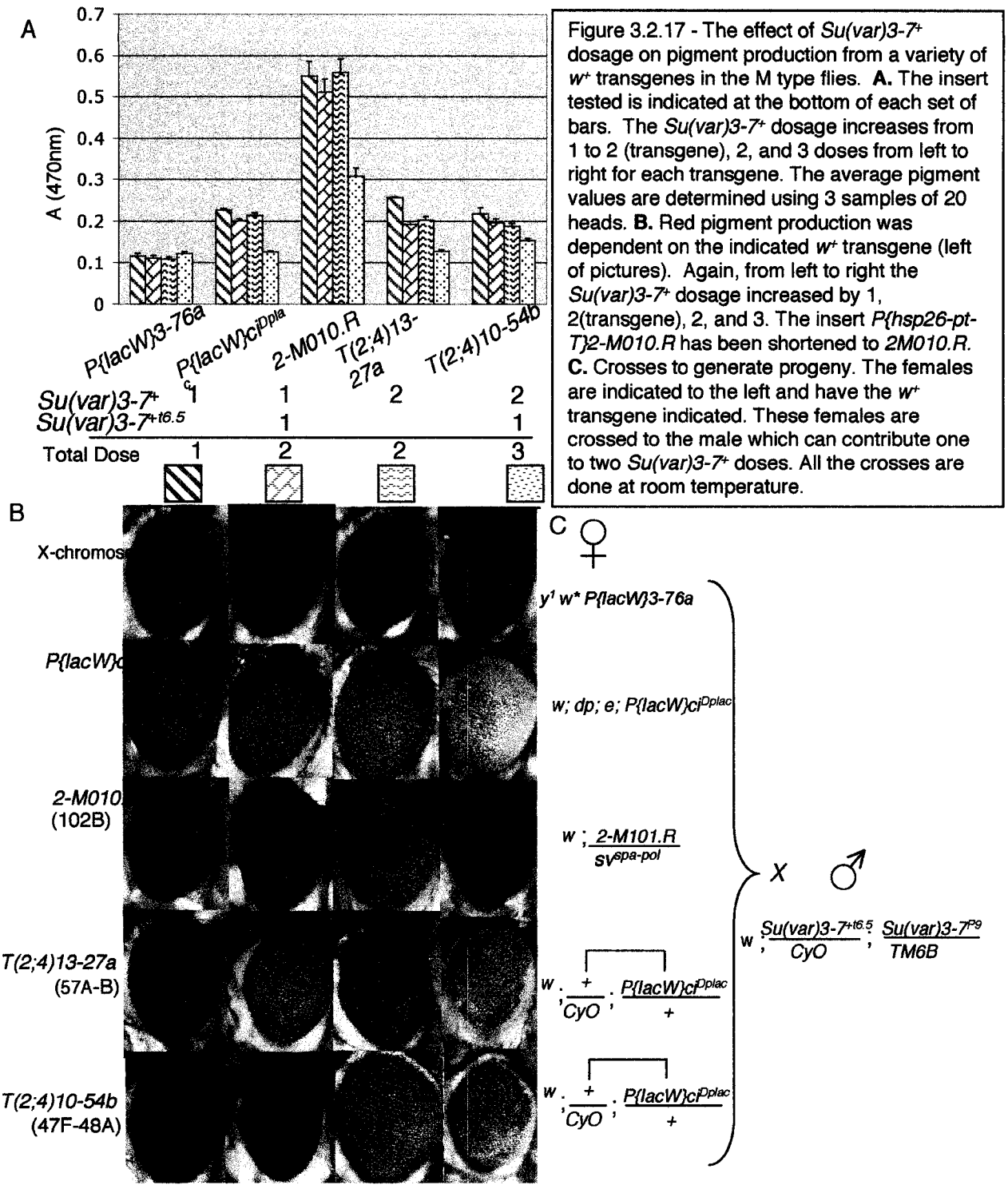


Figure 3.2.17 - The effect of *Su(var)3-7*$^{+}$ dosage on pigment production from a variety of *w*$^{+}$ transgenes in the M type flies. **A.** The insert tested is indicated at the bottom of each set of bars. The *Su(var)3-7*$^{+}$ dosage increases from 1 to 2 (transgene), 2, and 3 doses from left to right for each transgene. The average pigment values are determined using 3 samples of 20 heads. **B.** Red pigment production was dependent on the indicated *w*$^{+}$ transgene (left of pictures). Again, from left to right the *Su(var)3-7*$^{+}$ dosage increased by 1, 2(transgene), 2, and 3. The insert *P{hsp26-pt-T}2-M010.R* has been shortened to *2M010.R*. **C.** Crosses to generate progeny. The females are indicated to the left and have the *w*$^{+}$ transgene indicated. These females are crossed to the male which can contribute one to two *Su(var)3-7*$^{+}$ doses. All the crosses are done at room temperature.

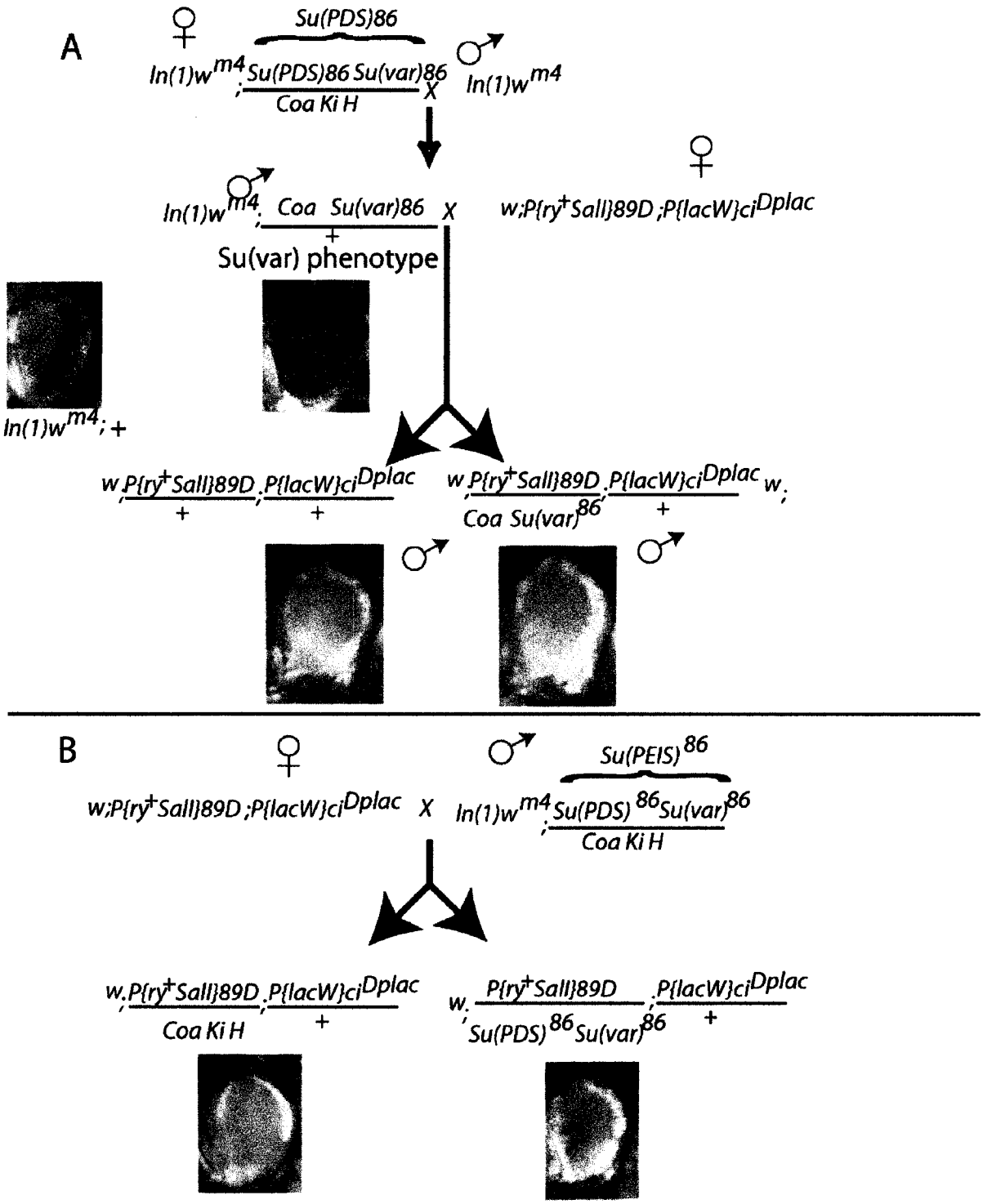


Figure 3.2.18 - Crossing scheme to generate *Su(var)P86*. **A.** The original *Su(PDS)P86* chromosome was crossed to a *In(1)w[m4], w[m4]* stock to find recombinants that could suppress *w[m4]* but not PDS. **B.** The original *Su(PDS)P86* chromosome suppressed PDS.

3.3 Two *P{lacW}ci^{Dplac}* alleles that are variably silenced in M type flies

During my examination of gene silencing at *P{lacW}ci^{Dplac}* in M strains, I recovered two spontaneous mutants that had a variegated eye phenotype in M strains instead of the usual full red eye. I investigated these alleles because they indicated that the *P{lacW}ci^{Dplac}* transgene was sensitive to variegation in the absence of *P* elements. Genetic analysis found that in both cases the variegated phenotype of the two alleles segregated with the *P{lacW}ci^{Dplac}* chromosome and both lines lacked *P* elements (other than the transgene itself). The alleles, called *P{lacW}ci^{E1}* and *P{lacW}ci^{E2}*, produced eyes that displayed red pigment variegation when heterozygous over a wild type chromosome 4 (Figure 3.3.1) In combination with *P{ry^{+} SalI}89D*, the *w^{+mC}* expression was completely silenced in both *P{lacW}ci^{E}* alleles as indicated by a completely white eye.

3.3.1 *Su(PDS)* mutants suppress the *P{lacW}ci^{E}* alleles

All the *Su(PDS)* mutants isolated in the screens above suppressed the *P{lacW}ci^{E}* alleles. All the *Su(var)2-5* mutant alleles weakly suppressed the *P{lacW}ci^{E}* alleles (Figure 3.3.1). The original *Su(PDS)P86* chromosome suppresses variegation from the *P{lacW}ci^{E}* alleles whereas the *Coa Su(var)P86* recombinant, derived from *Su(PDS)P86*, that acted as a Su(var) but no longer suppressed PDS, had no affect on the *P{lacW}ci^{E}* alleles(Figure 3.3.1). I assume the original *Su(PDS)* mutations was responsible for the suppression. *Su(var)3-7^{P9}* and *Su(var)3-7^{P13}* strongly suppressed the *P{lacW}ci^{E}* alleles (Figure 3.3.2-3). Together these results indicated that the same heterochromatic modifiers that dose dependently cause silencing at *P{lacW}ci^{Dplac}* in the presence of *P* elements also caused variable silencing of the *P{lacW}ci^{E}* alleles. Therefore, the *cis* acting *gypsy* element insertions (see below) appeared to trigger an equivalent heterochromatic change as the *trans*-acting *P* elements.

3.3.2 The insertion of a *gypsy* element is responsible for the *P{lacW}ci^{E}* alleles.

Molecular analysis of both *P{lacW}ci^{E}* alleles was undertaken and began with a genomic Southern blot probed with the *P* element sequences to see if there was a gross change in the *P* or adjacent sequences. A summer student, Christopher Oates, using Southern Blot analysis, found that that both *P{lacW}ci^{E1}* and *P{lacW}ci^{E2}* had a sequence alteration about 1 kbp from *P{lacW}ci^{Dplac}* towards *rpS3a*. PCR amplification and DNA sequencing found that both *P{lacW}ci^{E}* alleles had a *gypsy* element insertion that accounted for the change in restriction band sizes on the blot (Figure 3.3.4). Since the appearance of both of these insertions coincided with the silencing phenotype in each allele, I assumed that these insertions caused the variable silencing of *w^{+mC}* in both *P{lacW}ci^{E}* alleles.

Many *gypsy* induced mutations can be suppressed by *su(Hw)* and enhanced by *mod(mdg4)* (GDULA *et al.* 1996). I tested the *su(Hw)*

combinations *su(Hw)*^{2}/*su(Hw)*^{3} (Figure 3.3.5), *su(Hw)*^{2}/*su(Hw)*^{5} (Figure 3.3.6), *su(Hw)*^{2}/*su(Hw)*^{8} (Figure 3.3.7), and *su(Hw)*^{3}/*su(Hw)*^{8} (Figure 3.3.8) and found they weakly suppressed or did not affect the variegated phenotype produced by either *P{lacW}ci*^{E} allele. Also, the *su(Hw)*^{3}/*su(Hw)*^{5} combination did not alter the variegated phenotype of *P{lacW}ci*^{E1} (Figure 3.3.9). However, as a control, *su(Hw)*^{2}/*su(Hw)*^{3} (Figure 3.3.10), *su(Hw)*^{3}/*su(Hw)*^{5} (Figure 3.3.10), *su(Hw)*^{2}/*su(Hw)*^{5} (Figure 3.3.10), and *su(Hw)*^{2}/*su(Hw)*^{8} (Figure 3.3.12) did suppress known *su(Hw)* dependent mutations: *lozenge*^{1} (*lz*^{1}) and *diminutive* (*dm*^{1}) (MODOLELL *et al.* 1983). The *lz*^{1} allele is a recessive mutation that produces narrow irregular eye facets. The *dm*^{1} allele is a recessive mutant that produces small and slender bristles and body. I also tested an allele of *modifier of mdg4* (*P{PZ}mod(mdg4)*^{03582}) and found it weakly enhanced silencing from these *P{lacW}ci*^{E} alleles (Figure3.3.13). With either the *su(Hw)* or *mod(Mdg4)* mutations, the phenotype was too weak to map genetically (unlike the *Su(PDS)* mutations) and determine whether it segregates with the associated mutation. Modifiers on the same chromosome as the mutations studied could cause the weak changes to w^{+mC} expression. The weak response to *su(Hw)* indicated that the variegated phenotype produced by the *P{lacW}ci*^{E} alleles did not depend on the insulator function previously ascribed to *gypsy* element insertions.

3.3.3 Transvection occurs between *w*$^+$ transgenes inserted in the *ci* regulatory region

In the M type, *P{lacW}ci*^{Dplac} produced a full red eye, but when heterozygous with either *P{lacW}ci*^{E} allele, the eye was variegated (Figure 3.3.14). This demonstrated that both *P{lacW}ci*^{E} alleles silenced w^{+mC} expression not only in *cis* but also on the homolog containing the original *P{lacW}ci*^{Dplac} allele – a *trans*-acting function. By-itself, *trans*-silencing did not affect the expression from *P{hsp26-pt-T}ci*$^{2\text{-}M1021.R}$ because most *P{hsp26-pt-T}ci*$^{2\text{-}M1021.R}$/ *P{lacW}ci*^{E} heterozygotes had weak or no variegation in their eyes. The *P{hsp26-pt-T}ci*$^{2\text{-}M1021.R}$ insert was only a 140 bp distant from *P{lacW}ci*^{Dplac}.

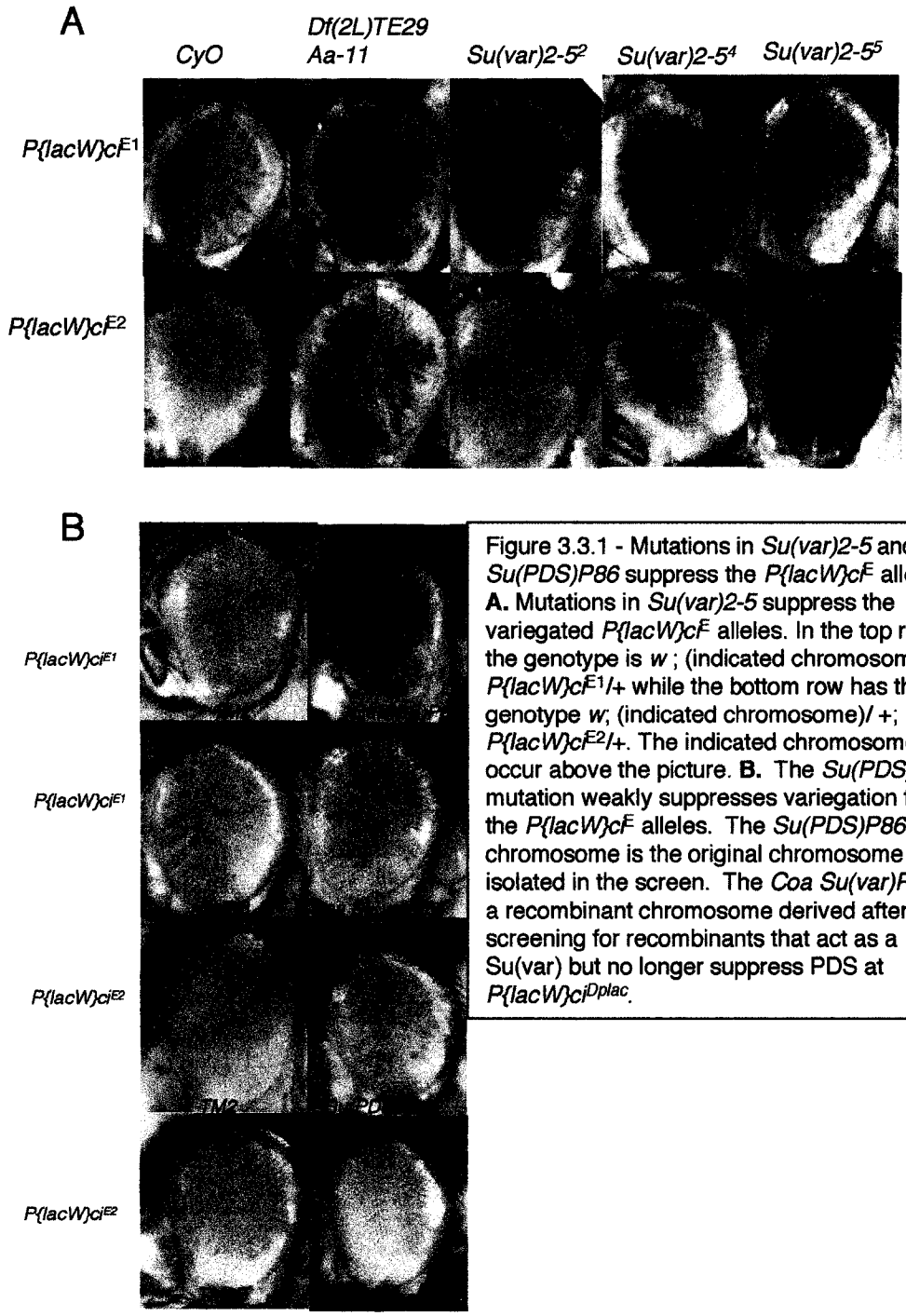


Figure 3.3.1 - Mutations in *Su(var)2-5* and *Su(PDS)P86* suppress the *P{lacW}cI*^{E} alleles. **A.** Mutations in *Su(var)2-5* suppress the variegated *P{lacW}cI*^{E} alleles. In the top row the genotype is *w* ; (indicated chromosome)/+; *P{lacW}cI*^{E1}/+ while the bottom row has the genotype *w*; (indicated chromosome)/ +; *P{lacW}cI*^{E2}/+. The indicated chromosomes occur above the picture. **B.** The *Su(PDS)86* mutation weakly suppresses variegation form the *P{lacW}cI*^{E} alleles. The *Su(PDS)P86* chromosome is the original chromosome isolated in the screen. The *Coa Su(var)P86* is a recombinant chromosome derived after screening for recombinants that act as a Su(var) but no longer suppress PDS at *P{lacW}ci*^{Dplac}.

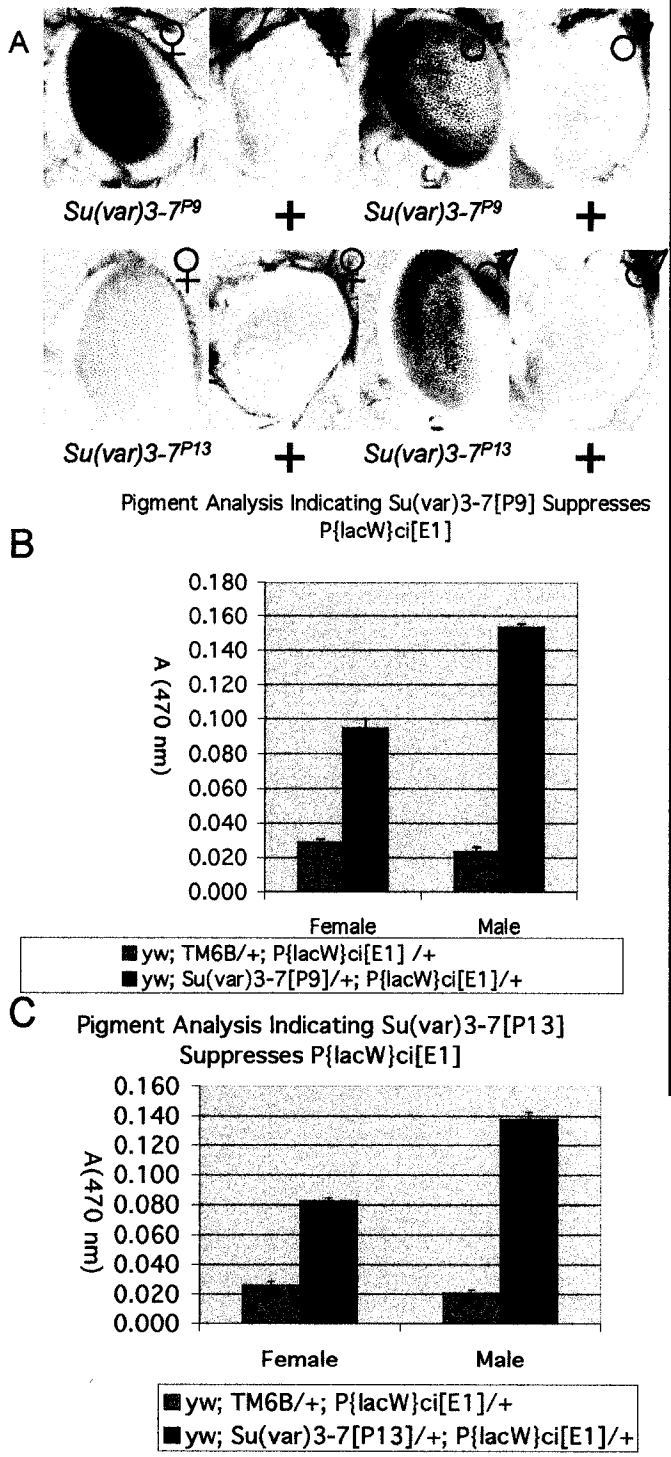
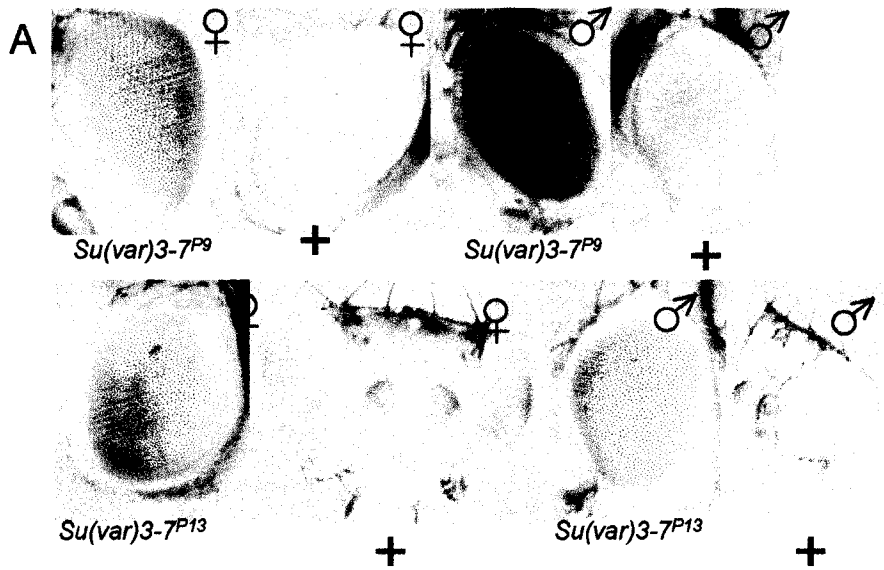


Figure 3.3.2 - Mutations in *Su(var)3-7* strongly suppress the *P{lacW}ci*^{E1} allele. **A.** Pictures of eyes with the following genotype *w*; allele/+; *P{lacW}ci*^{E1}/+. The *Su(var)3-7* allele is indicated below each picture. A (+) indicates a wild type allele. The sex of each fly is indicated in the right hand corner of each picture. **B.** Comparing pigment production in *Su(var)3-7*^{P9}/ + to wild type males and females when *P{lacW}ci*^{E1} is present. The values represent the average of 3 samples of 10 heads each. **C.** Comparing pigment production in *Su(var)3-7*^{P13}/ + to wild type males and females when *P{lacW}ci*^{E1} is present. The values represent the average of 3 samples of 10 heads each.



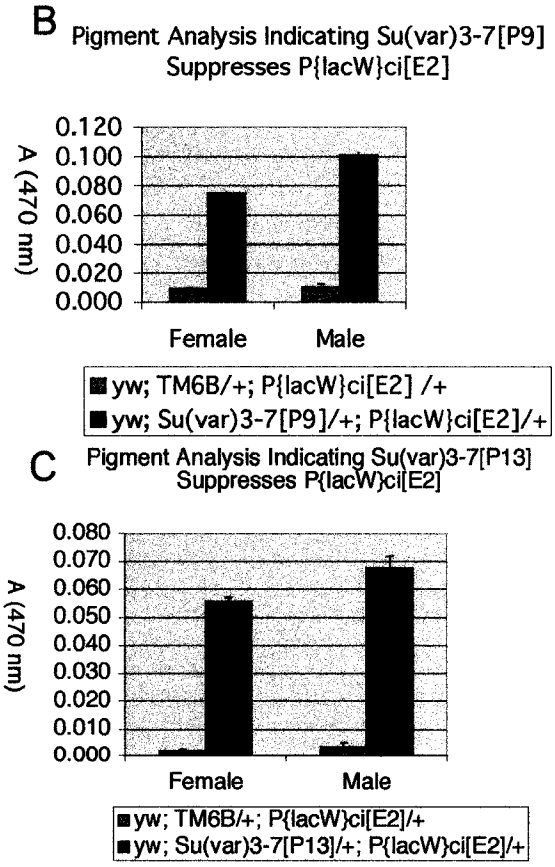


Figure 3.3.3 - Mutations in *Su(var)3-7* strongly suppress the *P{lacW}ci*^{E2} allele. **A.** Pictures of eyes with the following genotype *w*; allele/+; *P{lacW}ci*^{E2}/+. The *Su(var)3-7* allele is indicated below each picture. A (+) indicates a wild type allele. The sex of each fly is indicated in the right hand corner of each picture. **B.** Comparing pigment production in *Su(var)3-7*^{P9}/ + to wild type males and females when *P{lacW}ci*^{E2} is present. The values represent the average of 3 samples of 10 heads each. **C.** Comparing pigment production in *Su(var)3-7*^{P13}/ + to wild type males and females when *P{lacW}ci*^{E1} is present. The values represent the average of 3 samples of 10 heads each.

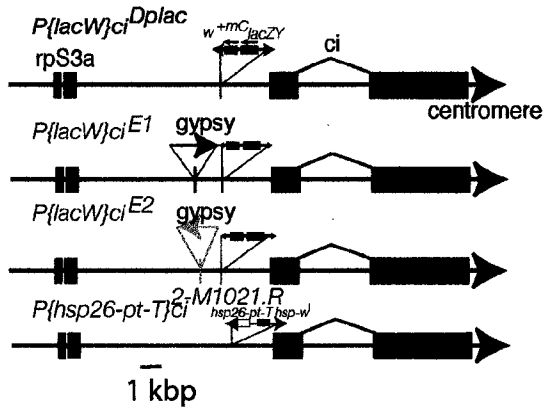


Figure 3.3.4- Location of the *gypsy* insertions in the *P{lacW}ci*^{E} alleles. *P{lacW}ci*^{E1} has a gypsy element inserted between a four base pair duplication (TACA) starting at 81294 (AE003854). *P{lacW}ci* ^{E2} has a gypsy element inserted between a four base pair duplication (TATA) starting at 80747 (AE003854). The arrows indicate the direction of the gypsy transcript.

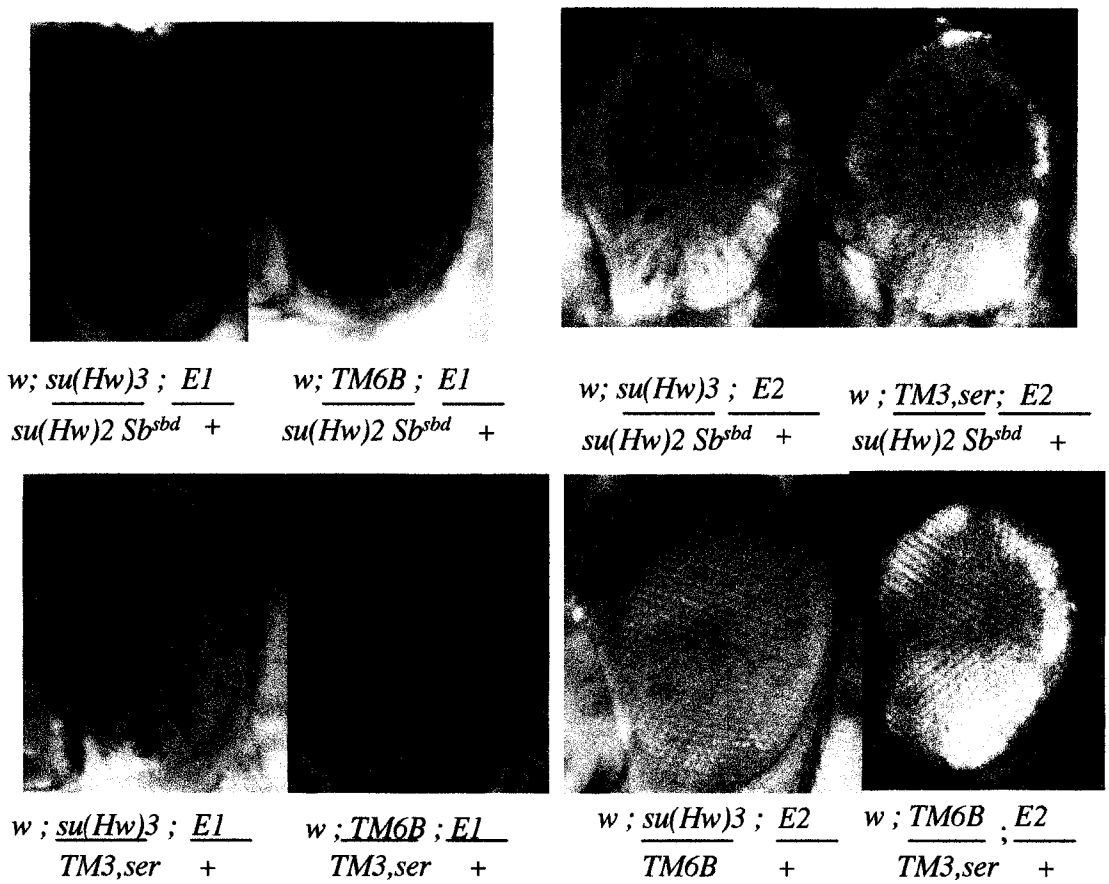


Figure 3.3.5 - *su(Hw)*2 and *su(Hw)*3 combinations slightly suppress variegation from the *P{lacW}ci*^{E} alleles. The *P{lacW}ci*^{E1} and *P{lacW}ci*^{E2} are abbreviated *E1* and *E2* in the genotypes underneath each eye picture.

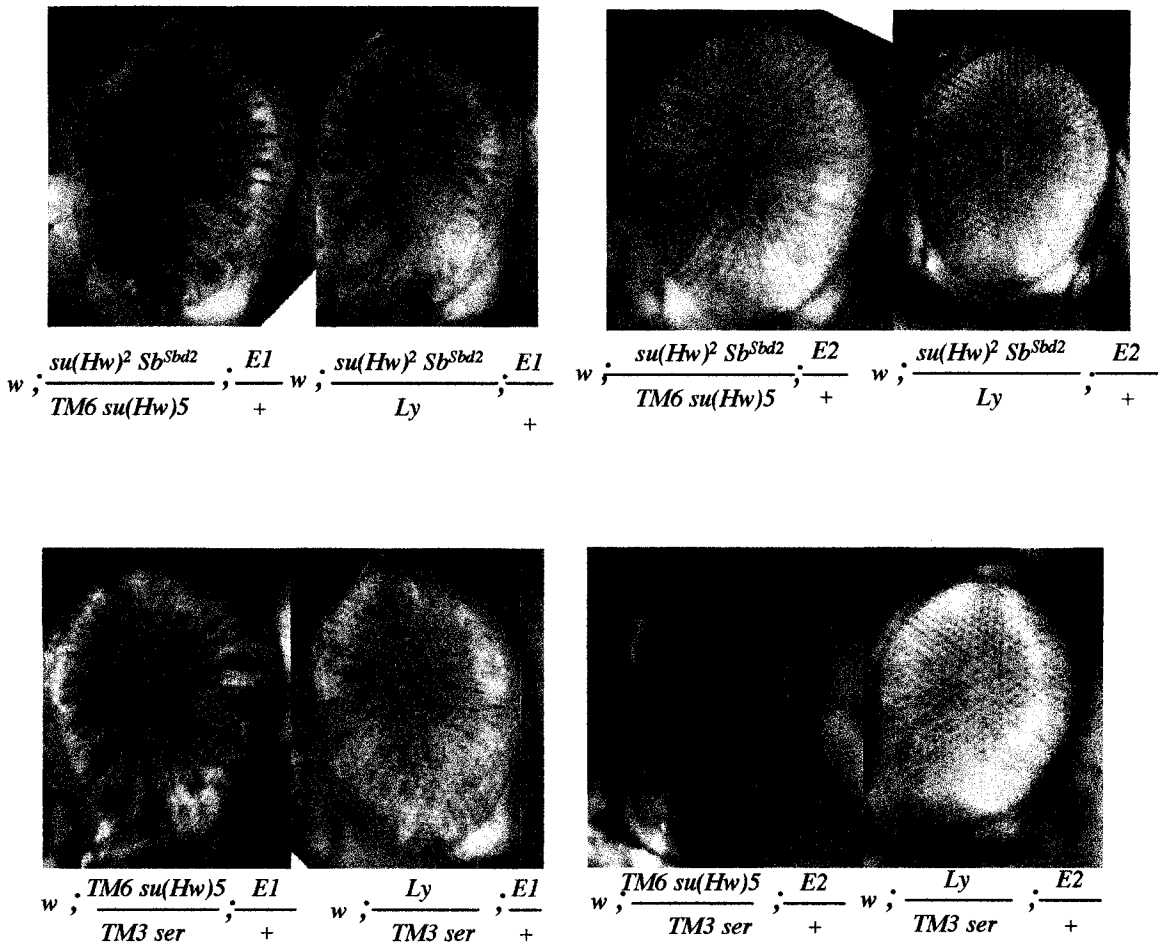


Figure 3.3.6 - *su(Hw)*2 and *su(Hw)*5 combinations do not suppress variegation from the *P{lacW}ci*^{E1} alleles. The *P{lacW}ci*^{E1} and *P{lacW}ci*^{E2} are abbreviated *E1* and *E2* in the genotypes underneath each eye picture.

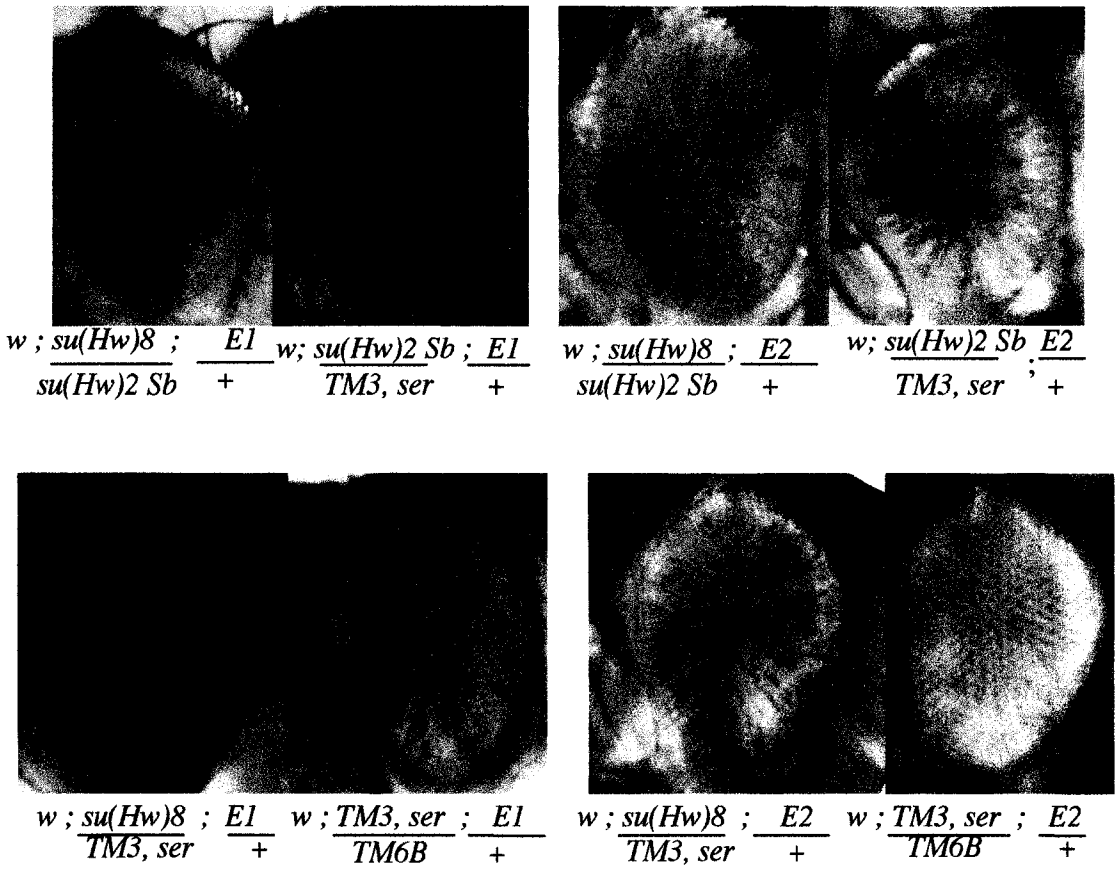


Figure 3.3.7 - *su(Hw)*2 and *su(Hw)*8 combinations slightly suppress variegation from the *P{lacW}ci*^{E} alleles. The *P{lacW}ci*^{E1} and *P{lacW}ci*^{E2} are abbreviated as E1 and E2 in the genotypes underneath each eye picture. The *Sb* allele is *Sb*^{Sbd2}.

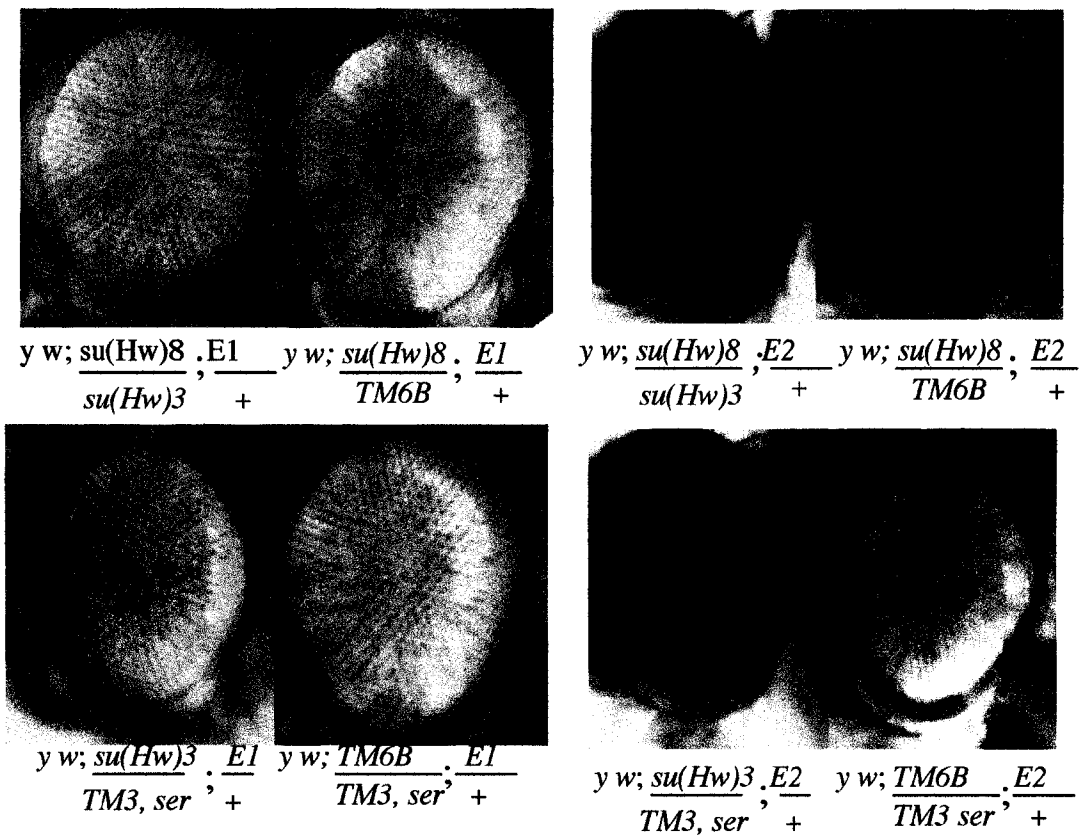


Figure 3.3.8 - *su(Hw)*3 and *su(Hw)*8 combinations do not suppress variegation from *P{lacW}ci*^{E1} but slightly suppress variegation from the *P{lacW}ci*^{E2} alleles. The *P{lacW}ci*^{E1} and *P{lacW}ci*^{E2} are abbreviated *E1* and *E2* in the genotypes underneath each eye picture.

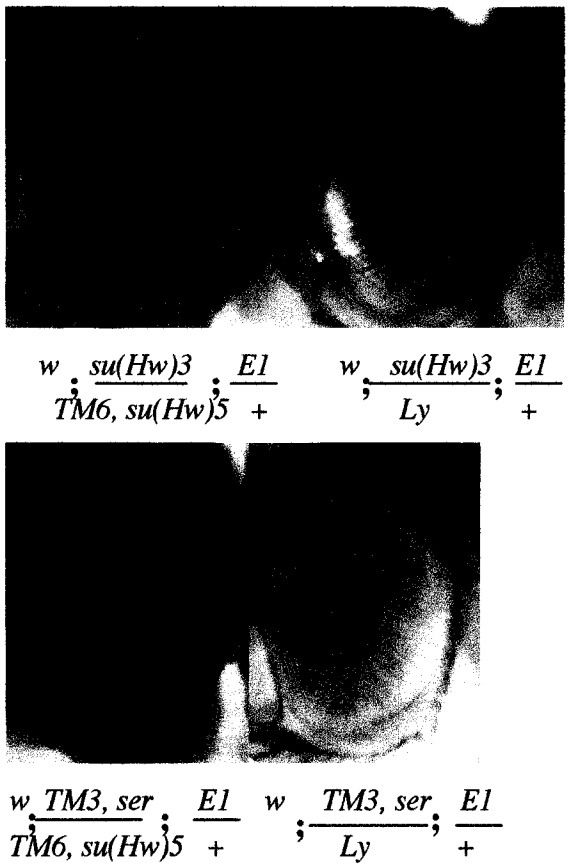


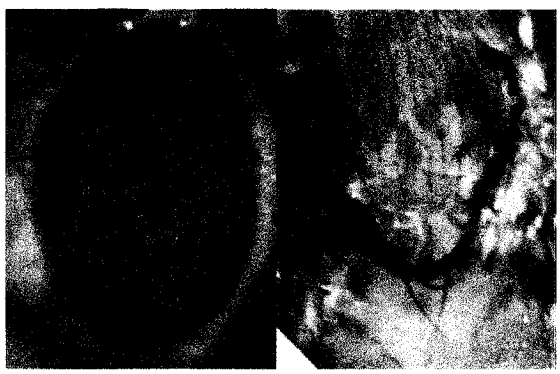
Figure 3.3.9 - The *su(Hw)3* and *su(Hw)5* combinations slightly suppress variegation from the *P{lacW}ci*^{E1} alleles. The *P{lacW}ci*^{E1} is abbreviated *E1* in the genotypes for each eye underneath each eye picture.

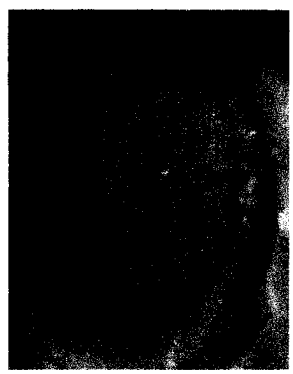
$y^1\ ac(Hw)^1\ dm^1\ lz^1$; $\frac{su(Hw)^3}{TM6,\ Ubx\ su(Hw)^5}$ $y^1\ ac(Hw)^1\ dm^1\ lz^1$; $\frac{su(Hw)^3}{su(Hw)^2\ Sb^{sbd2}}$

$y^1\ ac(Hw)^1\ dm^1\ lz^1$; $\frac{TM6,\ Ubx\ su(Hw)^5}{TM6B}$ $y^1\ ac(Hw)^1\ dm^1\ lz^1$; $\frac{su(Hw)^2\ Sb^{sbd2}}{TM6B}$

Figure 3.3.10 - The *su(Hw)2/su(Hw)3* and *su(Hw)3/su(Hw)5* combination suppresses *lz*^{1} and *dm*^{1} phenotypes in males.

$y^1\ ac(Hw)^1\ dm^1\ lz^1$; $\frac{su(Hw)^2\ Sb^{sbd2}}{TM6,\ su(Hw)^5}$

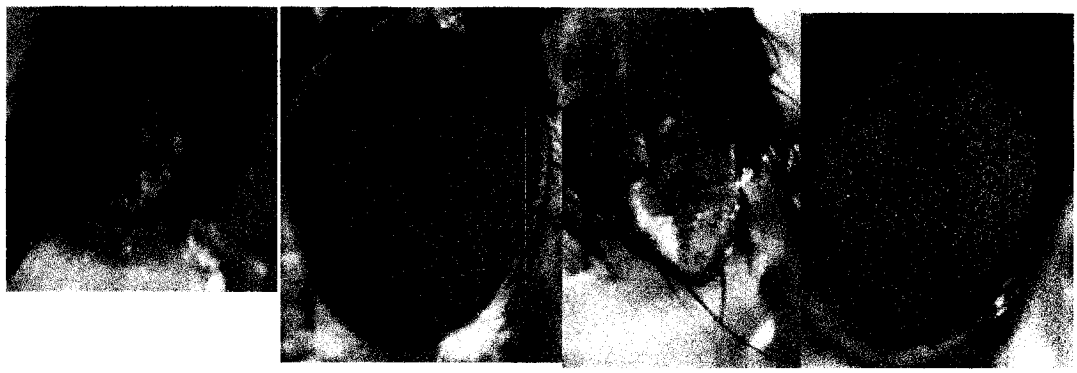




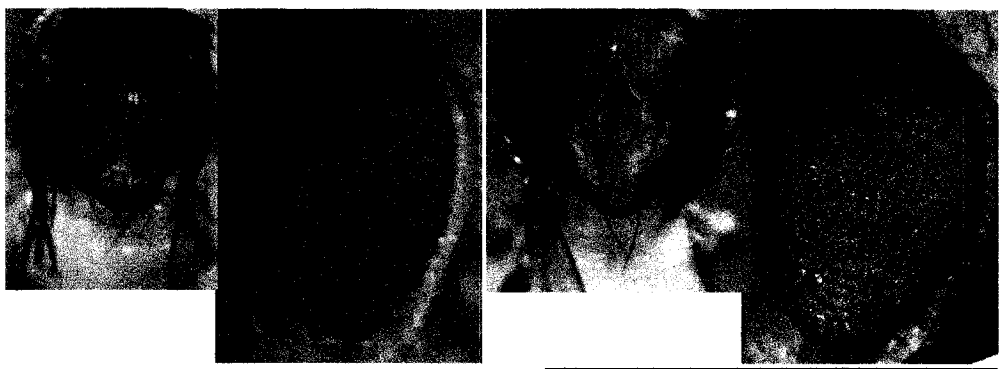
Bristle picture not taken same as Figure 3.2.12

$y^1\ ac(Hw)^1\ dm^1\ lz^1$; $\frac{su(Hw)^2\ Sb^{sbd2}}{TM6B}$ $\qquad$ $y^1\ ac(Hw)^1\ dm^1\ lz^1$; $\frac{TM6\ su(Hw)^5}{TM6B}$

Figure 3.3.11 - $su(Hw)^2/su(Hw)^5$ suppress lz^1 and dm^1 in males. Genotypes are indicated below each picture.

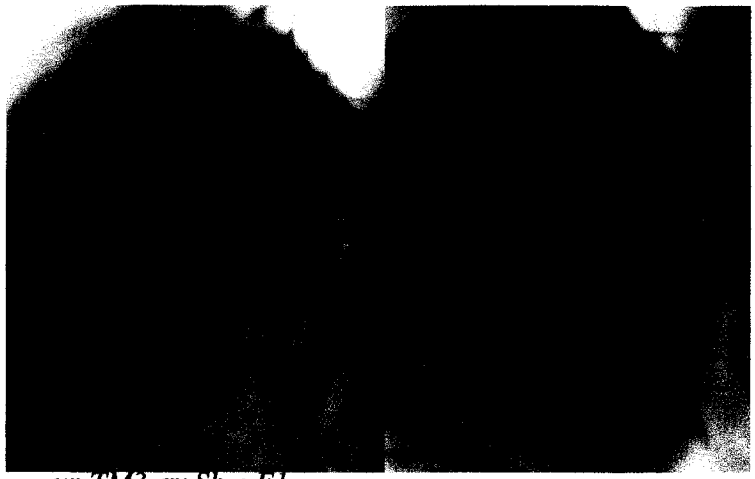


$y^1\ ac(Hw)^1\ dm^1\ lz^1$; $\frac{su(Hw)^2}{su(Hw)^8}$ $y^1\ ac(Hw)^1\ dm^1\ lz^1$; $\frac{TM6\ su(Hw)^5}{su(Hw)^8}$

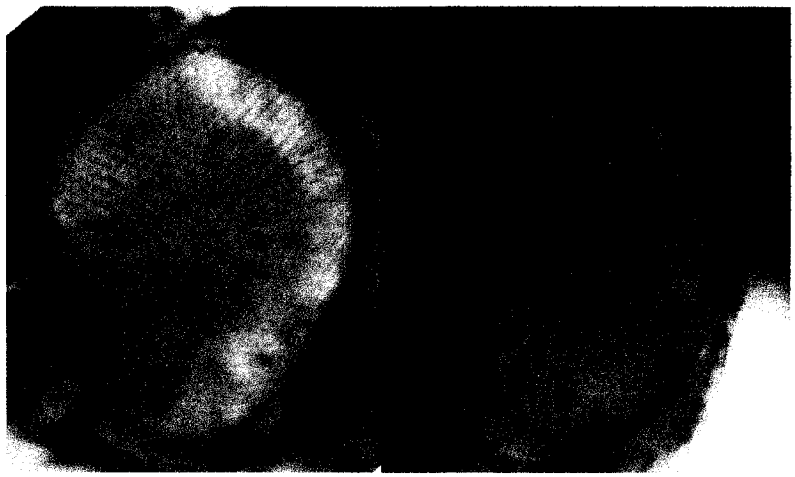


$y^1\ ac(Hw)^1\ dm^1\ lz^1$; $\frac{TM6,\ su(Hw)^5}{TM6B}$ $y^1\ ac(Hw)^1\ dm^1\ lz^1$; $\frac{su(Hw)^2}{TM6B}$

Figure 3.3.12 - su(Hw)^2/ su(Hw)^8 and su(Hw)^5/ su(Hw)^8 suppress *lz*^{1} and *dm*^{1}. The genotype is indicated below each picture.



w; *TM3, ry Sb* / + ; *E1* / + *w*; *ry*^{506} *P{PZ}mod(mdg4)*^{03582} / + ; *E1* / +



w; *TM3, ry Sb* / *e* ; *E2* / + *w*; *ry*^{506} *P{PZ}mod(mdg4)*^{03582} / + ; *E2* / +

Figure 3.3.13 - The *P{PZ}mod(mdg4)*^{03582} allele slightly enhances w^+ variegation from the *P{lacW}ci*^{E} alleles. The genotype is indicated below each picture. The *P{lacW}ci*^{E1} is abbreviated *E1* and *P{lacW}ci*^{E2} is abbreviated *E2*.

Although the *P{lacW}ci*^{E} alleles or *P{ry*$^{+}$ *SalI}89D* alone did not cause strong *w*$^{+}$ variegation from *P{hsp26-pt-T}ci*$^{2\text{-}M1021.R}$, the combination of both factors enhanced *w*$^{+}$ variegation (Figure 3.3.14). By-itself, *P{ry*$^{+}$ *SalI}89D* caused slight variegation of the *hsp70-w*$^{+}$ transgene in *P{hsp26-pt-T}ci*$^{2\text{-}M1021.R}$ (LOCKE *et al.* Submitted), but *w*$^{+mC}$ was more frequently silenced when heterozygous with a *P{lacW}ci* allele in a *P{ry*$^{+}$ *SalI}89D* background (Figure 3.3.14). These results indicated the *trans* effects and *P{ry*$^{+}$ *SalI}89D* were additive in their ability to cause silencing in this region.

To examine chromosomal positions affect on the *trans*-acting silencing of the *P{lacW}ci*^{E} alleles, I crossed the translocations of chromosome 4 containing *P{lacW}ci*^{Dplac} to the *P{lacW}ci*^{E} alleles (Figure 3.3.15-16). The translocations generally inhibited this *trans*-silencing and produced a suppressed phenotype compared to the original *P{lacW}ci*^{Dplac}/ *P{lacW}ci*^{E} heterozygote. This indicated that position or homolog pairing contributed to the *trans*-silencing. The combination of *P{ry*$^{+}$ *SalI}89D* with the translocations and a *P{lacW}ci* allele enhanced *w*$^{+}$ silencing. Neither of these factors alone caused this predominate silencing. Again, these results indicated the silencing of *P{ry*$^{+}$ *SalI}89D* and the *P{lacW}ci*^{E} alleles had an additive effect.

3.3.4 Strong *trans*-silencing correlated with strong *cis*-silencing by the *P{lacW}ci* alleles.

The three *P{lacW}ci* alleles could be ranked on a scale according to their variegated phenotype. On this scale starting at uniformly colored eyes to predominately silenced, the alleles would be ranked in the following order: *P{lacW}ci*^{Dplac}, *P{lacW}ci*^{E1}, and *P{lacW}ci*^{E2}. The *trans*-silencing effect reflects this order, since alleles such as *P{lacW}ci*^{E2}, which were strongly silenced in *cis*, also strongly silenced *P{lacW}ci*^{Dplac}, *P{hsp26-pt-T}ci*$^{2\text{-}M1021.R}$ and translocations of *P{lacW}ci*^{Dplac} in *trans* (Figure 3.3.14-16). There was one exception with *T(3;4)12-1* but otherwise this trend is consistent (compare Figures 3.3.15 and 3.3.16).

3.3.5 *Trans*-silencing only occurs between inserts within the *ci* region

Since translocations of *P{lacW}ci*^{Dplac} suppressed *trans*-silencing, this suggested that silencing was not a global effect that occurred ubiquitously throughout the genome. To test if *trans*-silencing was ubiquitous, *P w*$^{+}$ inserts at different positions throughout the genome were combined with *P{ry*$^{+}$ *SalI}*89D and the *P{lacW}ci*^{Dplac} alleles. When *P{lacW}ci*^{Dplac} and *P{ry*$^{+}$ *SalI}89D* was present, expression from *P{lacW}* inserts on chromosome X, 2, and 3 were not silenced (Figure 3.3.17). Dr. John Locke generated the *P{lacW}* inserts tested by mobilizing *P{lacW}ci*^{Dplac} using *Δ2-3*. In some cases, variegation from the *P{lacW}ci*^{Dplac} construct was seen but the coloration expected from the *P{lacW}* construct being tested was consistently expressed (Figure 3.3.17 [arrows]). Thus, the *P{lacW}ci*^{Dplac} and *P{ry*$^{+}$ *Sal}89D* combination did not affect expression from the other *w*$^{+}$ inserts.

I tested whether *trans*-silencing can occur between *P{lacW}ci*^{Dplac} or *P{lacW}ci*^{E1} and other w^+ inserts on chromosome 4. The w^+ transgenes in *P{hsp26-pt-T}39C-34* or *P{hsp26-pt-T}39C-12* variegated and the presence of *P* elements, such as *P{ry*$^+$ *SalI}89D*, suppressed this variegation (Figure 1.1.1) (HANNA S. PERSONAL COMMUNICATION). When *P{lacW}ci*^{Dplac} was heterozygous with either *P{hsp26-pt-T}39C-34* or *P{hsp26-pt-T}39C-12*, this did not change the variegated w^+ expression pattern from these transgenes (Figure 3.3.18). This result indicated that *P{lacW}ci*^{Dplac} did not *trans*-silence other w^+ transgenes on chromosome 4. However, heterozygous *P{hsp26-pt-T}39C-34/ P{lacW}ci*^{E1} flies consistently had less colored ommatidia compared to *P{hsp26-pt-T}39C-34/ ey*^{D} siblings when *P{ry*$^+$ *SalI}89D* was present (Figure 3.3.18 [B arrows]). This indicated that *trans*-silencing was occurring between *P{lacW}ci*^{E1} and the w^+ transgene in *P{hsp26-pt-T}39C-34*. However, w^+ expression from another chromosome 4 insert, *P{hsp26-pt-T}39C-12*, was not affected by the presence of *P{lacW}ci*^{E1}. Together, these results indicate that *trans*-silencing from *P{lacW}ci* alleles did not consistently occur throughout the nucleus but affected one other w^+ transgene on chromosome 4.

3.3.6 Factors that modify hPEV similarly modify w^{+mC} expression from the three *P{lacW}ci* alleles

Since an increased *Su(var)2-5*$^+$ and *Su(var)3-7*$^+$ dose silenced w^{+mC} expression from the *P{lacW}ci* alleles, the w^{+mC} insert behaved as if it was inserted in a heterochromatic region. However in one respect, this region behaved differently from classical heterochromatin because only a few *Su(var)*s enhanced w^{+mC} silencing. To determine if this region behaved like classical heterochromatin in respected to other modifiers, I examined the effect other well-characterized conditions, such as rearing temperature and an extra Y-chromosome, had on variegation. An increased rearing temperatures and an extra Y-chromosome suppressed w^+ variegation from classic heterochromatic regions, such as w^{m4} (PIRROTTA and RASTELLI 1994; SPOFFORD 1976).

Increasing the rearing temperature suppressed variegation in *P{ry*$^+$ *SalI}89D; P{lacW}ci*^{Dplac} individuals (Figure 3.2.12). I wanted to compare this response to M-type flies, which had no w^{+mC} variegation and therefore should not be heterochromatic. From *P{lacW}ci*^{Dplac}, the amount of pigment increased at higher rearing temperatures in the absence of *P* elements (Figure 3.3.19). These contrary responses to temperature suggested that *P* elements induced a fundamental change in chromatin structure, which alters sensitivity to temperature. This change in sensitivity matches the sensitivity to other heterochromatic regions, such as w^{m4}.

I also tested whether increasing the rearing temperature suppressed variegation with the *P{lacW}ci*^{E} alleles. I crossed females from the *P{lacW}ci*^{E} stocks to a *w; Su(var)3-7*$^{+t6.5}$*/ CyO; Su(var)3-7*^{P9}*/ TM2* male to generate flies with 1, 2, and 3 *Su(var)3-7*$^+$ doses that were reared at temperatures ranging from 18°C to 29°C. Increasing the rearing temperature weakly suppressed variegation from the *P{lacW}ci*^{E} alleles

in M type flies compared to the *P{ry⁺ SalI}89D ; P{lacW}ci*^{Dplac} combination (Figure 3.3.20). This weak suppression appeared to result from the weak heterochromatic silencing associated with *P{lacW}ci*^{E} alleles (M type) rather than a difference in the chromatin structure that caused silencing when *P{ry⁺ SalI}89D* was present. In terms of w^{+mC} expression, the *P{lacW}ci*^{E} alleles fell between the uniform expression from *P{lacW}ci*^{Dplac} and predominate silencing seen when *P{ry⁺ SalI}89D* was present. Increasing the temperature caused contradictory affects on w^{+mC} expression from *P{lacW}ci*^{Dplac} depending on the heterochromatic state within this region (presence/absence of P elements). Since the *P{lacW}ci*^{E} alleles fell between these states, the contradictory responses may cancel each other and this was the reason there was no visible change in variegation. Only when w^{+mC} silencing predominated and most of the ommatidia were white did increased rearing temperature increase w^{+mC} expression from *P{lacW}ci*^{Dplac}.

An extra Y-chromosome suppressed classic hPEV presumably by titrating out SU(VAR)s (SPOFFORD 1976). I generated flies with an extra Y-chromosome by crossing females with the *P{lacW}ci* allele to *C(1;Y)6,w*^{118} males (Figure 3.3.21 [A]). An extra Y-chromosome in female progeny weakly suppressed the variegated phenotype observed when *P{ry⁺ SalI}89D* caused silencing at *P{lacW}ci*^{Dplac} (Figure 3.3.21 [B]). Also, an extra Y-chromosome suppressed the variegated eye phenotype with both *P{lacW}ci*^{E1} and *P{lacW}ci*^{E2}. When *P{ry⁺ SalI}89D* was present, in combination with either *P{lacW}ci*^{E} allele, all progeny had predominately white ommatidia and there was no visible difference between X/XY and X/0 progeny. Presumably, an extra Y did not suppress the additive effects between *P{ry⁺ SalI}89D* and the silencing already occurring with the *P{lacW}ci*^{E} alleles. These results found that all three *P{lacW}ci* alleles respond similarly to factors that suppressed variegation at other heterochromatic regions.

3.3.7 *Su(PDS)* mutants suppress w^{m4} variegation

If our *Su(PDS)* mutants were deficient for the same function as *Su(var)* alleles then they should also suppress hPEV. Pigment analysis of our *Su(PDS)* derived mutants showed that they suppressed the classic PEV allele, *In(1)w*^{m4}, w^{m4} (referred to as w^{m4})(Figure 3.3.22). Both *Su(var)2-5* mutant alleles strongly suppressed w^{m4} variegation. However, our *Su(var)3-7* mutant alleles varied widely in their ability to suppress variegation, despite being mutant in the same gene. Both *Su(var)3-7*^{P9} and *Su(var)3-7*^{P12} acted as strong Su(var)s while *Su(var)3-7*^{P25}, *Su(var)3-7*^{P43}, *Su(var)3-7*^{P47}, and *Su(var)3-7*^{P71} did not suppress variegation. Both *Df(3)126c* and *Df(3R)ry615*, which delete *Su(var)3-7*(87E), also did not produce a Su(var) phenotype. Since other groups had reported that deficiencies that include 87E produce a Su(var) phenotype (REUTER *et al.* 1987), I assumed that other antagonistic PEV modifiers within these stocks were masking the effect.

To determine if temperature was affecting the variegation and confirm that mutations in *Su(var)3-7* did not consistently suppress w^{m4},

Su(var)3-7^{P9} and *Df(3R)126c* were crossed to w^{m4} stocks and raised at 25°C instead of 21°C. Again, the pigment analysis did not show a significant difference between flies that inherited the balancer and mutation when reared at 25°C (Figure 3.3.23).

3.3.8 w^{+mC} expression from the *P{lacW}* arrays is sensitive to the same heterochromatic modifiers that suppress PDS

In addition to *P{lacW}ci*^{Dplac}, *P* elements enhanced variegation from the *P{lacW}* arrays, *BX2* and *T1* (JOSSE *et al.* 2002). The w^+ transgenes within these tandem repeats were variably silenced and mutations in *Su(var)2-5*$^+$ suppressed this variegation (DORER and HENIKOFF 1994; DORER and HENIKOFF 1997). Since *Su(var)2-5*$^+$ also acted at *P{lacW}ci*^{Dplac}, a factor produced by *P* elements could interact with a similar chromatin structure found at *P{lacW}ci*^{Dplac} and the *P{lacW}* arrays. I extended this list and tested whether the *P{lacW}* arrays were sensitive to *Su(var)3-7*$^+$ dosage.

I found that increasing the *Su(var)3-7*$^+$ dosage strongly enhanced variegation from *BX2* and *T1* (Figure 3.3.24 [A]). This added another common chromatin factor that acted at both *P{lacW}ci*^{Dplac} and at the *P{lacW}* repeats. The P factor could enhance heterochromatic silencing at both *P{lacW}ci*^{Dplac} and within these arrays by interacting with a common heterochromatic structure found within both regions.

I wanted to determine whether a limited set of heterochromatic modifiers acted at *P{lacW}ci* and the *P{lacW}* repeats. I tested *Su(var)3-9* mutant alleles to determine their affect on w^+ expression from these loci. I specifically chose *Su(var)3-9* because it strongly suppressed variegation of w^{m4} and its protein associates with the chromocenter like SU(VAR)3-7 and HP1. *Su(var)3-9*^{1} and *Su(var)3-9*^{2} weakly suppressed variegation at *T1* and *BX2*, when compared to their wild type siblings (Figure 3.3.24 [B]). Neither *Su(var)3-9* allele modified w^{+mC} expression from the *P{lacW}ci* alleles. These results confirmed that a subset of *Su(var)*s dose-dependently suppressed variegation at loci where PDS occurred. These loci could appear insensitive to other *Su(var)s* because at these variegating loci the *Su(var)* dosage tested was not limiting or the product did not act at these loci to cause variegation.

In addition to *Su(var)3-9* mutants, I tested and found that *Su(var)3-4* weakly suppressed PDS at the *P{lacW}* arrays and *P{lacW}ci* (Figure 3.3.25). Mutations in this gene were not detected in my *Su(PDS)* screens possibly because of a weak effect. Therefore other modifiers possibly exist that weakly suppressed variegation at *P{lacW}ci*^{Dplac}. However, my *Su(PDS)* screen detected several alleles of two loci, indicating all the strong suppressors were identified.

3.3.9 Testing whether mutations that affect the RNA pathway also affect w^{+mC} expression from loci where PDS occurs

To test whether the RNAi pathway was involved in PDS at *P{lacW}ci*^{Dplac}, *T1*, and *BX2*. Mutations in *piwi* (*P{PZ}piwi*^{66843}) and *argonaute*^{1} (*P{PZ}AGO1*^{04845}) were crossed to *BX2*, *P{lacW}ci*^{E} alleles, *T1*

and *P{lacW}ci*^{Dplac} when *P{ry*$^{+}$ *SalI}SalI}89D* was present. In each case, males with either *P{PZ}piwi*^{66843} or *P{PZ}AGO1*^{04845} balanced over *CyO* were crossed to *w*$^{-}$ stocks that had one of the *w*$^{+mC}$ transgenes and male progeny were examined. With *P{lacW}ci*^{Dplac} and *BX2*, the *piwi* or *AGO1* mutations did not modify the variegated phenotype produced by these *w*$^{+mC}$ transgenes in the male progeny studied.

However, *T1* showed an interaction with *P{PZ}AGO1*^{04845} that reduced viability, enhanced variegation, and caused a Minute-like phenotype. In a cross between male *P{PZ}AGO1*^{04845} / *CyO* to female *w; T1/ Cy*, the number of progeny produced was 76 Cy females, 52 Cy male, 2 Cy+ females and 1 Cy+ male. The expected number of Cy+ progeny was 1/2 the Cy progeny in this cross. Furthermore, the Cy+ (*P{PZ}AGO1*^{04845}*/T1*) progeny had a Minute-like phenotype with short narrow bristles and reduced number of colored ommatidia compared to its Cy^{-} siblings.

While an absence of a dominant interaction by *piwi* and *ago* does not prove that RNAi was not involved, it suggested that silencing was not dependent on the gene dose of these loci.

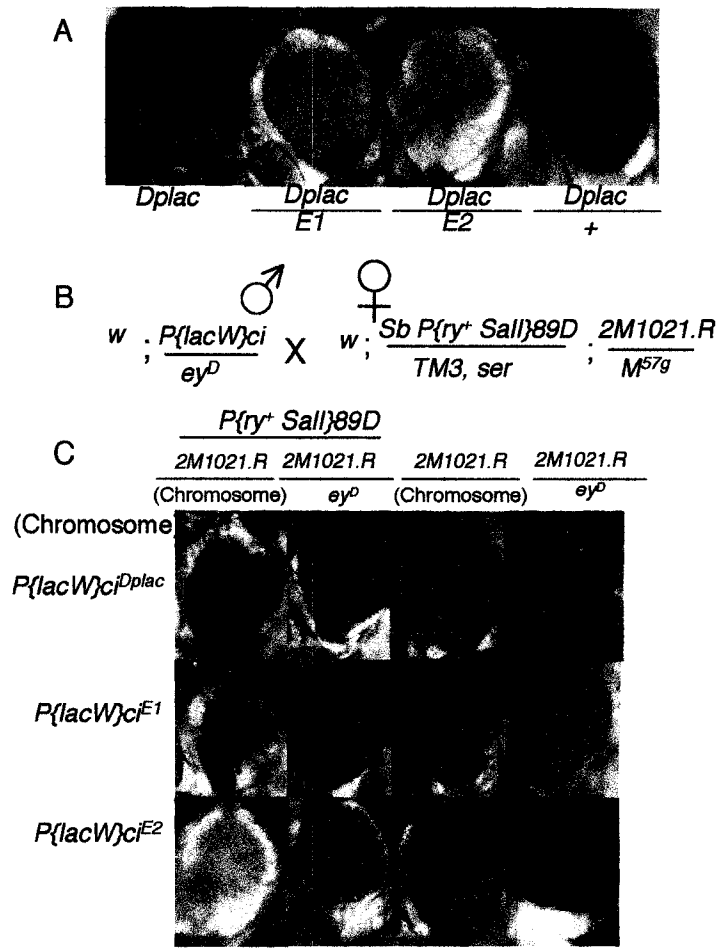


Figure 3.3.14 - *Trans*-silencing between *P{lacW}ci* derivatives and other inserts in the *ci* region. **A.** *P{lacW}ci*^{E} alleles *trans*-silence *w*$^{+mC}$ expression from the original *P{lacW}ci*^{Dplac} chromosome. Pigment produced depended solely on the *P{lacW}* allele inherited. The abbreviation for the *P{lacW}* is shown below each picture. A (+) indicates a wild type chromosome 4 that does not have a *P{lacW}* insertion. There are no *P* elements present in these flies. **B**.Crossing scheme to generate flies in C. **C.** *Trans*-silencing between *P{lacW}ci*$^{2\text{-}M1021.R}$ and *P{lacW}ci* alleles with and without *P{ry*$^{+}$ *SalI}89D* present As indicated above the first two columns, these flies inherited *P{ry+ SalI}89D* and the the last two columns inherited *TM3, ser.* The chromosome 4 combination is indicated above each column. Each row of pictures either inherited *ey*^{D} or the *P{lacW}ci* allele indicated to the left of each row. The *P{lacW}ci*$^{2\text{-}M1021.R}$ allele is abbreviated as *2M1021.R.*

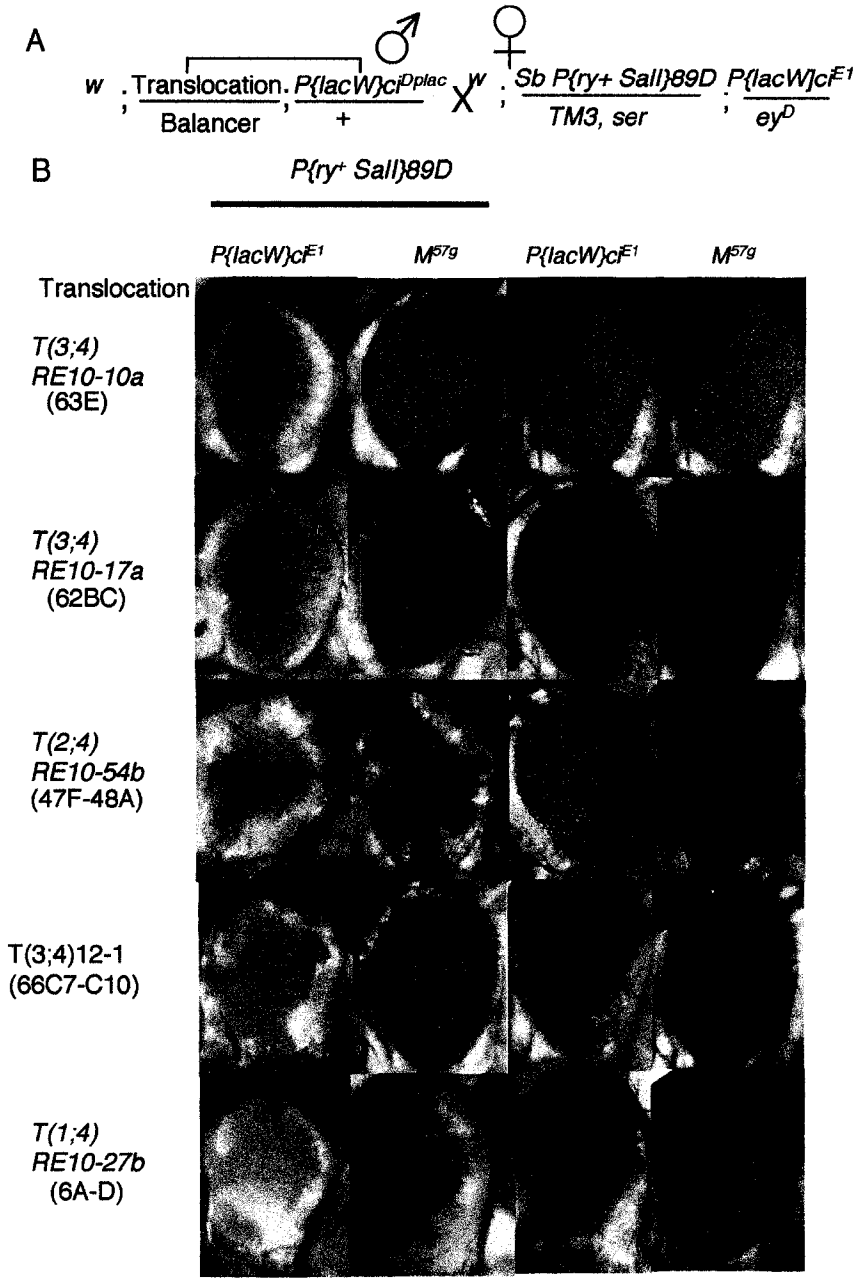


Figure 3.3.15 - *Trans*-silencing between *P{lacW}ci*^{E1} and the *P{lacW}ci*^{Dplac} translocations. **A.** Crossing scheme to generate progeny depicted in B. **B.** As indicated above the columns, the flies in the first two columngs inherited *P{ry*$^{+}$ *SalI}(89D)* and in the two last columns inherited *TM3, ser.* The translocation is heterozygous with either *ey*^{D} or *P{lacW}ci*^{E1} as indicated above each column. The translocation is indicated to the left of each row of pictures.

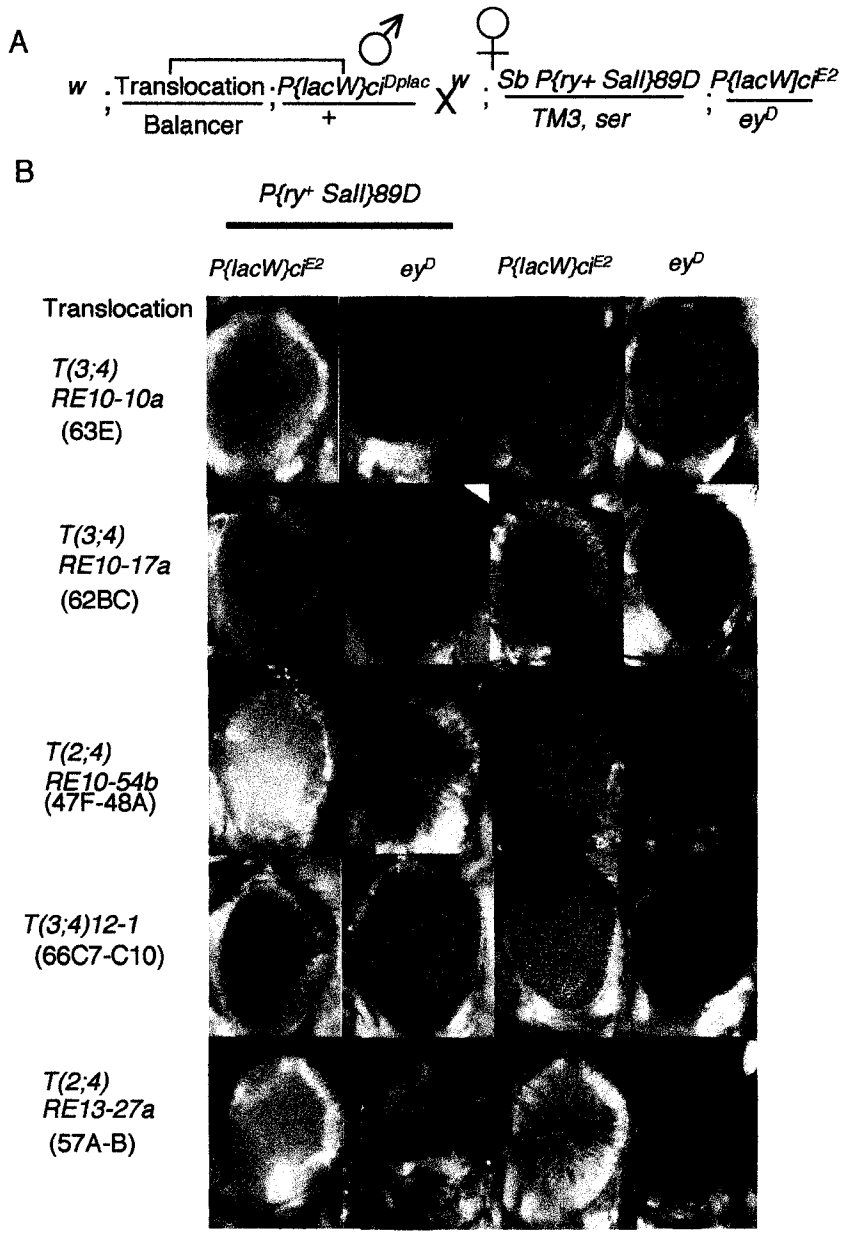


Figure 3.3.16 - *Trans*-silencing between *P{lacW}ci^E2* and the *P{lacW}ci^Dplac* translocations. **A**. Crossing scheme to generate progeny. **B.** As indicated above the columns, the flies in the first two columns inherited *P{ry+ Sall}89D* and in the last two columns inherited *TM3, ser*. The translocation is heterozygous with either *ey^D* or *P{lacW}ci^E2* as indicated above each column. The translocation is indicated to the left of each row of pictures.

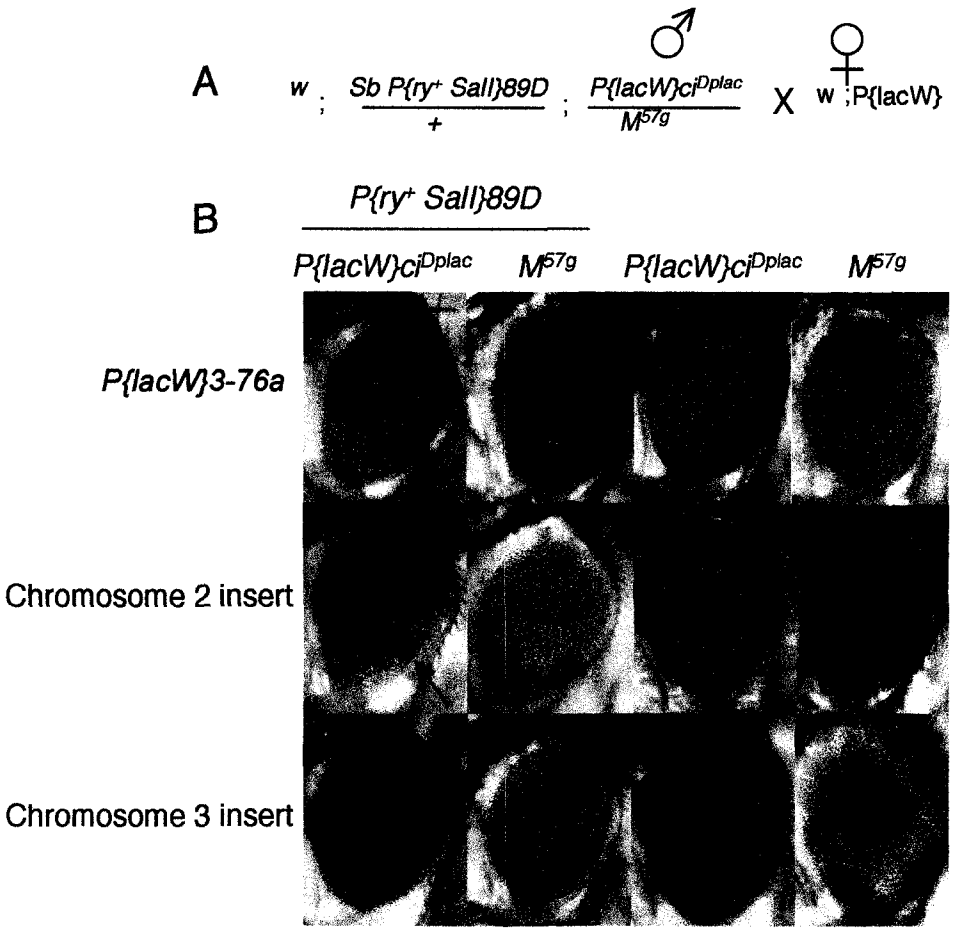


Figure 3.3.17 - The *P{lacW}ci*^{Dplac} allele does not *trans*-silence expression from the other *P{lacW}* inserts. **A** Crossing scheme to generate fly eyes photographed in B. **B.**The first two columns have *P{ry*$^{+}$ *Sal}89D* present and the last two columns have no *P* elements except for the *P* constructs tested. To the left of each row is the *P{lacW}* insert tested. The chromosome 2 and 3 inserts were generated by Dr. John Locke after mobilizing *P{lacW}ci*^{Dplac} using *Δ2-3*. The arrow points to variegation caused by *P{lacW}ci*^{Dplac} rather than than the insert being tested.

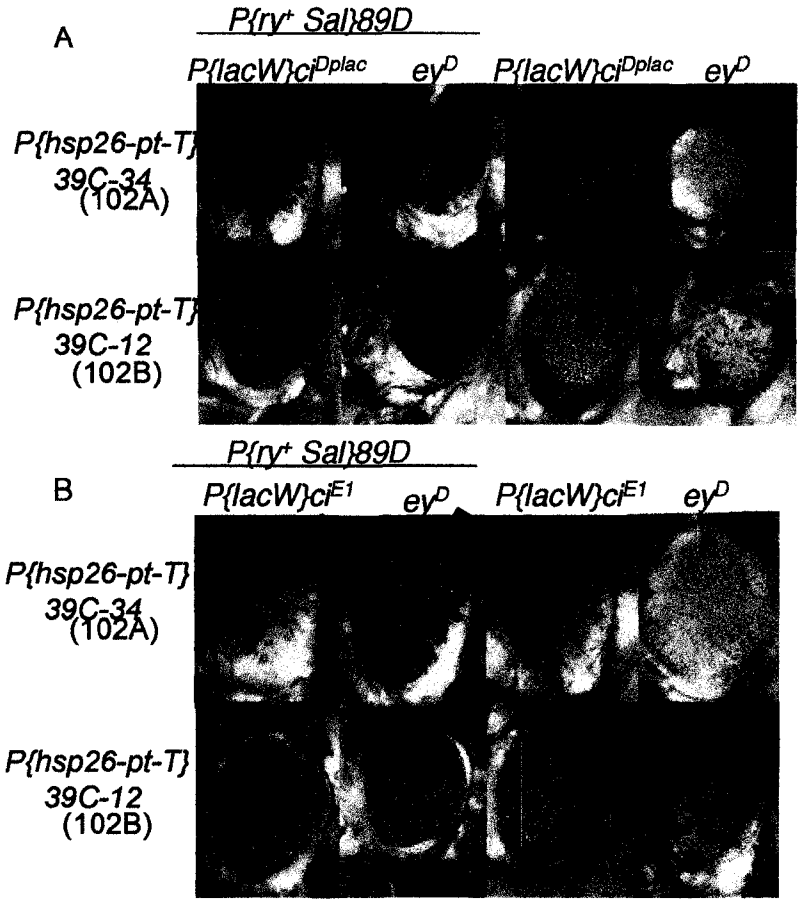


Figure 3.3.18 - *P{lacW}ci^{Dplac}* and *P{lacW}ci^{E1}* inconsistently *trans*-silence *P w$^+$* transgenes on chromosome 4 **A**. When heterozygous with *P{lacW}ci^{Dplac,}* expression from the *w$^+$* transgene in *P{hsp26-ptT}29C-34* (top row) or *P{hsp26-pt-T}39C-12* (bottom row) is not affected. The first two columns have *P{ry$^+$Sal}89D* present while the last two have no other *P* elements present except for the *w$^+$* transgene constructs. Rows that have *P{lacW}ci^{Dplac}* above them are *P{lacW}ci^{Dplac}/ w$^+$* heterozygotes while those that have *ey^D* are *ey^D/ w$^+$* transgene heterozygotes. **B**. When heterozygous with *P{lacW}ci^{E1}* expression from the *w$^+$* transgene from *P{hsp26-ptT}29C-34* (top row) is slightly reduced, and *w$^+$* expression *P{hsp26-pt-T}39C-12* is not affected (bottom row). Labeling and genotypes are the same as in A except *P{lacW}ci^{E1}* is tested rather than *P{lacW}ci^{Dplac}*. The arrows indicate the inconsistency in these results because *w+* expression from the *ey^D / P{hsp26-pt-T}39C-34* heterozygote appears to exceed the *P{lacW}ci^{Dplac}/ P{hsp26-pt-T}39C-34* heterozygote when *P{ry+Sal}89D* is present. This indicates that this combination *trans*-silences expression from the *w$^+$* gene in *P{hsp26-pt-T}39C-34*. The *w$^+$* expression from *P{hsp26-pt-T}39C-12* appears unaffected when *P{lacW}ci^{E1}* is present. To generate the fly eyes in the first row of A, *w; Sb P{ry+ Sal}89D/ TM6B; P{hsp26-pt-T}39C-34* females are crossed to *w; P{lacW}ci^{Dplac}* males. In A second row, these flies are generated by crossing *w; Sb P{ry+ Sal}89D/ TM6B; P{hsp26-pt-T}39C-12* to *w; P{lacW}ci^{Dplac}* males. Flies in B are generated in similar crosses as A except the males are *w; P{lacW}ci^{E1}* rather than *w; P{lacW}ci^{Dplac}*.

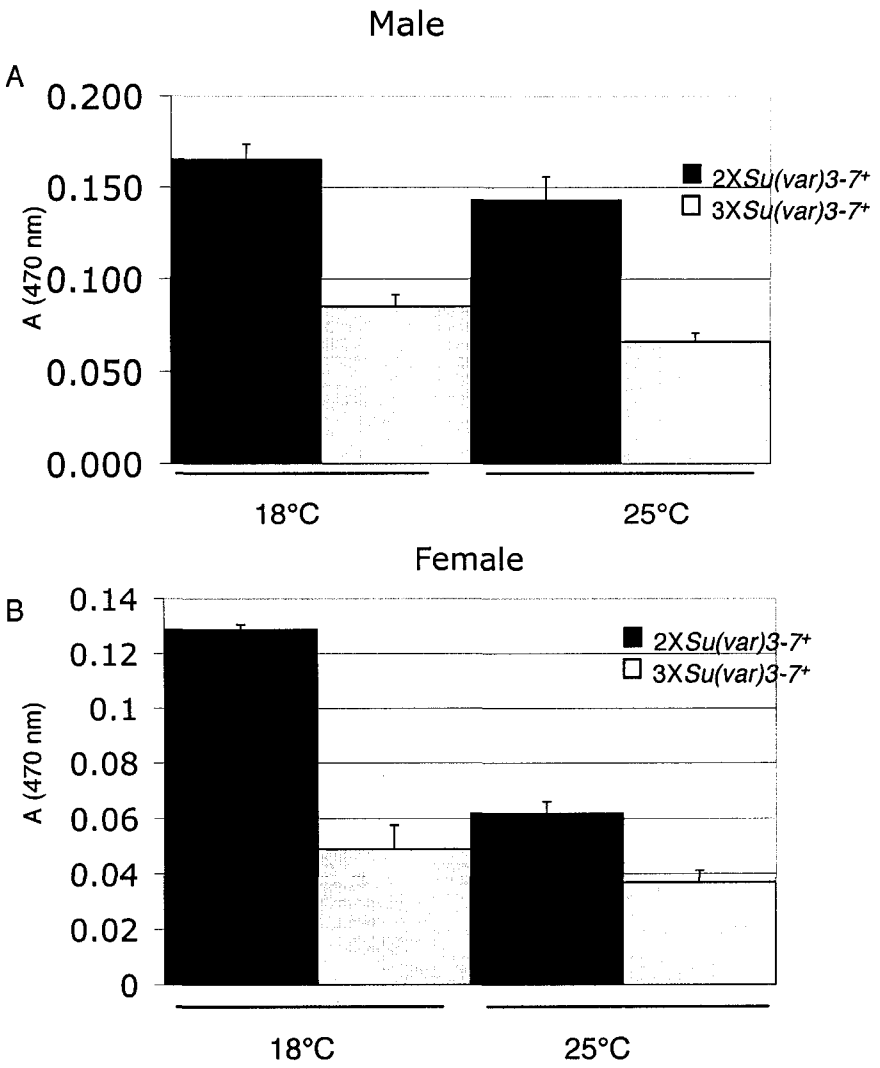


Figure 3.3.19 - As measured by pigment production, increasing the temperature decreases w^{+mC} transgene expression from *P{lacW}ci*^{Dplac} in the absence of *P* elements. Pigment is harvested from flies with the genotypes of *w*; *P{lacW}ci*^{Dplac}/+ (2X*Su(var)3-7*$^{+}$ black bar) and *w*; *Su(var)3-7*$^{+t6.5}$/+; *P{lacW}ci*^{Dplac}/+ (3X*Su(var)3-7*$^{+}$ grey bar) at the temperatures indicated. As indicated below the first two bars on the left, heads are collected from flies reared at 18°C and in the last two bars on the right from flies raised at 25°C. Measurements are the average of 3 samples each containing 20 heads. **A.** Heads are collected from males only. **B.** Heads are collected from females only.

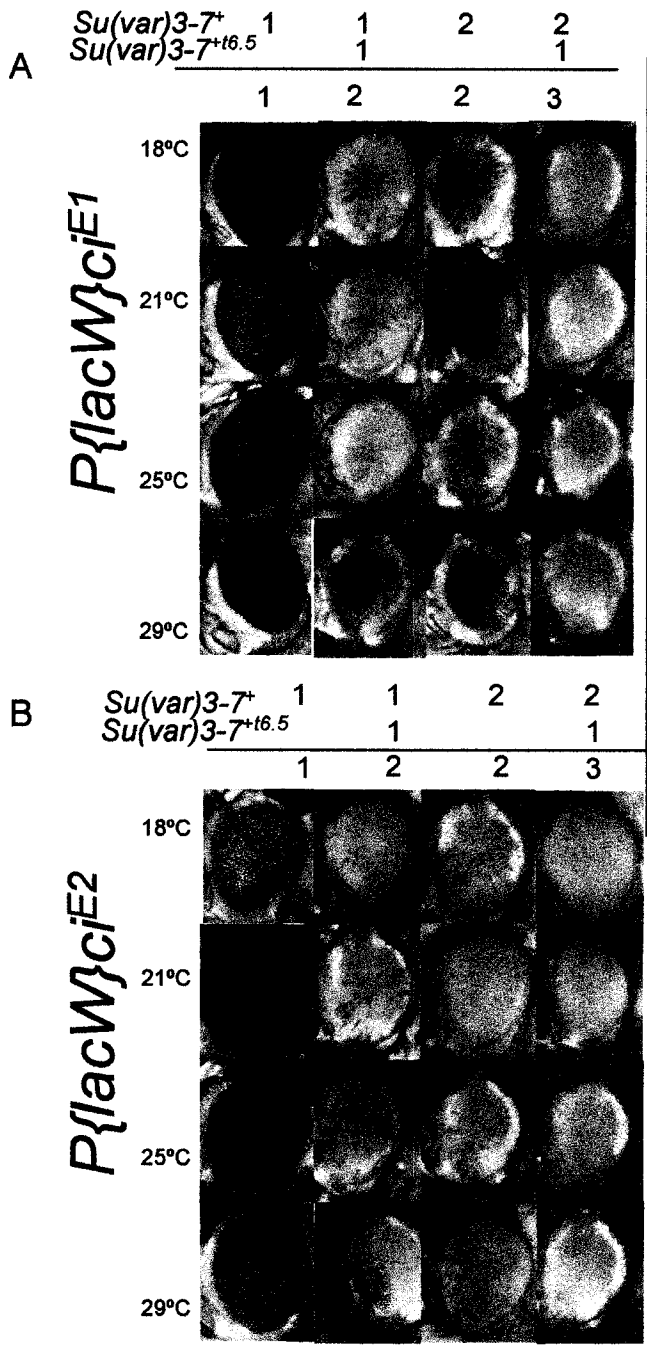


Figure 3.3.20 - Increasing the temperature weakly suppresses variegation from the *P{lacW}ci*^{E} alleles. The *Su(var)3-7*$^{+}$ dose increases from 1,2, 2, and 3 doses from left to right as indicated above each column of pictures. The rearing temperature is indicated to the left of each row of eyes. **A.** Pictures of eyes in which the w^{+mC} gene expression only occurs from *P{lacW}ci*^{E1}. **B.** Pictures of flies in which w^{+mC} gene expression occurs only from *P{lacW}ci*^{E2}.

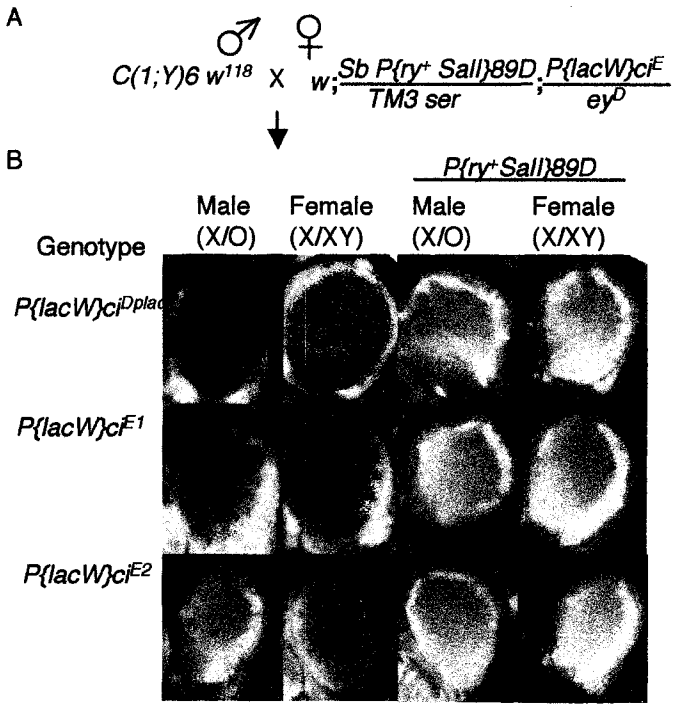


Figure 3.3.21 - An extra Y suppresses the *P{lacW}ci*^{E} alleles. **A.** Crossing scheme describing how the flies with an extra Y chromosome were generated to test the *P{lacW}ci*^{E} alleles. In the case of *P{lacW}ci*^{E1}, *M*^{57g} instead of *ey*^{D} is used as a dominant marker. Also, for *P{lacW}ci*^{Dplac} separate crosses generated the M and *P* type flies. The *C(1;Y)6 w*^{118} male was crossed to a *w; P{ry*$^{+}$ *SalI}89D; P{lacW}ci*^{Dplac} and *w; P{lacW}ci*^{Dplac} females to generate the *P* and M types respectively. **B.** Pictures of fly eyes that have one or zero Y doses as depicted above each column of pictures. The pigment is produced by the allele indicated at the beginning of each row of pictures.

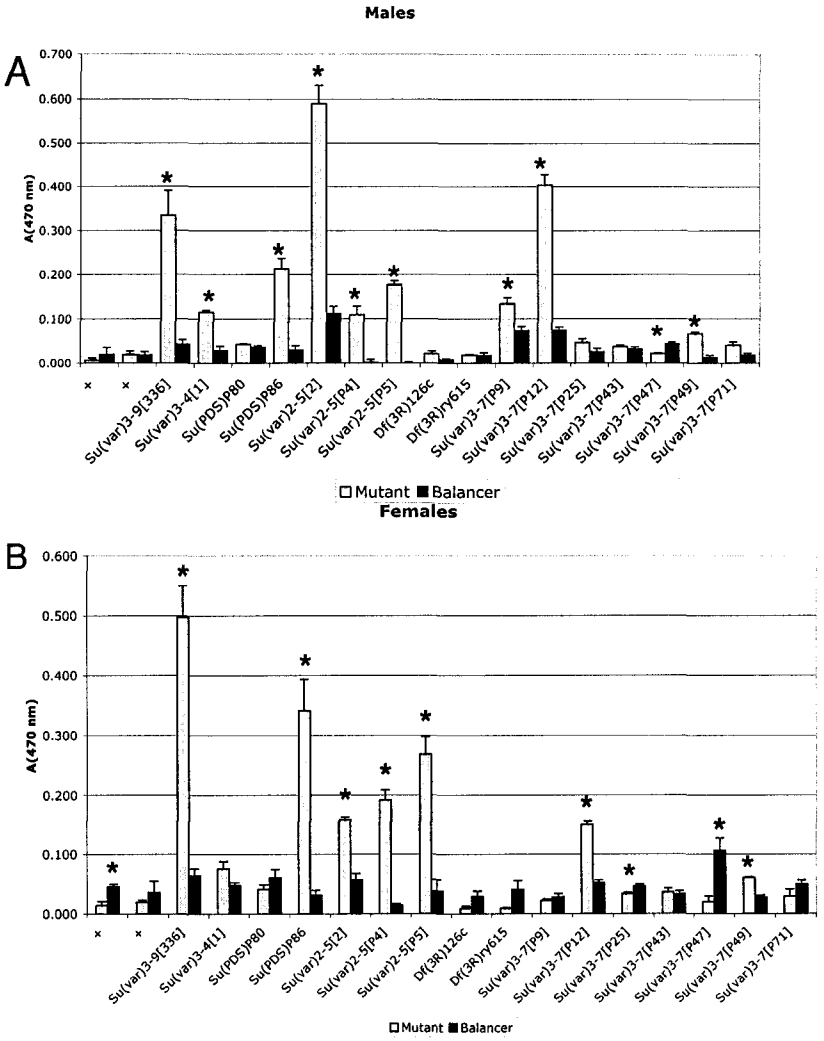


Figure3.3.22 – Pigment analysis testing whether the *Su(PDS)* mutants suppress *In(1)w^{m4}*, *w^{m4}* variegation at room temperature. The balancer chromosomes (black bar) do not substantially increase pigment production compared to wild type chromosomes from Oregon^{R} tested in the first two data sets: *TM2* (first grey bar) and *CyO* (second grey bar). The rest of the data sets compare pigment production between siblings that inherit the mutant chromosome or the balancer. Each measurement represents the average of three different samples and each sample used 20 heads. **A.** This bar graph compares males raised at room temperature (20°C). **B** This bar graph compares females raised at room temperature. See material and methods for a description of the crosses that produced these flies. A * indicates the mean value is significantly different from siblings that inherited the balancer chromosome according to t-test (P>0.05).

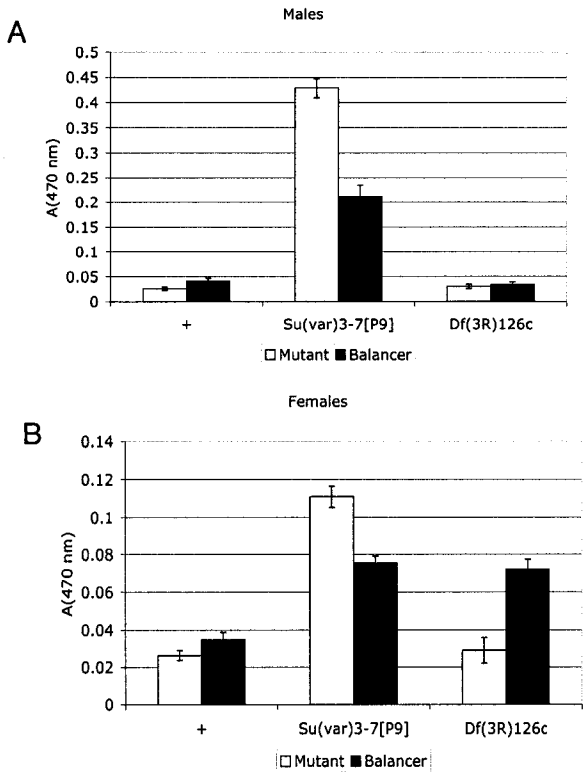


Figure 3.3.23 - Pigment analysis testing the *Su(PDS)* mutants reared at 25°C. Each bar is the average of 3 samples each containing 10 heads. The (+) bars compare a wild type chromosome from Oregon^R (grey bar) to the *TM2* balancer chromosome (black bar). All other bars compare mutant (white) to the same *TM2* balancer. **A.** Bar graph compares males raised at 25°C. **B.** Bar graph compares females raised at 25°C. Crosses to produce these flies are the same as Figure 3.3.22.

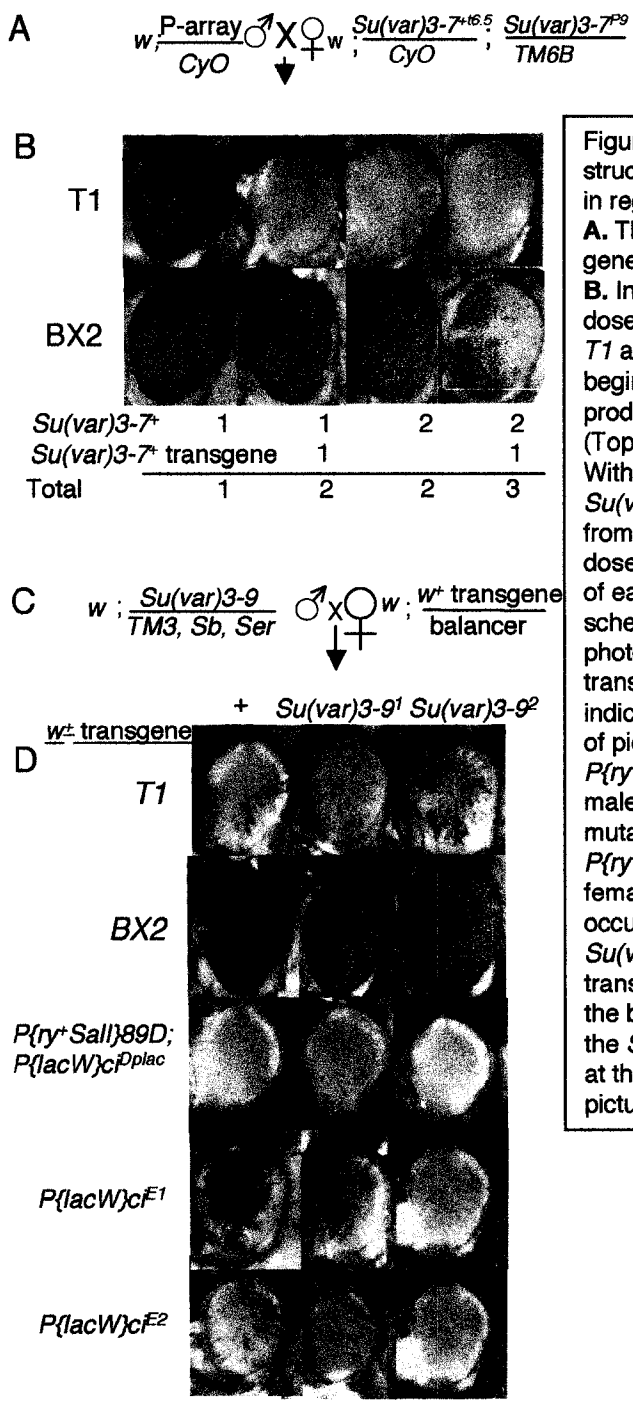


Figure 3.3.24 - Similar chromatin structure silences w^+ transgene in regions where PDS occurs. **A.** The crossing scheme to generate the files depicted in B. **B.** Increasing the *Su(var)3-7*$^+$ dose enhances variegation from *T1* and *BX2*. As labeled at the beginning of each row, pigment production is dependent on *T1* (Top row)and *BX2* (Bottom row). With each successive picture the *Su(var)3-7*$^+$ dosage increases from 1, 2 (transgene), 2, and 3 doses as indicated at the bottom of each column. **C.** Crossing scheme to generate the flies photographed in D. The w^+ transgene being tested is indicated to the left of each row of pictures. In the case of *P{ry*$^+$*Sall}89D; P{lacW}cl*^{Dplac}, males from the *Su(var)3-9*$^-$ mutant stocks are crossed to *w; P{ry*$^+$*Sall}89D; P{lacW}cl*^{Dplac} females. **D.** Loci at which PDS occurs are insensitive to *Su(var)3-9*$^+$ dosage. The w^+ transgenes tested is indicated at the beginning of each row and the *Su(var)3-9* allele is indicated at the top of each column of pictures.

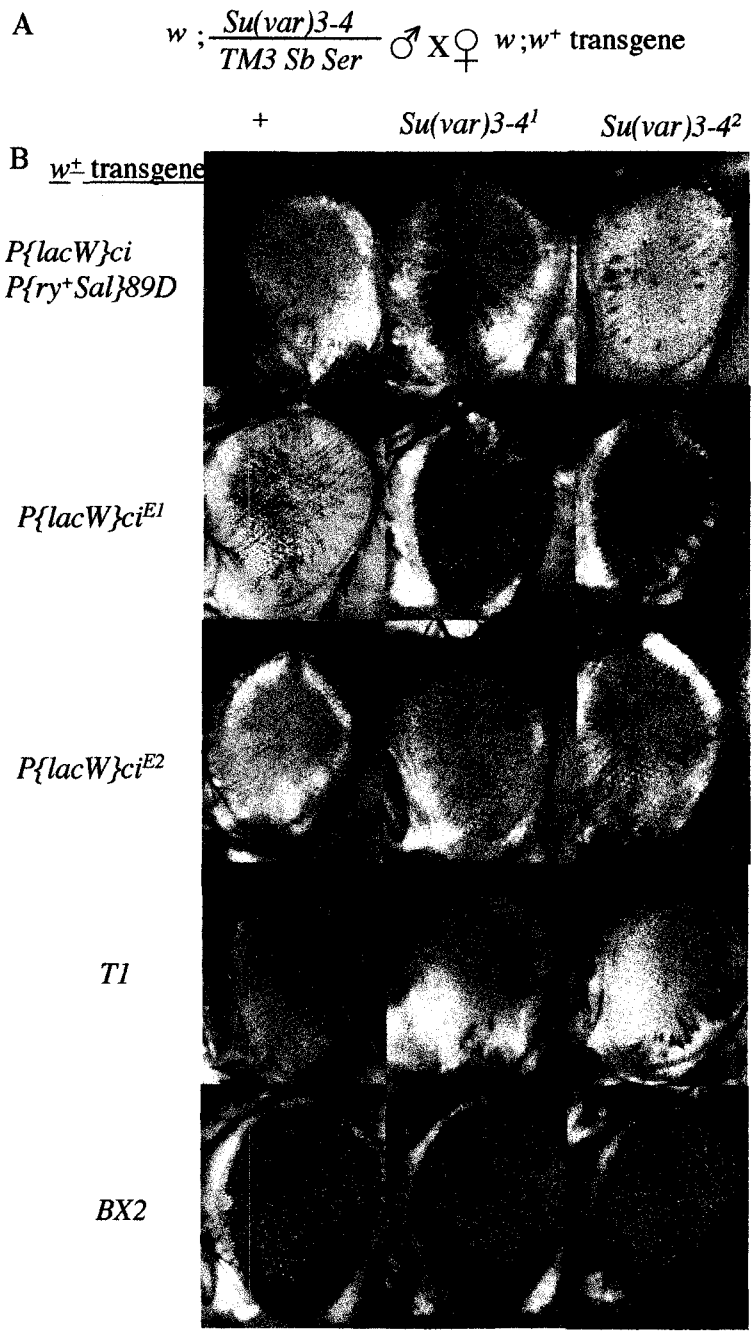


Figure 3.3.25 - *Su(var)3-4* weakly suppresses variegation at *P{lacW}ci* and the *P{lacW}* arrays. **A.** The crossing scheme to generate the flies photographed in B. **B.** The *Su(var)3-4* alleles tested are indicated above each column of eyes. The w^+ transgene tested is indicated before each row of eyes.

3.4 Molecularly characterizing and screening for suppressors of Minute alleles on chromosome 4

As mentioned earlier, a *Δ2-3* mutagenesis of *P{lacW}ci*^{Dplac} had generated a series of Minute mutants on chromosome 4 (HANNA S. PERSONAL COMMUNICATION). *P* elements, such as *P{ry*$^{+}$ *SalI}89D,* suppressed these Minute derivatives suggesting a connection to the PDS at this locus. I further investigated these Minute alleles by looking at the molecular changes caused by the mutagenesis and looking for mutations that suppressed the Minute phenotype.

3.4.1 Southern Blot of Minute Alleles

Southern blot analysis, found that the deletions in the Minute alleles occurred within the *P{lacW}ci*^{Dplac} insert using *Xho* I and *Bgl* II genomic digests from Minute stocks (Figure 3.4.1-2). The probe was isolated from a *Bgl* II digest of cosmid L7 (U66884) and spanned the 6 kbp between the *Bgl* II sites flanking *P{lacW}ci*^{Dplac}. All the bands in the Minute mutants had longer restriction fragments than the wild type bands for this region and this indicated that portions of the *P{lacW}ci*^{Dplac} insert were still there. In the *Bgl* II blot, the 2.2 kbp band was barely discernable but indicated that this restriction fragment had not changed. In the *Xho* I digest, all the bands were bigger than the 11 kbp wild type band from the balancer chromosome. Therefore the *P{lacW}ci*^{Dplac} insert had not been completely excised and portions of the insert remained.

Further analysis by a summer student, Jeff Berger, used primers that anneal within P{lacW} to determine where the breakpoints of the deletions occur. His estimate of the deletions fell within a ~1 kbp of my own estimate based on the Southerns for most of Minute mutants (Table 3.4.1). Only M^{F1} showed a large deviation from my own data. I do not know why there is a difference between our results.

Table 3.4.1: Comparing deletion size estimated from Southern and PCR analysis for the *P{lacW}ci*^{Dplac} derivatives

Stock#	Genotype	*Bgl* II	*Xho* I	*PCR*
$331	M^{A2}/ey^D	6	4	4.4
$332	$M^{B1}/l(4)13$	6.4	4	4.8
$333	M^{B3}/ey^D	7.2	7	6.2
$334	M^{C1}/ey^D	8.3	7	7.4
$335	M^{C2}/ey^D	7.4	6	6
$337	M^{F1}/ey^D	BNV	3	6.2
$338	M^{F2}/ey^D	9.9	9	8.9
$339	M^{G2}/ey^D	BNV	4	3.8
$340	M^{I1}/ey^D	6.4	6	5.4
$341	M^{J1}/ey^D	6.1	BNV	4.4
$342	M^{L1}/ey^D	9.2	8	8.6
$343	M^{M1}/ey^D	8.2	9	7.3

BNV stands for band not visible.

3.4.2 A screen to look for dominant mutations that suppress M^{F2}

I chose one Minute allele, M^{F2}, and screened for dominant mutations that suppressed the M^{F2} phenotype in both *cis* and *trans*. Four different crossing schemes were used to generate mutants (Table 3.4.2). In each crossing scheme, a M^{F2}/ ey^D male, treated with EMS, was crossed to a female homozygous for a marked chromosome 4. This allowed the isolation of a M^{F2} revertant. In screen 2, 3 revertants were isolated: *FR5-1*, *FR6-29*, and *FR7-59*.

Table 3.4.2: Results of a screen for M^{F2} revertants or suppressors

Cross		Scored	Putative	Revertants
1	M^{F2}/ ey^D male X female *y w; ci glv* ey^R *sv*	1048	5	0
2	M^{F2}/ ey^D male X female ci^1 ey^R	4770	11	3
3	M^{F2}/ ey^D male X female M^{F2}/ ey^D	1186	1	0
4	M^{F2}/ ey^D male X female *w; dp; e;* ci^1 ey^R	6604	10	0

The M^{F2}/ ey^D male was mutagenized in each cross.

Scored represents the number of M^{F2} (ey^+) flies counted in each cross. Putative revertants represents the number of wild type (bristle and eye) flies that were isolated from individual vials. Clusters of mutants from a single file were considered 1 putative. The number of revertants is the

number putative mutants that transmitted a wild type phenotype to their progeny.

The *FR5-1* mutant genetically acted as a translocation between chromosomes 3 and 4. Analysis of polytene chromosomes did not find a translocation of chromosome 4 to another chromosome. This could mean that the translocation occurred between the centromeres of chromosome 3 and 4. A translocation between the two centromeres would produce a polytene chromosome that would not look different from a wild type polytene chromosome.

Analysis of *FR7-59* found that it segregated with chromosome 4. However, the reversion was not stable and Minute flies would appear with crosses to a *w; dp; e; ci*^{1} *ey*^{R} stock. Do to its unreliable phenotype, *FR7-59* revertant was not studied further.

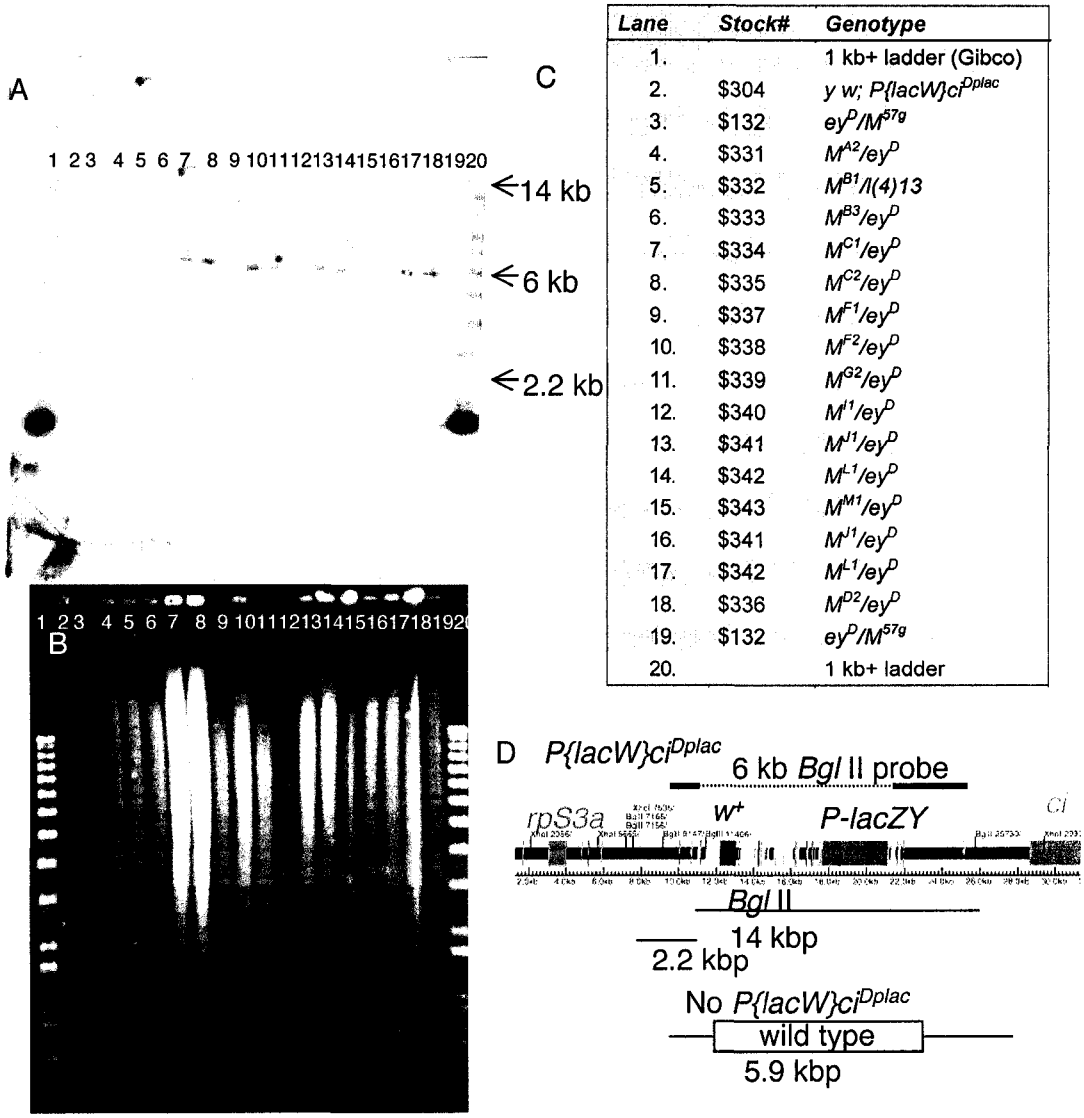
Analysis of FR6-29 found that it segregated with chromosome 4. The *FR6-29* allele was lethal when heterozygous with the original M^{F2} allele. Consequently, it was not a true revertant although it had a Minute phenotype. The *FR6-29* allele was not studied further.

3.4.3 Testing whether the Su(PDS) mutants suppress M^{F2}

Mutations in *Su(var)3-7* suppress PDS and I tested whether *Su(var)3-7* could affect the Minute phenotype of M^{F2}. In this test, M^{F2}/ eyD females were crossed to a *w; CyO/ T21A; TM2/ Su(var)3-7*^{P9} males. Neither an extra dose or loss of a *Su(var)3-7*$^{+}$ gene copy affected the Minute phenotype. Therefore mutations in *Su(var)3-7* do not alter the Minute phenotype caused by M^{F2}. As well, I crossed *Su(var)3-7*^{P25}, *Su(var)3-7*^{P47}, *Su(var)3-7*^{P49}, *Su(var)3-7*^{P71}, and *Su(var)3-7*^{P43} with M^{F2} but none of these alleles suppressed the Minute phenotype, either.

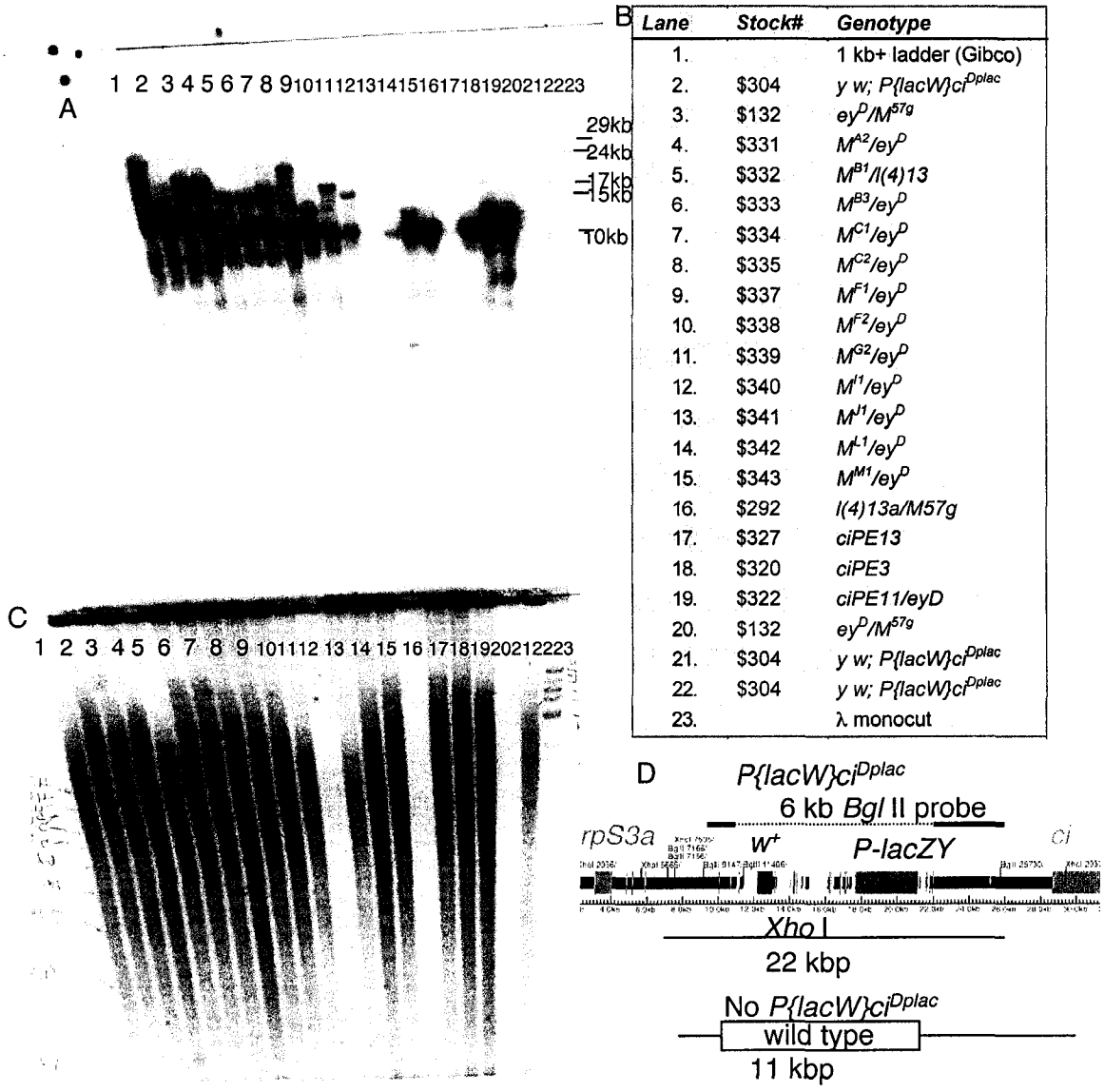
Other Su(var) genes were tested. These included *Su(var)2-5*^{P4}, *Su(var)2-5*^{P5}, *Su(var)2-5*^{5}, *Su(var)2-5*^{2}, *Su(var)3-9*^{1}, *Su(var)3-9*^{2}, *Su(PDS)P86*, and *Su(PDS)P80* but none of these affected the M^{F2} phenotype.

Mutations in *Su(var)3-7* did not prevent *P{ry*$^{+}$ *SalI}89D* from suppressing the Minute phenotype. I determined this by crossing a *w; Su(var)3-7*^{P13}*/ TM2;* M^{F2}/+ male to *w; P{ry*$^{+}$ *SalI}89D ; P{lacW}ci*^{Dplac} females. Half the progeny that inherited *TM2* and the other half that inherited *Su(var)3-7*^{P13} should have inherited the M^{F2} allele. There was 41 *Su(var)3-7*^{P13} and 41 *TM2* progeny scored and all had wild type bristles because *P{ry*$^{+}$ *SalI}89D* was present. The *Su(var)3-7*^{P13} did not prevent *P{ry*$^{+}$ *SalI}89D* from suppressing the Minute phenotype. This result was surprising because mutations in *Su(var)3-7* suppress PDS. Since the mutations that suppress PDS did not interrupt *P{ry*$^{+}$ *SalI}89D* suppression of the Minute alleles, *P* elements must trigger a different mechanism that influenced expression from both genes.



Lane	Stock#	Genotype
1.		1 kb+ ladder (Gibco)
2.	$304	*y w; P{lacW}ci^{Dplac}*
3.	$132	*ey^{D}/M^{57g}*
4.	$331	*M^{A2}/ey^{D}*
5.	$332	*M^{B1}/l(4)13*
6.	$333	*M^{B3}/ey^{D}*
7.	$334	*M^{C1}/ey^{D}*
8.	$335	*M^{C2}/ey^{D}*
9.	$337	*M^{F1}/ey^{D}*
10.	$338	*M^{F2}/ey^{D}*
11.	$339	*M^{G2}/ey^{D}*
12.	$340	*M^{I1}/ey^{D}*
13.	$341	*M^{J1}/ey^{D}*
14.	$342	*M^{L1}/ey^{D}*
15.	$343	*M^{M1}/ey^{D}*
16.	$341	*M^{J1}/ey^{D}*
17.	$342	*M^{L1}/ey^{D}*
18.	$336	*M^{D2}/ey^{D}*
19.	$132	*ey^{D}/M^{57g}*
20.		1 kb+ ladder

Figure 3.4.1 - Southern hybridization blot of *Bgl* II-digested genomic DNA from *P{lacW}ci*^{Dplac} derivatives that produce a Minute phenotype. **A.** Autoradiograph of a Southern blot probed with 6 kb *Bgl* II fragment from cosmid L7. **B.** 0.5% Agarose gel stained with ethidium bromide used to make the Southern blot. **C.** Table describing what is loaded into each lane in the agarose gel. **D.** Restriction map describing the *P{lacW}ci*^{Dplac} allele.



Lane	Stock#	Genotype
1.		1 kb+ ladder (Gibco)
2.	$304	*y w; P{lacW}ci^{Dplac}*
3.	$132	*ey^{D}/M^{57g}*
4.	$331	*M^{A2}/ey^{D}*
5.	$332	*M^{B1}/l(4)13*
6.	$333	*M^{B3}/ey^{D}*
7.	$334	*M^{C1}/ey^{D}*
8.	$335	*M^{C2}/ey^{D}*
9.	$337	*M^{F1}/ey^{D}*
10.	$338	*M^{F2}/ey^{D}*
11.	$339	*M^{G2}/ey^{D}*
12.	$340	*M^{I1}/ey^{D}*
13.	$341	*M^{J1}/ey^{D}*
14.	$342	*M^{L1}/ey^{D}*
15.	$343	*M^{M1}/ey^{D}*
16.	$292	*l(4)13a/M57g*
17.	$327	*ciPE13*
18.	$320	*ciPE3*
19.	$322	*ciPE11/eyD*
20.	$132	*ey^{D}/M^{57g}*
21.	$304	*y w; P{lacW}ci^{Dplac}*
22.	$304	*y w; P{lacW}ci^{Dplac}*
23.		λ monocut

Figure 3.4.2 - Southern Blot of *Xho* I digested genomic DNA from Minute and Dubinin effect *P{lacW}ci^{Dplac}* derivatives. **A.** Autoradiograph of a Southern blot probed with 6kb *Bgl* II fragment from cosmid L7 and exposed for 48 hours. **B.** 1% Agarose gel run by pulse field gel electrophoresis and stained with ethidium bromide used to make the Southern blot. **C.** Table describing the genotype of the *Xho* I digested genomic DNA loaded into each well. **D.** Restriction map indicating the expected sizes.

3.5 Testing *P* element constructs for the ability to cause silencing at *P{lacW}ci*$^{D_{plac}}$ and modify *P* dependent phenotypes

At the beginning of this project, *P{ry*$^{+}$ *SalI}89D* was the only single Type I element that could modify the *P* dependent phenotypes caused by *P* element insertions at *ci*. Further testing found that chromosomes with multiple *KP* elements also caused silencing. Since *KP*s fall into the Type II class, these results indicated that PDS behaved similarly to other *P* element dependent phenomenon and both Type I and II classes had an effect. However, based on this data, it could not be determined whether intermediate elements that range between the Type I and II elements have an effect. Testing for a difference was important to determine whether PDS behaved in a similar way to other *P* element dependent phenotypes.

Originally, Gloor *et al.* (1993) tested a large number of constructs that range from Type I into the intermediate elements to produce a statistically valid representation of their effects. Transformation resulted in random *P* insertions that varied in their ability to modify the *P* dependent phenotypes. After comparing many inserts, a general trend developed. Type I insertions frequently modified *P* element dependent phenotypes while intermediates rarely had any effect. He also mobilized *KP* elements and they frequently suppressed the $vg^{21\text{-}3}$ phenotype. This demonstrated that *P* elements with Type I or Type II sequence could modify *P* dependent phenotypes, but intermediate elements that fall between these two types had no effect.

To prevent position effect from confounding my results and forcing me to test many different inserts, I made the P{I SalI} construct that had FRT sequences flanking exon 2 (Figure 3.5.1). Except for the FRT sequences, this P{I Sal} construct was the same construct as P{ry^{+} SalI}. Since the FRT sequences are in the introns, they should not affect the coding capacity of this element. The FRT sequence within intron 2-3 occurs downstream from the stop codon for the P repressor and will not result in a truncated protein. In addition, the *Sal* I site in exon 3 was filled in and this resulted in a frameshift mutation in exon 3. This frameshift mutation prevented transposase from being produced but should not interfere with production of the P repressor protein. After building the P{I SalI} construct, sequencing confirmed that it had the appropriate sequences, including the FRT sites. This construct was then transformed into flies and the affect of Type I and intermediate elements on gene silencing was examined.

After transforming flies with the *P{I Sal}* construct, exon 2 was removed by crossing in a *hsFLP* transgene. The product from *hsFLP* induced recombination between the FRT sequences, which deleted exon 2. Loss of exon 2 changed the Type I construct into an intermediate insert (P{II SalI}) without changing the insertion site. Therefore, a position effect was not responsible for any change in phenotype. The *P{II Sal}* inserts closely resemble Type II inserts but are not equivalent because they have the end of exon 1 which is deleted in *KP* elements.

3.5.1 *P{ry⁺}*, *P{FRT ry⁺}* and *P{I SalI}* Transformation Results

Transformants were isolated with both the original *P{ry⁺}* construct, the transitional construct *P{FRT ry}* and final *P{I SalI}* construct (Table 3.5.1). The original construct and intermediate construct were transformed to make sure that these constructs could still transform and had transposase activity. A total of one *P{ry+}*, one *P{FRT ry⁺}*, and six *P{I SalI}* transformants were finally isolated.

Table 3.5.1: Transformation results with *P{ry⁺}* and derivatives

Injection#[1]	Embryos Injected	Eclosure	Transformants
P{ry⁺}			
J1	170	11	0
J4	326	15	1
P{FRT ry⁺}			
J3	258	40	1
J8	281	13	0
J9	499	69	0
P{I SalI}			
J2	157	9	0
J3	258	40	1
J6	436	41	0
J7	174	20	1
J12	219	19	3
J18	283	45	1

[1]Injection# is the injection session.

3.5.2 Testing a *P{FRT ry⁺}* insert for transposase activity

Placing FRT sequences within the introns between exons 1-2 and 2-3 could interrupt splicing of the immature RNA transcript produced by this construct and this could alter the interpretation of my results. I determined whether the FRT sequences interrupted splicing by testing the *P{FRT ry⁺}* transformants for transposase activity. The P{FRT ry+} construct was an intermediate construct that had the FRT sequences inserted in the introns flanking exon 2 but did not have the *Sal* I site filled in. There were no mutations in the P{FRT ry+} construct, except for possibly the FRT sequences, that would prevent transposase production. If the FRT sequences did not interrupt splicing, the *P{FRT ry⁺}* transformants should produce a mature transcript that encodes transposase.

I assayed for transposition events that transposed *P{lacW}3-76a* from the X chromosome to an autosome when *P{FRT ry+}* was present (Figure 3.5.2). Males that had *P{lacW}3-76a* produced only white males and red females when crossed to a w^- females (Figure 3.5.2 [A]). However, parents that had the *P{FRT* ry^+*}1b* insert present produced both red males, due to *P{lacW}* transposing to an autosome, and white eyed females, due to excision and loss of *P{lacW}3-76a* on the X chromosome (Figure 3.5.2 [B]). The seven new *P{lacW}* inserts, in red eyed males, were crossed to a *w; dp; e;* ci^1 ey^R and mapped to either chromosome 2 or 3.

The *P{FRT* ry^+*}* construct was autonomous and spontaneously excised and inserted itself to a new location. PCR using primers 2033F-3195R determined which parents inherited the *P{FRT* ry^+*}* insert (Figure 3.5.2 [C-D]). *P{lacW}3-76a* transposed only in parents that inherited a *P{FRT* ry^+*}* insert (Figure 3.5.2 [C]).

3.5.3 Testing *P{I SalI}* and its *P{II SalI}* derivative for the ability to modify *P* dependent phenotypes

From the original six transformants of *P{I SalI}*, eight chromosomes with at least one *P{I SalI}* insert were isolated. By tracking the ry^+ marker, I found that some of the transformants had inserts on multiple chromosomes (Table 3.5.2). Each chromosome bearing a *P{I SalI}* was crossed to determine whether the insert caused *P* element dependent silencing at *P{lacW}*ci^{Dplac}. Only the first transformant isolated, *P{I SalI}1a-3,* caused w^+ variegation at *P{lacW}*ci^{Dplac} (Table 3.5.2 and Figure 3.5.3). *P{I SalI}1a-3* also enhanced silencing with *BX2*, the *P{lacW}*ci^E alleles and suppressed $vg^{21\text{-}3}$. The seven other inserts tested with *P{lacW}*ci^{Dplac} or $vg^{21\text{-}3}$ did not suppress these *P* dependent phenotypes, thereby showing only some insert locations were able to induce silencing.

The stocks with the *P{I SalI}1a-3* insert and *P{I SalI}1b-3* were crossed to females with the *P{hsFLP}1* insert to remove exon 2 (see Materials and Methods for details). The *1a* and *1b* isolates were isolated from the same G_0 transformant and were assumed to be the same chromosome. Molecular analysis (described later) confirmed this assumption because these isolates had the exact same insertions. The *P{hsFLP}1; P{I SalI}1-3* flies were heat shocked during larval development and male flies with the insert were crossed to females from a *TM2* balancer stock. To determine if exon 2 had been removed, the males with putative *P{II SalI}1-3* insert/ *TM2* were mated back to the *TM2* balancer stock and then their DNA was extracted for PCR. A PCR test, that used primers 2033F-3195R tested whether exon 2 was removed (Figure 3.5.4 for molecular map). According to this PCR test, only isolates that had exon 2 removed were stocked and tested with the *P* dependent alleles.

Table 3.5.2: Testing *P{I Sal}* and the derivative *P{II Sal}* inserts for the ability to modify *P* dependent phenotypes

#Insert	P{ISal}	Chrom.[1]	*P{lacW}ci^{Dplac}*		*BX2*		*T1*		*vg$^{21-3}$*		*E1*[2]		*E2*[3]		Location
			Type I[4]	IN[5]	I	IN	I	IN	I	IN	I	IN	I	IN	
1	1a	3	40-80%[6]	100%	10-40%[7]	80-99%	-	-	+	-	+	-	+	-	91D and unknown
	1b	3	20-60%	100%	+	-	-	-	+	-	+	-	+	-	91D and unknown
2	2a	2	100%												Repetitive DNA
3	2a	3	100%						-						86D1E1
	2b	2	100%												
	2b	3	100%												
4	3a	Y	100%						-						Repetitive DNA
5	3b	3	100%						-						2 inserts
6	4a	2													
	4b	2	100%												
7	5a	2	100%												
8	6a	2	100%												

[1]Chromosome is shortened to chrom. Indicates which chromosome the insert is on.

[2]*P{lacW}ci^{E1}* is shortened to E1.

[3]*P{lacW}ci^{E2}* is shortened to E2.

[4]The Type I element tested is the *P{I SalI}* inserts

[5]IN stands for intermediates element *P{II SalI}*, which has exon 2 deleted.

[6]For *P{lacW}ci^{Dplac}* percent represents an estimate of the number of colored ommatidia. The minimum and maximum values represent the range seen from crosses to *P{lacW}ci^{Dplac}*. In the absence of a P element, 100% of the ommatidia are colored when *P{lacW}ci^{Dplac}* is present.

[7]For *BX2*, percent represents an estimate of the number of colored ommatidia. The minimum and maximum values represent the range seen from crosses to *BX2*. In the absence of a *P* element, 80-99% of the ommatidia are colored when *BX2^{c}* is present.

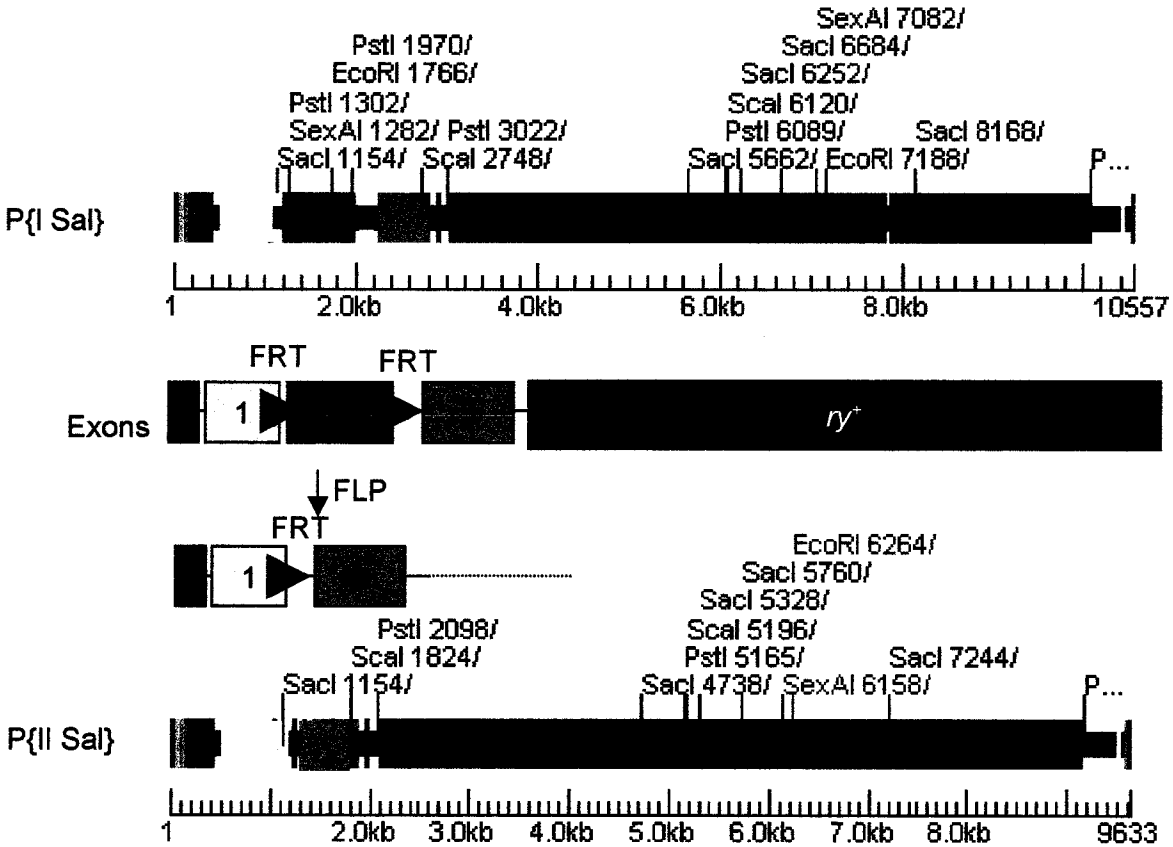


Figure 3.5.1 - Diagram depicting P{I Sal} and P{II Sal} constructs. The FRT sequences are inserted in the introns between exons 1-2 ad 2-3. When FLP is present it catalyzes a recombination reaction between these sequences that deletes exon 2. The P{II Sal} construct has exon 2 removed.

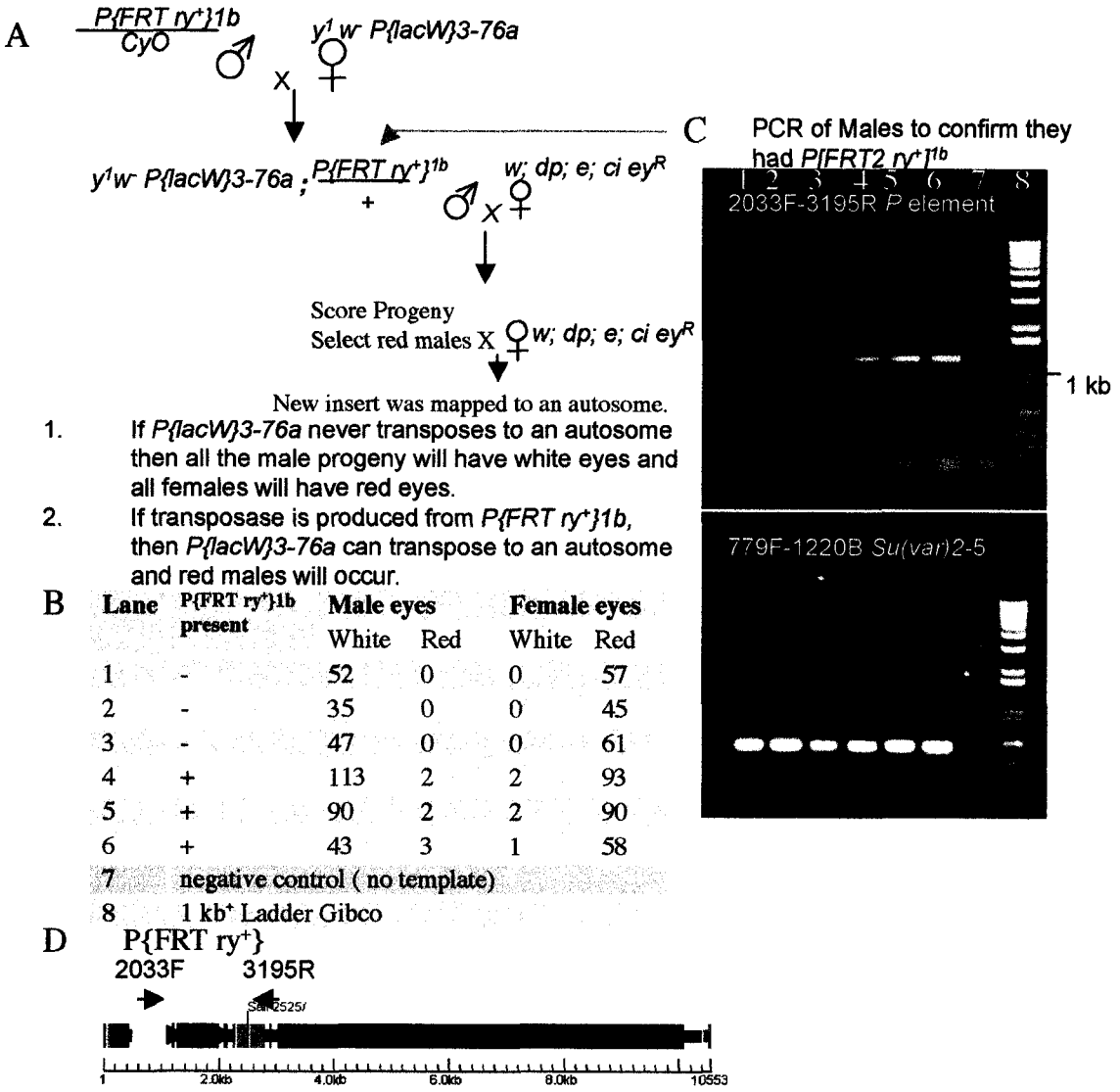


Figure 3.5.2 - Assaying for transposase activity from *P{FRT ry+}1b*. **A**. Crossing scheme to detect a transposition event. **B**. Table summarizing PCR and crossing results **C**. PCR results detecting a *P{FRT ry+}* insert. Primers 2033F-3195R are specific to the *P* element sequence. The primer pair 779F-1120B is a positive control to confirm that template can be amplified from another location within the *Su(var)2-5+* gene. **D**.Diagram displaying the location of primers used in P{FRT ry+}.

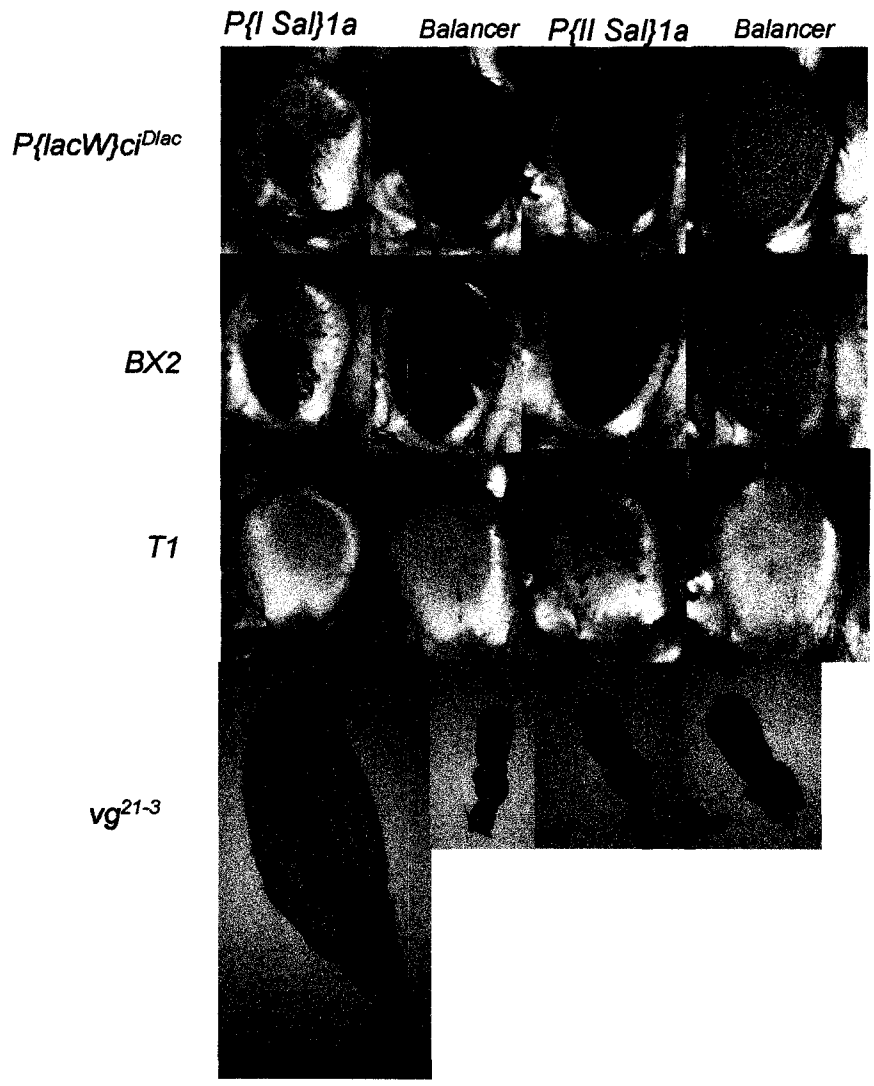


Figure 3.5.3 - *P{I Sal}1a-3* consistently modifies *P* dependent phenotypes but a *P{II Sal}1a-3* derivative does not. Above each column of pictures is the chromosome tested. The balancer in the eye pictures was *TM2* and for $vg^{21\text{-}3}$ is *TM6B.* These balancers have no affect on the *P* dependent phenotypes. To the left of each row is the allele tested. For $P\{lacW\}ci^{Dplac}$, *BX2*, and *T1*, these transgenes have the only functional w^+ transgenes in the flies studied. The $vg^{21\text{-}3}$ row is homozygous for $vg^{21\text{-}3}$. The original *P{I Sal}1a-3* chromosome has two *P{I Sal}* inserts. The *P{II Sal}1a-3* derivative tested has exon 2 deleted in both inserts. This *P{II Sal}1a-3* derivative is referred to as J16.7-2 in the PCR and Southern blot analysis.

The *P{I SalI}1-3* inserts (both *1a-3* and *1b-3*) modified *P* dependent phenotypes and after FLP treatment, independently isolated intermediate (*P{II SalI}1-3*) derivatives either continued to modify *P* dependent phenotypes or did not modify these phenotypes. This variation between the different derivatives occurred because the original *P{I SalI}1-3* chromosome had two inserts. The original PCR test, used primers 2033F-3195R that produced a product that spanned exon 2 (Figure 3.5.4). Deletion of exon 2, produced a *P{II SalI}* template that produced a smaller (~350 bp) fragment that amplified preferentially to the larger fragment (~1.2 kbp) (Figure 3.5.4). Consequently, a chromosome that had a *P{I SalI}* and a *P{II SalI}* insert produced a ~350 bp fragment and not a 1.2 kbp fragment.

An additional PCR reaction used primers 2632F-3413B and confirmed that those isolates that continued to modify *P* dependent phenotypes still had an insert with exon 2 (Figure 3.5.5). The primer 2632F anneals within exon 2 and whenever exon 2 was present a PCR product was produced. All the putative *P{II SalI}1-3* chromosomes (*1a-3* and *1b-3*) that continued to modify *P* dependent phenotypes had at least one *P{I SalI}* because PCR that used 2632F-3414B produced the appropriate size product (Figure 3.5.5). Since these chromosomes also produced a 350 bp band in a PCR reaction with primers 2033F-3195B, these *1-3* chromosomes also had a *P{II SalI}* insert (Figure 3.5.4).

The *1-3* chromosomes that had only P{II Sal} inserts, as determined using both PCR tests with the exon 2 specific primer (2632F), never modified *P* dependent phenotypes (Figure 3.5.3).

3.5.4 Southern blot Analysis of insert *P{I SalI}1a-3*

To confirm these results, I did Southern blot analysis of the original *P{I Sal}1a-3* and *1b-3* chromosomes and its *P{II Sal}* derivatives. These Southern blots confirmed that the original *P{I Sal}1a-3* and *1b-3* isolates, which where not treated with *hsFLP*, were the same chromosome because the different digests always gave the same restriction pattern. When referring to this chromosome, I will use the *P{I SalI}1-3* designation from here on. In addition, the Southern blot analysis confirmed that there were two *P{I Sal}* inserts on chromosome *1-3*. Lastly, the Southern blots confirmed the PCR results that showed that after *hsFLP* treatment exon 2 was being deleted.

Southern blots were made using genomic DNA digested with either *EcoR* I, *SexA* I, *Sac* I, or *Pst* I . I will only discuss the Southern blots that had the *Pst* I and *Sac* I digests. The other Southern blots, which were not included, supported the conclusions based on the *Pst* I and *Sac* I Southern blots.

The *Pst* I and *Sac* I Southern blots were probed with a PCR fragment (PLW-2-3413B) that was complementary to the 5' end of *P{I Sal}* (Figure 3.5.6 [D] for the molecular map). In the *Pst* I digest, I expected a 0.668 and 0.998 kbp internal fragments. The 0.668 kbp was produced from two *Pst* I cut sites that flanked exon 2 (Figure 3.5.6 [D]). The 0.998 kbp fragment was an internal fragment that included exon 3. The original *P{I*

Sal}1-3 chromosome produced these two bands. In addition, two fragments (6.5 kbp and 3.8 kbp Lanes 1 and 3) specific to two different insertions also occurred (Figure 3.5.6). As expected, if exon 2 was deleted from both inserts on the *1-3* chromosome the 0.668 kbp band disappeared (Figure 3.5.6 Lane 2). As well, when exon 2 was deleted the *Pst* I sites were also deleted. When these *Pst* I sites were lost the external fragments and internal (0.998 kbp) fused to become one fragment. Consequently the 6.5 kbp and 3.8 kbp band increased by ~1 kbp (0.998 kb) to 7.5 kbp and 4.8 kbp respectively (Figure 3.5.6 Lane 2). *P{I Sal}1-3* derivatives were isolated that had reciprocal *P{I Sal}* and one *P{II Sal}* inserts at the two different locations (Figure 3.5.6 Lanes 4 and 5).

As well, FLP treatment also deleted exon 2 in the single insert *P{I Sal}2a-3* (Figure 3.5.6 Lanes 7 and 8). The original *P{I Sal}2a-3* produced the 0.668 kbp and the 0.998 kbp fragment (Lane 8). In contrast, the *P{II Sal}2a-3* fragment had exon 2 deleted because the 0.668 kbp fragment disappeared and the 3.3 kbp band, which was specific to the inserts location, increased by 1 kbp to 4.3 kbp (Lane 7).

Analysis of the *Sac* I digests confirmed that exon 2 was flipped out in the *P{I SalI}1-3* inserts and in the *P{I SalI}2a-3* insert. In this case, *Sac* I cut before exon 2 and loss of exon 2 generated a smaller internal fragment. The internal fragment decreased from 4.8 kbp in lanes 12 and 14 to the 3.7 kbp in lane 13 after exon 2 was deleted from both inserts on chromosome *1-3* (Figure 3.5.6). This was the expected size decrease if exon 2 was removed. The same trend occurred in lanes 17 and 18 for insert *P{II SalI}2a-3* and *P{I SalI}2a-3* respectively.

Together, these results confirmed that exon 2 was being deleted after *FLP* treatment. The *1-3* chromosome had two *P{I Sal}* inserts. When exon 2 was deleted in one of the *P{I Sal}* inserts the ability to modify P dependent phenotypes was lost. The other *P{I Sal}* did not modify *P* dependent phenotypes.

3.5.5 Inverse PCR determined the insertion sites for some of the *P{I SalI}* inserts

Inverse PCR was used to determine where the *P{I SalI}* inserts for *1-3*, *2a-2*, *2a-3*, and *3b-3* occurred. Originally, using an *Hha* I digest, ligation, and then inverse PCR the insert for *P{I SalI}1-3* was determined to be in 91D (Figure 3.5.7). However, the PCR results and Southern blot analysis determined there was a second insert. Since there was no second band in the inverse PCR after an *Hha* I digest, inverse PCR was done after a *Sau3A* I digest. *Saus3A* I digestion and inverse PCR produced two bands that ran at ~300 and ~800 bp. The ~800 bp band was produced from the insert at 91D and the ~300 bp was sequenced. Sequencing showed a *Sau3A* I restriction site at the 5′ end of the *P* sequence (See Appendix). Consequently, there was no sequence with which to do a BLAST search and the insertion site remained unknown. The insert at 91 D was confirmed using PCR that used primers specific to the 91D locus (Figure 3.5.8). Southern blot analysis (see appendix) determined that the insert

at the unknown location was responsible for modifying the *P* dependent phenotypes.

By digesting the genomic DNA with *Sau3A* I, ligation and then inverse PCR, flanking sequence was recovered from *2a-2* and *3a-Y* (See Appendix). In the case of *2a-2*, BLAST searches found homologous sequence on chromosomes X and 2. Therefore, this sequence was repetitive and probably occurred on chromosome 2 with which this insert segregates. The *3a-Y* insert occurred in a *Juan* element on the Y chromosome. Using inverse PCR after an *Hha* I digest, flanking sequence was recovered from *2a-3* (See Appendix). A BLAST search identified homologous sequence in 86D-E.

3.5.6 Generating and testing new *P{I SalI}* inserts

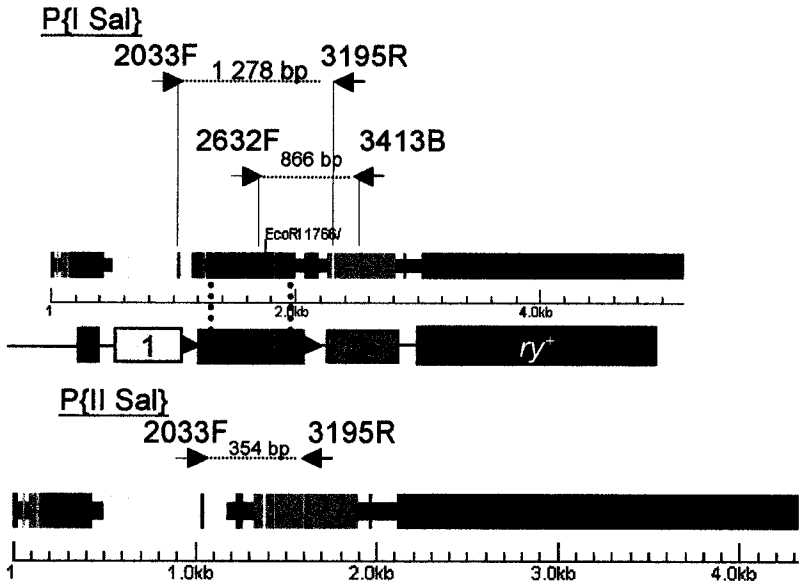
To determine if position effect was the reason why only one insert caused variegation while the rest did not, inserts that did not cause variegation were remobilized using *P{ry⁺ Δ2-3}99B*. The chromosome with *P{I SalI}* inserts chosen include *2a-2*, *2a-3*, *3b-3*, *4b-2*, and *3a-Y* (Table 3.5.3). All inserts, except *4b-2*, generated new inserts that caused variegation at *P{lacW}ci^{Dplac}* after remobilization. In the case of *4b-2*, too few progeny were probably scored to isolate a new insert.

Table 3.5.3: Mobilizing *P{I Sal}* inserts

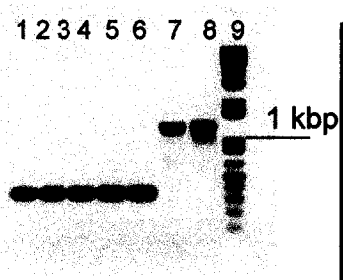
Insert Mobilized	Total Scored	Isolates[1]	Frequency[2]
2a-2	512	1	512
2a-3	284	1	284
3b-3	161	3	54
4b-2	178	0	0
3a-Y	2484	11	226

[1] The number of chromosomes identified with at least one insert causing variegation.
[2]The average number of flies scored for every variegating fly recovered

A



B



C

Lane#	Stock#	PCR Result	Description	Modifies P dependent phenotype
		Top		
1.	J17.11-19	~350 bp	Flipped out[1]	no
2.	J16.7-2	~350 bp	Flipped out	no
3.	J16.7-2	~350 bp	Flipped out	no
4.	J16.6-3	~350 bp	Flipped out	no
5.	J16.6-3	~350 bp	Flipped out	no
6.	J16-3	~1.2 kbp	Original	yes
7.	J16-3	~1.2 kbp	Oiriginal	yes
8.	1 Kb+ ladder			
9.	No fly			

Figure 3.5.4 - PCR used to detect flip-out of exon 2. **A**. Primers and expected product from the *P{I Sal}* and *P{II Sal}* inserts. **B.** 1% Agarose gel stained with ethidium bromide and loaded with PCR product from reactions using primers 2033F-3195R to test for the presence of exon 2. The 1.2 kbp product occurs from the *P{I Sal}* insert and 350 bp product is generated after exon 2 is deleted from the *P{II Sal}*. **C**. Table describing what is loaded in each lane of the agarose gel. "Flipped out" indicates that previous PCR reactions using primers 2033F-3195B had indicated that exon 2 was removed.

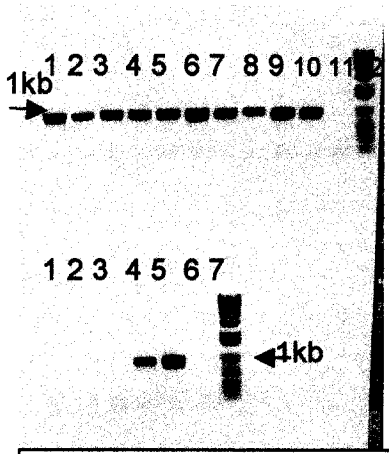
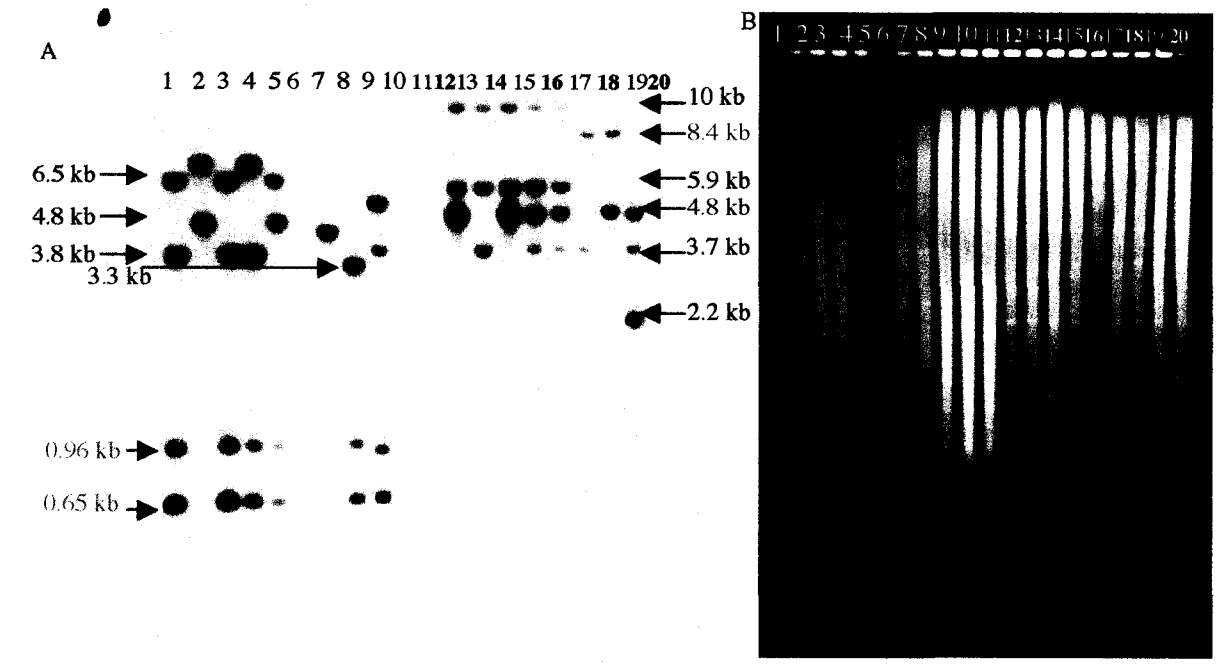


Figure 3.5.5 - PCR test using primers 2632F-3413B to test for the presence of exon2. "[1]Flipped out" constructs had been tested previously using primers 2033F-3195 to confirm that exon 2 had been flipped out. However, further tests found that some of the flipped out constructs could still modify *P* dependent phenotypes. This indicated that there was a second insert that was not flipped out. [2]ND stocks not tested. Template J16.7-2 and J16.6-3 was used in the previous PCR reaction described in Figure 3.5.4 and produced the 350 bp band when exon 2 is deleted.

Lane#	Stock#	PCR Result	Description	Modifies P dependent phenotype
		Top		
1.	J17.10-11	~900 bp	Flipped out[1]	ND[2]
2.	J17.10-11	~900 bp	Flipped out	ND
3.	J17.11-21	~900 bp	Flipped out	no
4.	J17.11-21	~900 bp	Flipped out	no
5.	J17.11-22	~900 bp	Flipped out	yes
6.	J17.11-22	~900 bp	Flipped out	yes
7.	J17.11-18	~900 bp	Flipped out	ND
8.	J17.11-18	~900 bp	Flipped out	ND
9.	J17.11-19	~900 bp	Flipped out	no
10.	J17.11-19	~900 bp	Flipped out	no
11.	J16.7-2	-	Flipped out	no
12.	1kb+ Ladder Gibco			
		Bottom		
1.	J16.7-2	-	Flipped out	no
2.	J16.6-3	-	Flipped out	no
3.	J16.6-3	-	Flipped out	no
4.	J16-3	~900	Original	yes
5.	J16-3	~900	Original	yes
6.	No fly	-	Negative control	
7.	1 kb+ Ladder (Gibco)			



C

Lane	Insert	Stock#	Digest
1	P{I Sal}1a	J16-4	PstI
2	P{II Sal}1a	J16.7-2	PstI
3	P{I Sal}1b	J17.11-25	PstI
4	P{I/II Sal}1b	J17.11-22	PstI
5	P{I/II Sal}1b	J17.11-19	PstI
6	1 Kb+ ladder		
7	P{II Sal}2a-3	J26.6-1	PstI
8	P{I Sal}2a-3	J26.5-3	PstI
9	P{ry+Sal}89D	$390	PstI
10	no insert	ry506	PstI
11	no insert	TM2 ubx	PstI
12	P{I Sal}1a	J16-4	SacI
13	P{II Sal}1a	J16.7-2	SacI
14	P{I Sal}1b	J17.11-25	SacI
15	P{I/II Sal}1b	J17.11-22	SacI
16	P{I/II Sal}1b	J17.11-19	SacI
17	P{II Sal}2a-3	J26.6-1	SacI
18	P{I Sal}2a-3	J26.5-3	SacI
19	P{ry+Sal}89D	$390	SacI
20	no insert	ry506	SacI

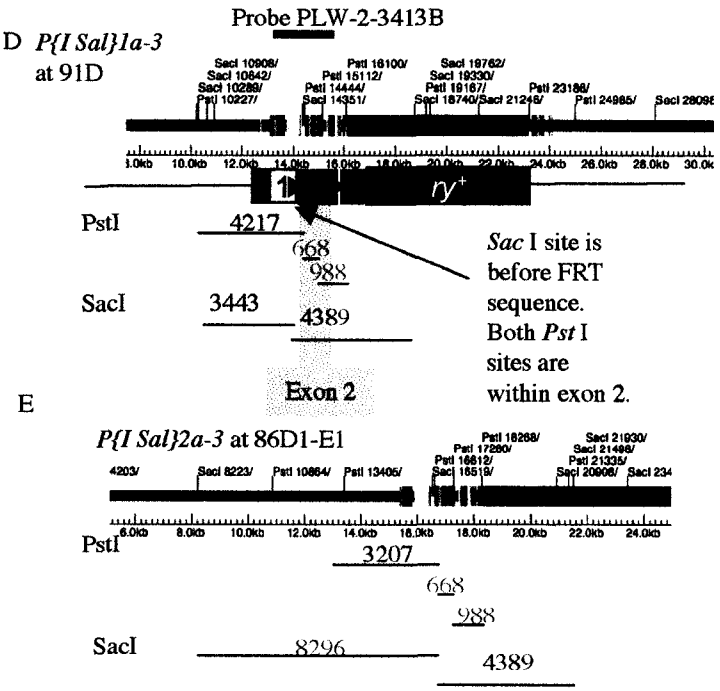


Figure 3.5.6 - *Pst* I and *Sac* I hybridization Southern blot probed with PLW-2-3413B to study inserts *1a-3*, *1b-3*, and *2a-3*. **A.** Autoradiograph of a Southern blot probed with PLW-2 - 3413B. **B.** Ethidium bromide stained agarose gel used to make the Southern blot. **C.** Table describing the insert and restriction digest. **D.** Restriction map of the insert expected at 91D in inserts *1a-3* and *1b-3*. **E.** Restriction map expected for the insert on chromosome *2a-3*. The fragment sizes for both maps are in bp.

A. Inverse PCR results for *P{I Sal}1a-3*

```
      3269F     5'CGATGAGATGTTAAGCAATAT
3269F           ccntgaagngccc-------------CCNAAATGGATGAGTTGACGGAGGATGCGATGGA    47
11RPT-5         nggggtgttaagcaatatagatttcaCCGAAATGGATGAGTTGACGGAGGATGCGATGGA    60
The blue sequence has homology to the original P element pi25.1

3269F           ATATATCGCGGGCTATGTCATTAAAAAATTGAGAATCAGTGACAAAGTAAAAGAAAATTT   107
11RPT-5         ATATATCGCGGGCTATGTCATTAAAAAATTGAGAATCAGTGACAAAGTAAAAGAAAATTT   120
                          Sal I site is filled in
3269F           GACATTTACATACGTCGATCGACGAGGTGTCTCACGGCGGACTTATTAAGCCGTCCGAAA   167
11RPT-5         GACATTTACATACGTCGATCGACGAGGTGTCTCACGGCGGACTTATTAAGCCGTCCGAAA   180

3269F           AATTTCAAGAGAAGTTAAAAGAGCTAGAATGTATTTTTTTGCATTATACAAATAATAATA   227
11RPT-5         AATTTCAAGAGAAGTTAAAAGAGCTAGAATGTATTTTTTTGCATTATACAAATAATAATA   240
                                                    Hha I site
3269F           ATTTTGAAATTACAAATAATGTAAAGGAAAAATTAATATTAGCAGCGCAATTTCGTTGTA   287
11RPT-5         ATTTTGAAATTACAAATAATGTAAAGGAAAAATTAATATTAGCAGCGCAATTTCGTTGTA   300

3269F           TTGTTTGCTTAGCTAGAAATTTAATCTGTTGACACCGCAACACTTGCCAATGAAATTGGT   347
11RPT-5         TTGTTTGCTTAGCTAGAAATTTAATCTGTTGACACCGCAACACTTGCCAATGAAATTGGT   360
                                                              31bp repeat
3269F           AAACAAAGGCGACCACTGCAAGTGCGGGACCATGATGAAATAACATAAGGTGGTCCCGTC   407
11RPT-5         AAACAAAGGCGACCACTGCAAGTGCGGGACCATGATGAAATAACATAAGGTGGTCCCGTC   420

3269F           GATAGCCGANGCTTACCGAAGTATACACTTGAATTCANCGCACGTNTGNTNNTTGAGANG   467
11RPT-5         GATAGCCGAAGCTTACCGAAGTATACACTTAAATTCAGTGCACGTTTGCTTGTTGAGAGG   480

3269F           AANGGTNGTGTGCNGcacgaatnnttnnttgaaaacatcaacccccc               515
11RPT-5         AAAGGTTGTGTGCGGacgattatg------------------------              504
```

B. Blast search results

>gi|13096036|gb|AC007813.4|AC007813 Drosophila melanogaster, chromosome 3R, region 91C-91D, BAC clone
BACR40J10, complete sequence
Length = 160089

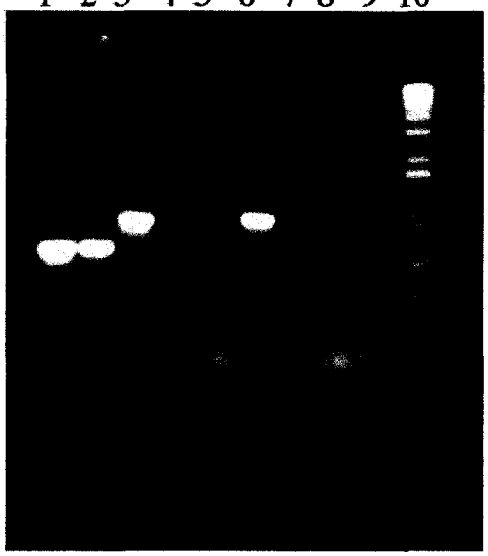
```
 Score =  204 bits (103), Expect = 9e-50
 Identities = 106/107 (99%)
 Strand = Plus / Plus


Query: 115    gtcccgcacttgcagtggtcgcctttgtttaccaatttcattggcaagtgttgcggtgtc 174
              ||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct: 146893 gtcccgcacttgcagtggtcgcctttgtttatcaatttcattggcaagtgttgcggtgtc 146952


Query: 175    aacagattaaatttctagctaagcaaacaatacaacgaaattgcgct 221
              |||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 146953 aacagattaaatttctagctaagcaaacaatacaacgaaattgcgct 146999
```

Figure 3.5.7 – Inverse PCR results for *P{I Sal}1a-3*. **A**. Sequence using primers 3268F and 11RPT-5 to read the different strands generated by inverse PCR. **B**. BLAST search using the sequence proximal to the *P{I Sal}1a-3*. Later analysis demonstrated that there are two *P{I Sal}* inserts in the *1-3* isolate.

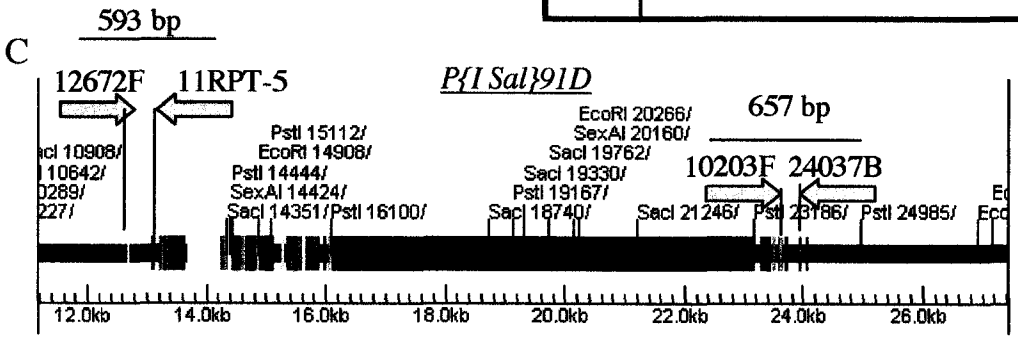
A



B

Lane	PCR Product	Template
1	12672F-11RPT-5	*P{I Sal}1a-3*
2	10203F-24037B	*P{I Sal}1a-3*
3	12627F-24037B	*P{I Sal}1a-3*
4	12672F-11RPT-5	*Su(var)2-5^5/ CyO*
5	10203F-24037B	*Su(var)2-5^5/ CyO*
6	12627F-24037B	*Su(var)2-5^5/ CyO*
7	12672F-11RPT-5	no template
8	10203F-24037B	no template
9	12627F-24037B	no template
10	1 kb$^+$ ladder Gibco	

C



D

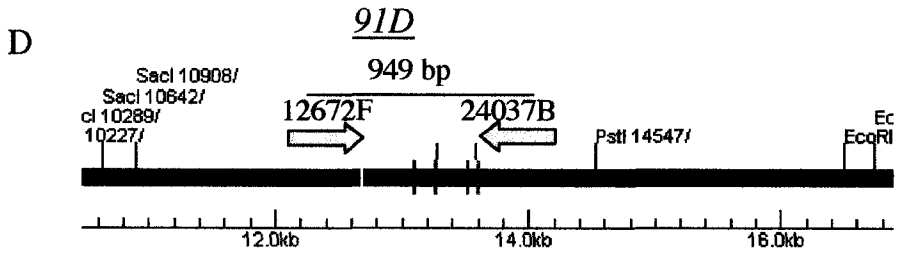


Figure 3.5.8 - PCR using primers specific to the insert in 91D to determine if *P{I Sal}1a-3* has an insert in this region. **A.** Ethidium bromide stained agarose gel separating the PCR product. **B.** Table describing the PCR reaction loaded in each lane in the agarose gel. *Su(var)2-5^5* is used as a negative control for an insert at 91D. **C.** A diagram depicting the relative position of the primers in 91D with the *P{I Sal}91D* insert in the *P{I Sal}1a-3* chromosome. **D.** A diagram depicting the relative position of the primers in 91D without the P{I Sal} insert. Both 12672F and 24037B are primers complementary to sequence within the 91D region. The 11RPT-5 and 10203F primers are complementary to sequence within the P{I Sal} construct.

P{II SalI} inserts were mobilized to determine if an insertion could be isolated that caused variegation at *P{lacW}ci*^{Dplac}. The *P{II SalI}* inserts mobilized using *Δ2-3* included *2a-2*, *2a-3*, and *3a-Y* (Table 3.5.4). No *P{II SalI}* inserts were recovered that caused a variegated eye phenotype after screening 2490 progeny in crosses mobilizing these various inserts. In comparison, mobilizing the original *P{I SalI}* inserts, from which the *P{II Sa}* inserts were derived, identified 13 variegating flies after scoring 3280 flies (Table 3.5.3-4). This could be because loss of exon 2 reduced the transposition rate. However, *P* inserts that only have the *P* ends can be mobilized if transposase is produced. Further, I tested, using the *ry*$^{+}$ phenotype, rather than PDS, for transposition of *P{II SalI}3a-Y* and it mobilized as frequently as the *P{I SalI}3a-Y* insert (Table 3.5.5). This mobilization involved crossing *P{I or II SalI}3a-Y; Sb Δ2-3(99B)/ry506* males to *ry506* females and selecting wild type (for the rosy phenotype) females. The *3a-Y* insert was in the Y chromosome. Any females wild type for the rosy phenotype had a *P{I or II SalI}* insert transposed to another chromosome. Two inserts of each Type were further analyzed and the *P{I SalI}* insert mapped to the X-chromosome and the other *P{II SalI}* insert mapped to chromosome 2. This result indicated that *P{I SalI}* and *P{II SalI}* had the same transposition rate and any difference was caused because insertions of *P{II SalI}* rarely caused variegation at *P{lacW}ci*^{Dplac}. Therefore, I failed to identify any intermediate elements that caused PDS because new inserts were not causing the variegated phenotype.

Table 3.5.4 : Mobilizing *P{II Sal}* inserts

Insert Mobilized	Total Scored	Isolates[1]
2a-2	186	0
2a-3	414	0
3a-Y	1890	0

[1] The number of chromosomes identified with at least one insert causing variegation.

Table 3.5.5: Mobilizing *3a-Y* insert and screening for new inserts using the *ry*$^{+}$ marker

Insert Type	Scored	Isolates	Frequency
P{I Sal}	85	2	43
P{II Sal}	132	4	33

[1] The number of chromosomes identified with at least one insert causing variegation.
[2] The average number of flies scored for every variegating fly recovered

3.5.7 Testing the new *P{I SalI}* and *P{II SalI}* inserts

Of the 16 new *P{I SalI}* inserts, 14 were treated with FLP and *P{II SalI}* inserts were recovered. Only inserts on chromosomes 2 and 3 were tested because testing inserts on chromosomes 1 and 4 required a more complex crossing scheme. PCR using primers 2632F-3413B detected whether exon 2 was present. The 2632F primer anneals within exon 2 and, even if multiple inserts existed, then all of them must lose exon 2 to prevent a 2632F-3413B product from being amplified in the PCR test. As a positive control, primers 2033F-3195 were used in another PCR that produced a smaller product when exon 2 was removed.

In all 14 cases when exon 2 was deleted in the inserts on these chromosomes, the chromosomes also lost the ability to cause silencing at *P{lacW}ci*^{Dplac} (Table 3.5.6). In each case, it did not matter how strong the variegation was before exon 2 was flipped out, because after flipping out exon 2, no variegation could be seen from *P{lacW}ci*^{Dplac}. Column 3 in Table 3.5.6 gave a qualitative estimate of how many ommatidia were colored when *P{I SalI}* was present. Individual inserts varied widely in their expressivity and consequently a range was given for each insert.

Many inserts were tested to demonstrate that loss of exon 2 consistently resulted in the loss of the variable silencing at *P{lacW}ci*^{Dplac}. The second reason to study many inserts was to compare the effect different inserts had on the *P* dependent phenotypes and determine whether all the inserts that consistently caused silencing at *P{lacW}ci*^{Dplac} also modified the other *P* dependent phenotypes tested. The *P* dependent phenotype studied were caused by the following alleles: *BX2*, *vg*$^{21-3}$, and *M*^{P2}. The effect of *P{I SalI}* inserts and their *P{II SalI}* derivatives had on these *P* dependent phenotypes was summarized in Table 3.5.6. Figure 3.5.9 (chromosome 3 inserts) and Figure 3.5.10 (chromosome 2 inserts) display the phenotypic effects caused by these inserts.

Table 3.5.6: Summarizing the effect each *P{I Sal}* insert and its *P{II Sal}* derivative have on the *P* dependent phenotypes test.

General Information			*P{lacW}ci*^{Dplac}		*BX2*		Vg^{21-3}		M^{F2}	
Insert[1]	Donor[2]	Chrom.[3]	I[4,5]	IN[5]	I	IN	I[6]	IN[6]	I	IN
M10	2a-3	2	30-60%	100%	80%	80-100%			M+	M-
M14	3b-3	3	10-20%	100%	40%	80-100%	1	21-24	M+	M-
M20	3a-Y	3	30-80%	100%	5-80%	80-100%	1	21-24	M+	M-
M21	3a-Y	3	30-60%	100%	5-70%	80-100%	1-7	21-24	M+	M-
M23	3a-Y	2	80-90%	100%	5-40%	80-100%			M+	M-
M24	3b-3	3	5-20%	100%	60-70%	80-100%	6-11	21-24	M+	M-
M25	3b-3	2	30-80%	100%	90-99%	80-100%			M+	M-
M26	2a-2	3	70-90%	100%	80-90%	80-100%	**27**	21-25	**M-**	M-
M29	3a-Y	2	60-90%	100%	10-80%	80-100%			M+	M-
M30	3a-Y	2	80-90%	100%	80%	80-100%			M+	M-
M31	3a-Y	3	80-90%	100%	80%	80-100%	1	21-24	M+	M-
M32	3a-Y	2	70-90%	100%	80%	80-100%			M+	M-
M34	3a-Y	3	80-99%	100%	60-90%	80-100%	18-22P		M+	M-
M35	3a-Y	2	85-99%	100%	60-80%	80-100%			M+	M-
M35	3a-Y	4	5-20%						M+	
M36	3a-Y	1	40-60%							

[1]Insert column indicates the designated number for the insert.
[2]The original G_0 mobilized to produce the new insert.
[3]The chromosome the insert is isolated on.
[4]The I column refers to test using the *P{I Sal}* insert. The II column refers to tests using the *P{II Sal}* insert.
[5]The percent represents the number of colored ommatidia based on an estimate from visual inspection of 20-30 flies.
[6]The vestigial phenotype is based on the scale of Nakashima and Tanaka (1967). 1 is wild type and 31 is strong vestigial.

The *P* inserts affect on *BX2* was determined by comparing *BX2* flies that inherited the *P{I SalI}* insert with the original parents rather than the siblings that inherited the balancer chromosome. The *CyO* chromosome, with which the *P{I SalI}* inserts on chromosome 2 were balanced, was semi-lethal with *BX2* and therefore there were often not enough progeny to compare. When there was adequate numbers, the Cy class was often slightly suppressed compared to the class with the

insert. However, these classes were often significantly enhanced compared to the original w; *BX2 / Cy* stock in both males and females.

This problem also occurred if the insert was on chromosome 3. After crossing to a *w; BX2/ Cy* females, the progeny that inherited the chromosome 3 balancer appeared slightly suppressed compared to progeny that inherited the *P{I SalI}* insert. Both the *P{I SalI}* and balancer classes appeared significantly enhanced compared to the original *w; BX2 / Cy* stock. When testing the *P{II SalI}* inserts there was no difference between progeny that inherited the balancer and the insert. Furthermore, there was no difference between these progeny and the original *w; BX2 / Cy* stock. These results suggested that there may be a parental effect with the *P{I SalI}* inserts, which was not lost within a single generation.

Each *P{I Sal}* tested that caused PDS at *P{lacW}ci*^{Dplac} also enhanced variegation from the *BX2* array. However, there was not a strong correlation between a *P{I SalI}* insert enhancing variegation at *P{lacW}ci*^{Dplac} and *BX2*. For instance *P{I SalI}M23-2*, strongly enhanced variegation from *BX2* but weakly enhanced variegation at *P{lacW}ci*^{Dplac} (Figure 3.5.10). With most of the other inserts, *P{I SalI}* caused strong variegation at *P{lacW}ci*^{Dplac} but weakly enhanced variegation from the BX2 array. When exon 2 was deleted, none of the *P{II Sal}* derivatives had an effect on w^+ expression from *BX2*.

3.5.7.1 Suppression of *P{lacW}ci*^{Dplac} did not correlate with *vg*$^{21-3}$

P{I SalI} inserts that caused strong variable silencing at *P{lacW}ci*^{Dplac} did not necessarily suppress the vestigial phenotype caused by *vg*$^{21-3}$. For example insert *P{I SalI}M26-3*, which caused PDS, did not suppress the vestigial phenotype (Figure 3.5.9). Also, *P{I SalI}M21-3* that strongly caused PDS did not completely suppress *vg*$^{21-3}$. In comparison, *P{I SalI}M20-3* strongly suppressed *vg*$^{21-3}$ but weakly caused PDS. These results indicated that each insert affected each *P* dependent phenotype independently and that this probably related to the insertion site.

As with the *vg*$^{21-3}$, the *P{I SalI}* inserts varied in their ability to suppress M^{F2}. There appeared to be a correlation between the ability to suppress *vg*$^{21-3}$ and M^{F2} because in no case was there an insert that suppressed one but not the other. *P{I SalI}M26-3* did not suppress M^{F2} as well as *vg*$^{21-3}$ but did cause PDS. All the other inserts suppressed both M^{F2} and *vg*$^{21-3}$. These results indicated that *P{I SalI}M26-3*'s expression pattern could determine whether an insert suppressed a phenotype. This model would predict that *P{I SalI}M26-3* insert was expressed in the eye imaginal disc and not in the wing imaginal disc that forms the scutellar bristles and wings. Whereas, all the other inserts were expressed in both imaginal discs to various extents and suppressed the *P* dependent phenotypes in these discs.

3.5.8 Exon 2 contributes to *P* element silencing and the other *P* element dependent phenotypes.

Together these results found that *P* silencing effects that occurred at *BX2* and *P{lacW}ci*^{Dplac} respond to a change in the *P* element sequence. This confirms that *P* elements are producing a product that causes silencing at *P{lacW}ci*^{Dplac}. Only the Type I and Type II inserts were able to enhance w^{+mC} variegation at *P{lacW}ci*^{Dplac} . None of the *P{I SalI}* inserts after being changed to a *P{II SalI}* modified the *P* dependent phenotypes. This demonstrated that intermediate *P* elements, which fall between Type I and Type II, cannot cause silencing at *P{lacW}ci*^{Dplac} or modify other *P* dependent phenotypes. Since deleting exon 2 changes the protein coding sequence in the *P* element this suggests that the P repressor protein is causing the change in chromatin structure at *P{lacW}ci*^{Dplac} which results in silencing.

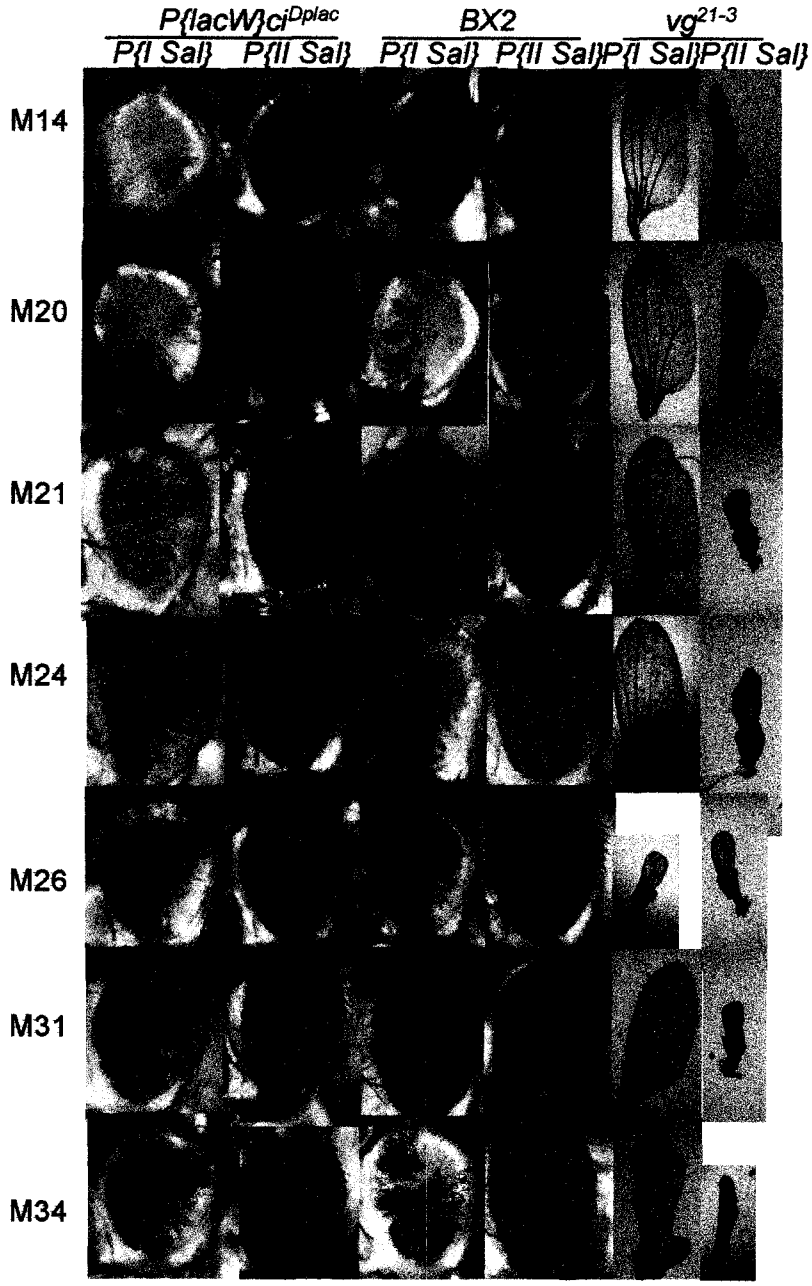


Figure 3.5.9 - Testing the effect *P{I Sal}* and derivative *P{II Sal}* inserts on chromosome 3 have on *P* dependent phenotypes. In the first two columns, w^+ pigment production occurs from *P{lacW}ci*^{Dplac}. In the middle two columns, w^+ pigment production is solely from the *BX2* array. Wings in the last two columns come from flies homozygous *vg*$^{21-3}$ with the indicated insert present. The insert tested is indicated to the left of each row of eyes.

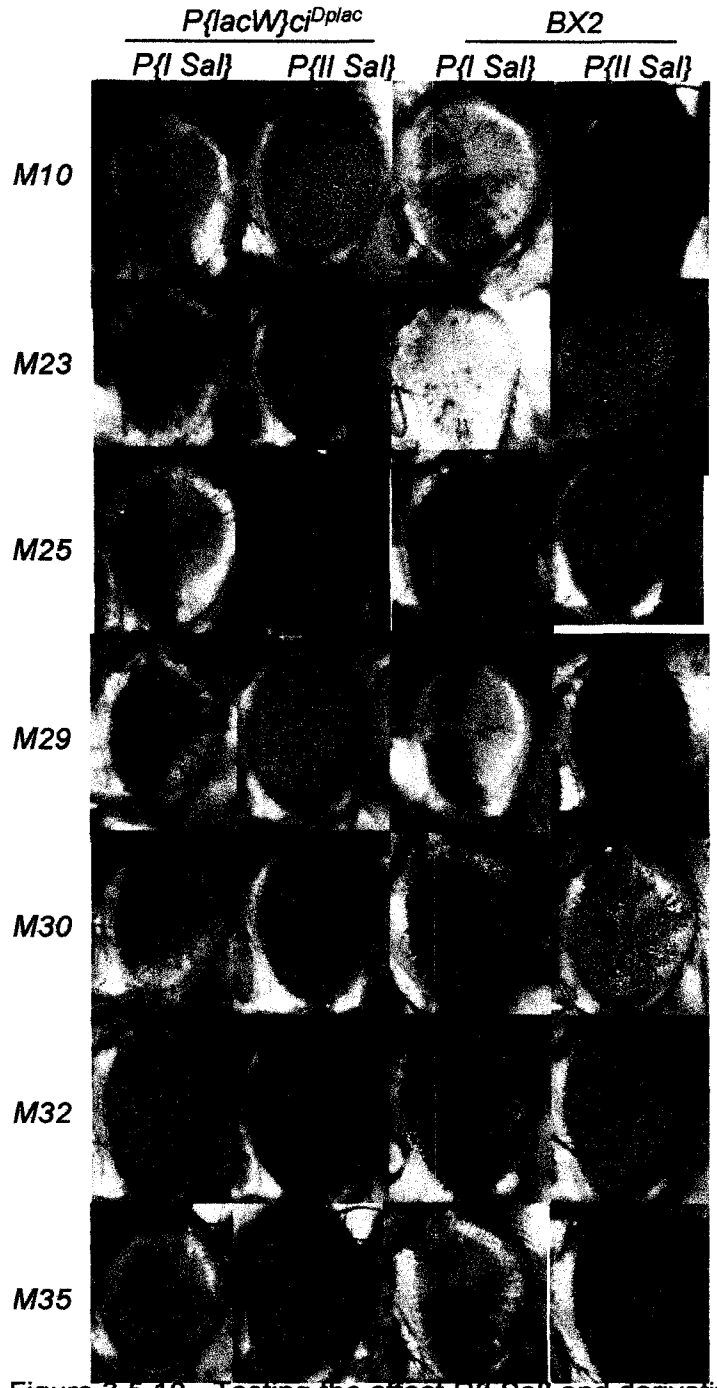


Figure 3.5.10 - Testing the effect *P{I Sal}* and derivative *P{II Sal}* inserts on chromosome 2 have on *P* dependent phenotypes. In the first two columns, w^+ pigment production occurs from the *P{lacW}ci*^{Dplac}. In the last two columns, w^+ pigment production is solely from the *BX2* array. The insert tested is indicated to the left of each row of eyes.

4 Discussion

4.1 Summary

I discuss my study of *P* element dependent phenotypes in three parts.

First, I discuss my successful screen for mutations that suppress the PDS at *P{lacW}ci*^{Dplac}. Finding two *Su(var)* loci indicates that PDS involves heterochromatic silencing and this has implications for the evolutionary relationship between heterochromatin and TEs.

Second, I discuss the difference between Type I and intermediate elements in their ability to modify the *P* element dependent phenotypes at *ci*. By testing different inserts at the same position, I circumvented the problems associated with position effect, which confounded previous analyses. *P* elements with an intermediate sequence falling between Type I and Type II do not cause silencing or modify the other *P* dependent phenotypes tested. This leads into discussion trying to explain the different phenotypic caused by Type I and Type I element compared to intermediate elements.

Together these results start to explain the interactions that occur between the *P* element and the *Drosophila* genome, which is part three of my discussion. Similar interactions may occur at other locations within the genome and lead to the domestication of the *P* element. Although the *P* element dependent phenotypes I have studied involve the somatic tissues, these findings may be applicable to *P* cytotype effects in the germ line. What I have learned about P elements at the *ci* locus may have broader implications in terms of the evolutionary relationship between TEs and their host. TEs account for most of the spontaneous mutations that cause morphological changes in *Drosophila*. They represent a means to generate genetic diversity within a population without challenging individual fitness.

4.2 A Subset of *Su(var)* loci silence *w*$^{+mC}$ expression from *P{lacW}ci*

In contrast to genetic screens that have identified about 150 *Su(var)* and *E(var)* modifiers of *w*^{m4} (SCHOTTA *et al.* 2003; WALLRATH 1998), my genetic screen repeatedly identified mutations primarily in two *Su(var)* loci that suppress PDS at *P{lacW}ci*^{Dplac}: *Su(var)2-5* and *Su(var)3-7*. Both *in vivo* and *in vitro* evidence indicates that SU(VAR)3-7 and the HP1 protein product of *Su(var)2-5* directly interact (CLEARD *et al.* 1997; DELATTRE *et al.* 2000). Similar evidence indicates HP1 also interacts with SU(VAR)3-9, (HWANG *et al.* 2001; SCHOTTA *et al.* 2002; SCHOTTA and REUTER 2000) and mutations in *Su(var)3-9* suppress PEV caused by telomeric and centromeric position effects at other loci (DONALDSON *et al.* 2002). However, PDS at *P{lacW}ci* is insensitive to mutations in *Su(var)3-9*. This insensitivity to *Su(var)3-9*$^{+}$ dose probably reflects chromosome 4's chromatin structure, since mutations in *Su(var)3-9* do not affect the nucleosome methylation pattern of chromosome 4 but do

disrupt methylation within the centromeric heterochromatin (SCHOTTA *et al.* 2002).

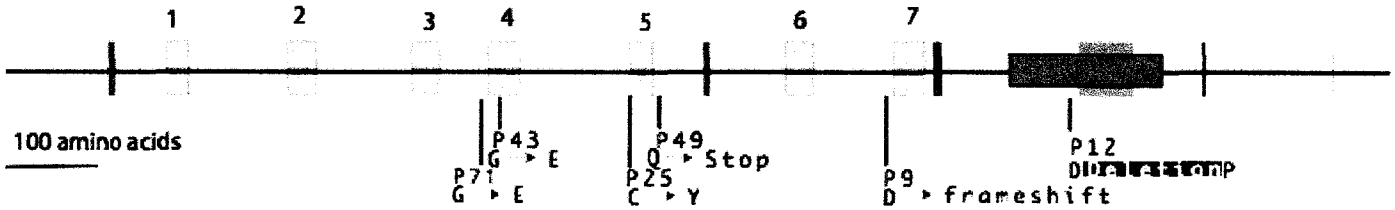
Mutations in *Su(var)2-5* and *Su(var)3-7* show a contrast in their ability to suppress variegation in the w^{m4} and *P{lacW}ci*^{Dplac} assays. Both previously isolated *Su(var)2-5* mutations, and those isolated in our screen, weakly suppress PDS at *P{lacW}ci*^{Dplac} but act as strong Su(var)s in w^{m4} variegation (EISSENBERG *et al.* 1990). In contrast, *Su(var)3-7* has the opposite effect in that these mutations have a strong Su(PDS) phenotype, but in our tests using w^{m4}, they acted as weak Su(var)s. Since the number and expressivity of modifiers depends upon the assay, different assays will isolate modifiers missed in other screens (BALASOV 2002; CRYDERMAN *et al.* 1999a; DONALDSON *et al.* 2002). Although previous assays using w^{m4} have isolated mutations affecting the *Su(var)3-7*$^{+}$ locus (REUTER *et al.* 1990), these screens did not isolate single base-pair mutations within the *Su(var)3-7* gene, only deletions that included the locus. However, targeted mutagenesis of *Su(var)3-7* has produced mutations within this gene (SEUM *et al.* 2002). In my EMS based PDS screen, single base pair changes in *Su(var)3-7* were the most common mutation. The strong PDS phenotype may have permitted me to recover these mutations, which would have been missed in a w^{m4} screen.

My *Su(var)3-7* mutants offer further insights into the domains needed for protein function. The mutations are either point mutations in the zinc finger consensus sequences, deletions resulting in the production of a truncated product, or a deficiency for the entire gene (Figure 4.2.1). SU(VAR)3-7 has 7 zinc fingers (CLEARD *et al.* 1995) and mutations affecting either zinc finger 4 or 5 (*Su(var)3-7*^{P25}, *Su(var)3-7*^{P43}, and *Su(var)3-7*^{P71}) strongly suppress PDS. *Su(var)3-7*^{P12} has a deletion that removes the BESS motif, which is necessary for SU(VAR)3-7 self association (JAQUET *et al.* 2002). Therefore self-association, as well as zinc fingers 4 and 5, are necessary for SU(VAR)3-7 to silence w^{+mC} from *P{lacW}ci*^{Dplac}. These results support the conclusion that only the full length SU(VAR)3-7 protein enhances heterochromatic silencing (JAQUET *et al.* 2002) and suggest this is also true for PDS.

Mutations in *Su(var)3-7* produce a maternal effect that can be zygotically rescued (SEUM *et al.* 2002). Complementation testing using *Su(var)3-7*^{P9}'s maternal effect permitted me to identify our *Su(PDS)* mutants as *Su(var)3-7* alleles. None of the mutant *Su(var)3-7* alleles could zygotically rescue offspring produced by *Su(var)3-7*^{P9} homozygous females. Even hypomorphic mutants, such as *Su(var)3-7*^{9}, failed to zygotically rescue the maternal effect and this mutant can be kept as a homozygous stock through successive generations (SEUM *et al.* 2002). Thus, testing for zygotic rescue using *Su(var)3-7*^{P9} provides a sensitive test to detect and recover new mutations in *Su(var)3-7*. The fact that the *Su(var)3-7*$^{+t6.5}$ transgene could rescue *Su(var)3-7*^{P9} maternally and zygotically confirms that mutations within *Su(var)3-7* cause this phenotype. Although these mutations interrupted zygotic activity, alleles with mutations in one of the zinc fingers, such as *Su(var)3-7*^{P25},

Su(var)3-7^{P43}, and *Su(var)3-7*^{P71} had a weak maternal effect and hemizygous mothers (*Su(var)3-7*$^{-}$/ Deficiency) produced *Su(var)3-7* *(Su(var)3-7*$^{-}$*/ Deficiency)* mutant progeny at a reduced frequency. In comparison, *Su(var)3-7* alleles encoding truncated proteins, such as *Su(var)3-7*^{P9} and *Su(var)3-7*^{P49} had a strong maternal effect and hemizygous mothers never produced *Su(var)3-7* mutant progeny.

In general, mutations in *Su(var)3-7* that produce a maternal effect phenotype, also suppress PEV at *In(1)w*^{m4}, *Heidi* (SEUM *et al.* 2002), and *P{lacW}ci.* This suggests the mutations are interrupting a function common to these phenotypes. Referring to the maternal effect, hypomorphic *Su(var)3-7* mutants die later in development than amorphic *Su(var)3-7* mutations (SEUM *et al.* 2002). The same hypomorphic alleles that die later in development, such as *Su(var)3-7*^{9}, produce a weaker Su(PDS) phenotype than amorphic alleles, such as *Su(var)3-7*^{14}, which died earlier in development. An exception to this general trend is the suppression of *w*^{m4}, because *Su(var)3-7* mutants that produce a strong Su(PDS) phenotype did not consistently act as a strong *Su(var)*s. The insensitivity of *w*^{m4} to *Su(var)3-7*$^{+}$ dosage may be due to modifiers within the stocks masking the effect.



Domain		Amino Acid Range		Type
FT	ZN_FING	136	155	C2H2-TYPE.
FT	ZN_FING	238	262	C2H2-TYPE.
FT	ZN_FING	344	365	C2H2-TYPE.
FT	ZN_FING	406	431	C2H2-TYPE.
FT	ZN_FING	526	548	C2H2-TYPE.
FT	ZN_FING	656	680	C2H2-TYPE.
FT	ZN_FING	748	771	C2H2-TYPE.
FT	DOMAIN	86	92	POLY-ASP.
FT	DOMAIN	589	592	POLY-GLU.
FT	DOMAIN	780	788	POLY-ALA.
FT	DOMAIN	1008	1011	POLY-ASN.
FT	DOMAIN	1113	1116	POLY-ASN.

Motif reponsible for self association (Jacquet et al. 2002)

BESS Motif

Figure 4.2.1 - Diagram depicting the mutations and motifs in *Su(var)3-7*$^{+}$. The abbreviation for each mutation is listed as well as the amino acid change. The ranges are taken from Swiss-pro protein database.

4.2.1 *P* elements enhance heterochromatic characteristics at *P{lacW}ci*

The genetic screens indicate that heterochromatic proteins are necessary for silencing at $P\{lacW\}ci^{Dplac}$. This leads to speculation as to whether *P* elements initiate heterochromatinization or enhance pre-existing heterochromatic silencing at *P* inserts. A *P* element product, such as the P repressor, could initiate heterchromatin formation at *P* elements by producing a product that directly recruits HP1 and SU(VAR)3-7 to this region. For example, the *P* repressor protein could directly bind HP1 and SU(VAR)3-7 through protein-protein interactions while also binding to DNA sequences in the $P\{lacW\}ci^{Dplac}$ insert. Previous work has shown that ectopically recruiting SU(VAR)s to a locus can cause variable silencing. For example, a hybrid HP1 protein fused with the GAL4 DNA binding domain, can cause variegated w^+ transgene expression from a *P* construct containing the GAL4 binding site (SEUM *et al.* 2000). Another example is the *polyhomeotic*^{P1} (ph^{P1}) allele, which encodes a chimeric protein as a result of a KP element insert at the *ph* locus (BELENKAYA *et al.* 1998). This KP-PH peptide recruits POLYCOMB and POSTERIOR SEX COMBS to *P* elements and represses a *yellow* allele that has a *P* element insert.

There are two main arguments against a direct interaction between a *P* product and SU(VAR)s. First, such a direct interaction should consistently enhance heterochromatic gene silencing at all *P* inserts. However, *P* w^+ inserts, such as *P{hsp26-pt-T}2-M010.R*, which are sensitive to *Su(var)3-7*$^+$ dosage, do not variegate in the presence of *P* elements. In contrast, *P* elements can suppress variegation from *P* w^+ inserts at other locations, other than *ci*, on chromosome 4. Also, *P* elements increase w^{+mC} expression from translocations that include $P\{lacW\}ci^{Dplac}$ (LOCKE *et al.* Submitted). *Su(var)3-7*$^+$ continues to dose-dependently repress w^{+mC} expression from these translocations although it no longer causes variegation. The second argument is explained in detail below and argues that the *P* factor is not recruiting new heterochromatic modifiers to this region. Instead, these heterochromatic modifiers already can act in this region in the absence of *P* elements because factors other than *P* elements can cause w^{+mC} silencing in this region.

Although a simple model where a *P* factor directly recruits heterochromatic modifiers to *P* inserts cannot explain my results, I cannot rule out a more complex interaction where protein-protein interactions could occur at certain nuclear positions. Local chemical modifications occur within certain regions of the nucleus. For example, nucleosomes are chemically modified in certain regions. Similar chemical modifications could occur to the P factor and or the heterochromatic proteins with which it interacts. Such chemical modifications could change the affinity these factors have for one another.

Increasing the *Su(var)3-7*$^{+}$ dose to three doses causes w^{+mC} variegation from *P{lacW}ci*^{Dplac} in the absence of any additional *P* elements. This indicates that heterochromatic modifiers already act in this region in the absence of *P* elements. This w^{+mC} transgene behaves differently from inserts in typical euchromatic regions, which are insensitive to *Su(var)3-7*$^{+}$ dosage.

The *P{lacW}ci*^{E} alleles also indicate that these heterochromatic modifiers already act in this region. In both, *P{lacW}ci*^{E1} and *P{lacW}ci*^{E2} there is a proximal *gypsy* insertion that is coincident with the w^{+mC} variegation. Although *gypsy* insertions at *yellow* (*y*) can cause y^{+} variegation when there are mutations in *mod(mdg4)*, *gypsy* insertions usually act as insulators and do not normally produce heterochromatic changes at other loci (GDULA *et al.* 1996; GERASIMOVA *et al.* 1995). Mutations in *su(Hw)*$^{-}$ suppress both the variegation in *mod(mdg4)*$^{-}$ mutants and insulation. I tested *su(Hw)*$^{-}$ combinations that interrupt this insulation and found that these combinations do not consistently suppress the variegated phenotype. However, the same *Su(PDS)* mutants isolated in my screen, such as *Su(var)3-7* and *Su(var)2-5*, suppressed variegation from the *P{lacW}ci*^{E} alleles. This indicates that changes in local *cis* DNA sequence trigger the same heterochromatic silencing that *P* elements do in *trans*.

I have identified a limited number of heterochromatic modifiers that act at *P{lacW}ci* and this suggests the chromatin structure differs within this region from the chromatin structure at other heterochromatic regions. General conditions that modify hPEV also modify w^{+mC} expression at *P{lacW}ci*^{Dplac}. For example, changing the chromosomal position (LOCKE *et al.* Submitted), increasing the rearing temperature, and an extra Y-chromosome, suppress the variable silencing in the *P{lacW}ci* region. This confirms that the variegated phenotype results from heterochromatic silencing.

This heterochromatic region includes *P{hsp26-pt-T}ci*$^{2\text{-}M1021.R}$, which is inserted ~140 bp away from the insertion site of *P{lacW}ci*^{Dplac}. Sun *et al.* (2000) compared chromosome structure at *P{hsp26-pt-T}2-M010.R* and *P{hsp26-pt-T}ci*$^{2\text{-}M1021.R}$. *P{hsp26-pt-T}2-M010.R* is located much farther from the centromere on chromosome 4 (102B) compared to *P{hsp26-pt-T}ci*$^{2\text{-}M1021.R}$ (101F). Both these inserts were defined as occurring in a euchromatic-like region, because they uniformly express w^{+} throughout the eye in M type flies (SUN *et al.* 2000). However, *P{hsp26-pt-T}ci*$^{2\text{-}M1021.R}$ behaves like w^{+} transgenes inserted in heterochromatin because it is less accessible to endonuclease digestion and heat shock induction (SUN *et al.* 2000). Therefore, *P{hsp26-pt-T}ci*$^{2\text{-}M1021.R}$ could be considered heterochromatic despite being uniformly expressed. This contrasts with *P{hsp26-pt-T}2-M010.R*, which is accessible to endonuclease digestion and heatshock induction as are the other lines that are not variegated. Although our tests with w^{+} transgenes on chromosome 4 indicate both *P{lacW}ci*^{Dplac} and *P{hsp26-pt-T}2-M010.R* are sensitive to *Su(var)3-7*$^{+}$ dosage, other tests studying chromatin structure indicate a difference in chromatin structure at different w^{+} inserts on chromosome 4. This

difference in chromatin structure, as well as the position next to the centromere, may explain why PDS occurs at *ci* but not at the w^+ transgenes inserted at other locations.

My observation that the *P{lacW}ci*^{E}/ *P{lacW}ci*^{Dplac} heterozygote is more silenced than the *P{lacW}ci*^{Dplac}/ + indicates that silencing also acts in *trans* and affects the other homologue. Transvection occurs between other heterochromatic regions (DORER and HENIKOFF 1997) and rearrangements suppress *trans*-repression (HENIKOFF 1997; HENIKOFF and COMAI 1998). Similarly, translocations that reposition *P{lacW}ci*^{Dplac} inhibit the *trans*-silencing.

4.2.2 Are *P* elements modifiers of PEV?

Since *P* strains do not modify expression from w^{m4}, *P* elements are not considered to be general modifiers of hPEV, but the P factor acting in *trans* has attributes associated with chromatin modifiers. *P* elements are enhancing heterochromatic silencing at *P{lacW}ci* and traditionally only increasing *Su(var)* dosage or changing chromosomal position could trigger such an event. The fact that P elements can increase or decrease expression from *P* w^+ transgenes, depending on their location, does not argue against the P factor being a chromatin modifier. Other heterochromatic modifiers, such as *Su(var)2-5*, can also suppress or enhance expression depending on the location of the locus (BALASOV 2002; CRYDERMAN *et al.* 1999a; PIACENTINI *et al.* 2003). The relationship between the identified heterochromatic modifiers and *P* elements could resemble the epistatic relationship described between other *Su(var)* genes (SCHOTTA *et al.* 2003). In terms of heterochromatic silencing at *P{lacW}ci*, *Su(var)3-7* is epistatic to the silencing caused by *P* elements. By itself, *Su(var)3-7*$^+$ can cause variegation at *P{lacW}ci* and occurs downstream in the pathway that leads to variegation.

Thus far, *P* elements have only been shown to modify expression from w^+ transgenes inserted within *P* constructs. This means that a sequence within the *P* element may be necessary for the *P* elements to affect expression in *trans*. This differs from classic heterochromatic modifiers that act on many loci and rearrangements without requiring a specific sequence. I would not expect *P* elements to silence a w^+ transgene inserted in the ci distal regulatory that has no flanking *P* element sequence. Further, we have not tested whether P elements act dose-dependently to cause silencing at *P{lacW}ci*^{Dplac}. Heterochromatic modifiers act dose dependently to silence expression from heterochromatic regions, such as w^{m4}. Whether the P factor should be classed with other heterochromatic modifiers, depends on further biochemical and genetic characterization. However, mutations within other TEs, such as *nomad*, can modify variegation from w^{m4}. A mutation within a single *nomad* insertion, which reduces transcript expression, enhances heterochromatic silencing at w^{m4} and bw^{VDe2} (WHALEN *et al.* 2003). Therefore, other TEs appear to encode factors that can modify heterochromatin structure.

It has been suggested that *P* element transgenes and their derivatives could act as "protosilencers " (FOUREL *et al.* 2002). Combinations of protosilencers, either by themselves or with other *cis* acting sequences form stable heterochromatin. Individually, however, protosilencers activate transcription or act as insulators. This description fits the characteristics of individual *P* elements and *gypsy* elements inserted in other regions while also explaining their effect at *ci*. Both elements could be protosilencers that interact with other cis-acting sequences near *ci* to form stable heterochromatin complexes.

In the case of *P{lacW}ci*^{Dplac} and *P{hsp26-pt-T}ci*$^{2\text{-}M1021.R}$, these sequences in combination with local sequences are not strong enough to stabilize heterochromatin formation by themselves, but the addition of a *P* product (or more SU(VAR)3-7^{+}) helps stabilize heterochromatin formation. The *P{lacW}* arrays act similarly and a threshold number of repetitive sequences are necessary to cause heterochromatic silencing (DORER and HENIKOFF 1994). This suggest a certain repeat threshold or configuration of *cis* sequence is necessary to stabilize the heterochromatin composed of both HP1 and SU(VAR)3-7. Once past this threshold the chromatin structure is stable and enhances silencing. Specific sequences within the repeats appear to play a role because tandem repeats of *P[ry; Prat:brown]* do not result in heterochromatic silencing (CLARK *et al.* 1998). Chromosome position also affects this stability and proximity to heterochromatin enhances heterochromatic silencing at *P{lacW}* repeats (DORER and HENIKOFF 1997) and PDS at *P{lacW}ci*^{Dplac} (LOCKE *et al.* Submitted).

As well, there is a weak relationship between proximity to the heterochromatic chromocenter and *P* element effects on the *P{lacW}ci*^{Dplac} translocations. Instead of silencing expression from translocations that include *P{lacW}ci*^{Dplac}, pigment analysis indicates *P* elements increase expression from the w^{+mC} gene as distance increases from the centromere. In comparison, expression in the absence of *P* elements only increases slightly as distance increases from the centromere. This dual effect may reflect a dual role of the P factor. TEs, like the P element, must balance replication with senescence. *P* elements that replicate unchecked will not be found in *Drosophila* populations because they kill their host. In the reverse, if *P* elements do not replicate then they will eventually be lost as a mobile element in the genome because random mutation will eventually knockout all the autonomous copies. Therefore, *P* elements could produce a single factor or multiple factors that could induce or repress transcription at the appropriate times in development to allow replication without reducing the fitness of their host. The P factor may interact with the host's chromatin structure to balance these requirements. Since chromatin structure changes along the length of the chromosome, the P factor could interact with the unique chromatin structure in these various regions to either promote or repress transcription.

In conclusion, the *P* elements act indirectly to silence expression from the w^{+mC} transgene in *P{lacW}ci*^{Dplac} by changing chromatin structure at

this locus. This is evident because within this region, changes in cis-DNA sequence can initiate silencing through the same heterochromatic modifiers. This region is already primed for heterochromatic silencing by *P* elements or the other changes that tip the balance towards silencing rather than expression. However, these results do not demonstrate how *P* elements interact with chromatin structure. To study this interaction, the *P* element portions that promote silencing at *P{lacW}ci*^{Dplac} have to be determined and studied.

4.2.3 Examples of both Type I and II elements exist that modify *P* dependent phenotypes

Thus far, both Type I and II elements affect the *P* element dependent phenotypes I have studied. The *KP-D* and *KP-U* chromosomes, that only contain Type II elements, modify the same phenotypic effects as *P{ry*$^{+}$ *SalI}89D*, which is a Type I element. Both types cause silencing at *P{lacW}ci*^{Dplac} but conversely, increase expression from other w^{+} transgenes on chromosome 4. Both types suppress the *P* element dependent phenotypes associated with *ci* and *RpS3a* expression. Both types also suppress the vestigial phenotype caused by $vg^{21\text{-}3}$ but do not have a strong effect on the vestigial vg^{2a33} allele.

The difference between vg^{2a33} and other *P* element dependent *vg* alleles that are *P* element dependent is a 50 bp deletion at the 3' end of vg^{2a33} (HODGETTS and O'KEEFE 2001). This deletion includes the 11 bp sequence to which the KP repressor can bind *in vitro* at high protein concentrations (LEE *et al.* 1996). Since the site with the highest affinity for transposase (KAUFMAN *et al.* 1989) and KP proteins (LEE *et al.* 1996) is retained in vg^{2a33}, the loss of a weak binding site would not seem to offer a sufficient change to account for the loss of *P* element influence. If *P* element suppression of $vg^{21\text{-}3}$ is solely mediated through the P repressor protein binding, then the 50 bp deletion should still be able to bind with high affinity to the remaining sequence. Thus, a P repressor based mechanism does not satisfactorily explain these results.

According to the literature, the only somatic phenotype that appears to react differently to the P Types is *P* promoter expression using the *P-lacZ* reporter (LEMAITRE and COEN 1991). *P* elements cause the repression from *P-lacZ* inserts in somatic cells. Since there are degrees to repression, *P-lacZ* acts as a qualitative assay rather than a quantitative assay with a on/off result. I have not used this as a test in my analysis because of the difficulty in interpreting a qualitative assay and because the difference in the literature is probably due to only a few Type II elements being tested. Lemaitre *et al.* (1991) tested Type II elements that suppress hybrid dysgenesis but did not test Type II elements that affect somatic phenotypes. The *KP* elements I tested had somatic effects and if these elements were tested using this assay, I expect they would repress *P-lacZ* expression in somatic tissues.

The original experiments that divided *P* elements into two sequence types is based on the frequency with which the insertion Types could modify P element dependent phenotypes. Gloor *et al.* (1993) transposed

P constructs representing both types and found that Type I and Type II inserts frequently suppressed $vg^{21\text{-}3}$. Originally, they tested many inserts because the insertions occur randomly and each location will result in a different level and pattern of expression. This position effect results in *P* inserts modifying the *P* dependent phenotypes to different extents. They constructed a continuous series of P constructs each encoding a successively smaller P repressor and tested 34-124 different insertions of each type of construct to determine whether the position or the sequence was responsible for the change in phenotype. A sequence change that prevented the *P* sequence from modifying *P* dependent phenotypes should affect all the inserts, whereas position effect will result in inserts modifying the *P* dependent phenotypes to different extents. These experiments demonstrated that Type I inserts that had exons 0, 1, and 2, including the first 9 amino acids in intron 2-3, frequently modified the *P* dependent phenotypes tested. A similar experiment would be needed to determine whether the same classification applies to the *P* element dependent phenotypes at *ci*. Further, multiple *KP* elements exist on the chromosomes tested and thus far, a single *KP* element has not modified *P* element dependent phenotypes in my tests.

4.3 Type I and intermediate elements are not equivalent

The ability of *KP* elements to modify *P* dependent phenotypes in somatic tissues demonstrates that exon 2 is not necessary for this effect. To determine if this is true for all *P* inserts, I constructed a *P* element construct that has the FLP recombination target (FRT) (GOLIC and LINDQUIST 1989) sequence inserted in the introns flanking exon 2. I refer to this construct as P{I Sal} and it encodes the full length P repressor protein. After transforming *D. melanogaster* with this insert, exon 2 could be removed after crossing in a recombinase (FLP) transgene that targets the FRT sequence for recombination. Removal of exon 2 converts the insert into an intermediate element that lacks exon 2 and is now referred to as *P{II SalI}*. Since Type I and intermediate elements at the same location can be tested, position effect should not confound the results. This intermediate element P{II Sal} has more sequence than a Type II element but has less sequence than a Type I element. Such *P* inserts that fall between the two types do not modify somatic *P* dependent phenotypes.

Only one of the original eight *P{I SalI}* transformants caused w^+ variegation at $P\{lacW\}ci^{Dplac}$. This frequency is expected because previous transformation with similar constructs did not always produce inserts that modify other *P* dependent phenotypes such as $vg^{21\text{-}3}$ (GLOOR *et al.* 1993). Molecular and genetic analysis found that this transformant, referred to as *P{I SalI}1-3*, had two inserts on chromosome 3. "Flipping out" exon 2 from both inserts in the *P{I SalI}1-3* completely removed the ability to cause PDS at *P{lacW}ci* and *BX2*, as well as the ability to suppress $vg^{21\text{-}3}$. Deleting exon 2 from one of these inserts, at 91D, didn't affect PDS, while deleting exon 2 in the other insert, at an unidentified

location, correlated with loss of silencing. The insert at the unidentified location appears to be solely responsible for modifying the *P* dependent phenotypes because loss of exon 2 from only this insert removed the ability for this chromosome to modify *P* dependent phenotypes. These results clearly demonstrated that the presence of exon 2 in inserts at specific locations is needed for silencing.

Using *Δ2-3*, I mobilized non-silencing *P{I SalI}* inserts and transpositions to new locations created sixteen new inserts that caused w^{+mC} silencing at $P\{lacW\}ci^{Dplac}$. This confirms that the insert's position rather the element's sequence is responsible for the lack of PDS. Flipping out exon 2 always removes the ability to cause silencing. This confirms Gloor *et al.*'s (1993) original results that divided the *P* elements into two types and indicates that *P* element dependent phenotypes at *ci* respond similarly to the other somatic *P* dependent alleles that have been characterized.

Almost all the *P{I SalI}* selected for PDS at $P\{lacW\}ci^{Dplac}$ modified the other *P* dependent phenotypes. The exceptions indicate that an insert that modifies one *P* dependent allele does not necessarily modify another. Often the strength with which one insert strongly modified a *P* dependent phenotype did not always correlate with its ability to modify another P dependent phenotype. For example, *P{I SalI}M23-2* weakly silences $P\{lacW\}ci^{Dplac}$ but strongly silences *BX2*. However, most of the other inserts had an opposite affect and strongly silence $P\{lacW\}ci^{Dplac}$ and weakly silence BX2. Additionally, the *P{I SalI}* inserts usually suppress both M^{F2} and vg^{21-3} but there is one exception: *P{I SalI}M26-3*. The *P{I SalI}M26-3* insert suppresses neither the Minute nor vestigial phenotypes. This result could be easily explained if *P{I SalI}M26-3* is inserted at a location that results in expression in the eye imaginal disc but not in the wing imaginal disc. The ability of these Type I inserts to modify the *P* dependent phenotypes could rely on their expression pattern.

Three *P{II SalI}* inserts were mobilized but none could generate inserts that cause PDS at $P\{lacW\}ci^{Dplac}$. This indicates the inability of *P{II Sal}* to modify the *P* dependent is due to its sequence rather than position effect. This phenotypic difference was not due to a differences in transposition rate because *P{I SalI}3a-Y* and *P{II SalI}3a-Y* inserts jumped from the Y chromosome to another chromosome at the same frequency. Therefore, Type I inserts frequently modify *P* dependent phenotypes, while intermediate elements had no effect. The sequence within exon 2 is not necessary for silencing at $P\{lacW\}ci^{Dplac}$. Instead, the difference between Type I and intermediate sequences may involve protein stability or change in protein activity.

4.3.1 What constitutes the P factor that mediates *P* effects?

The P repressor appears to mediate *P* effects because changes in to protein coding portions of the P element prevent these effects. A protein model predicts that expression rate and pattern from these inserts should correlate with their ability to cause their effect. In the case of

PDS, expression from the *P{I SalI} inserts* should be inversely proportional to silencing at *P{lacW}ci*^{Dplac}. Since silencing and expression can be quantified using pigment analysis and RT-PCR, future experiments can try to find this correlation.

Other tests can be done with these inserts. Although *P* elements cause silencing at *P{lacW}ci*^{Dplac}, they also increase expression from w^{+} transgenes inserted at other locations. This activity may be the result of the same mechanism that silences w^{+mC} expression at *P{lacW}ci*^{Dplac}. If it is part of the same activity then exon 2 should be necessary within the *P{I SalI}* inserts to increase expression from other w^{+} transgene as well as silence w^{+mC} expression from *P{lacW}ci*^{Dplac}.

As explained in the following section, P repressor alone cannot be the only molecule that mediates *P* effects. The P repressor protein is not necessary for producing P cytotype in the germ line. Another mechanism could be an RNA molecule, such as molecules produced in RNAi. However, a protein model rather than an RNA based model best explains why converting the same insert from *P{I SalI}* to *P{II SalI}* completely removes the effect strongly. This change in DNA sequence does not change any homology between the *P{I SalI}* and the *P{lacW}ci*^{Dplac} target. A homology dependent (RNA) model would predict the opposite effect and removing non-homologous sequence between the two inserts should have no effect. Only removing homologous sequence should have an effect because it prevents production of an RNA molecule that could bind in *trans* at *P{lacW}ci*^{Dplac} .

4.3.2 Arguments for a second mechanism that is not protein based but due to the location of the *P* inserts: RNAi?

Researchers believed that the P repressor was responsible for P cytotype, but *P* inserts, which do not encode the P repressor, have been isolated that reproduce P cytotype effects: *LK-P(1A)* and *T1* (MARIN *et al.* 2000; ROCHE *et al.* 1995; RONSSERAY *et al.* 2001; RONSSERAY *et al.* 1991; RONSSERAY *et al.* 1996; RONSSERAY *et al.* 1997; RONSSERAY *et al.* 1998). The ability of these inserts to reproduce P cytotype is attributed to their location in TAS repeats at the telomere or in repetitive *P* sequence. Although these inserts do no modify *P* dependent phenotypes in somatic tissues, the *P* inserts that do modify *P* dependent phenotypes in somatic tissue could be acting through a similar mechanism. If this is true, expression from the *P* inserts in somatic tissues will not correlate with silencing at *P{lacW}ci*^{Dplac} just as expression from the *P* inserts, such as *LK-P(1A)*, that cause P cytotype does not correlate with cytotype strength.

Why *P* inserts at certain locations should trigger a sequence homology based silencing system is not understand. This sequence homology based system could be mediated by an RNA molecule, or pairing between *P* element inserts (STUART *et al.* 2002). The nature of these mediators does not explain why only certain locations evoke this effect. The locations that cause P cytotype effects have heterochromatic characteristics. Perhaps heterochromatin formation triggers the P

cytotype effects. However, it should be noted that not all *P* inserts in heterochromatic regions reproduce P cytotype effects (RONSSERAY *et al.* 1998).

Some TEs act as foci for heterochromatin formation. Research in *S. pombe* has found that mutations in genes involved in RNA interference (RNAi) and heterochromatin formation suppress heterochromatin formation at the long terminal repeats (LTR) of retro-transposons and silencing of adjacent genes (SCHRAMKE and ALLSHIRE 2003). An RNAi pathway, similar to the one in *S. pombe*, could be working in *Drosophila* and causing heterochromatin formation at TEs. In this RNAi system, RNAi produced from TEs at certain locations would seed heterochromatin formation at TEs with complementary sequence. An alternative possibility is that high expression from multiple TEs could trigger RNAi production, which could act similarly (PAL-BHADRA *et al.* 2002). RNAi described thus far in *Drosophila* acts at the post-transcriptional level, as well as the transcriptional level. The PDS in *Drosophila* acts only at certain locations at the transcriptional level because *P* elements only silence w^+ transgene expression at *ci* or in the *P{lacW}* arrays. However, RNA mediated chromatin remodeling does occur specifically on the X-chromosome in *Drosophila* (KELLEY *et al.* 1999). Therefore other RNA systems could exist that could act and whose effect is position specific to the *ci* region.

RNAi only explains the processing of dsRNA into 23-26 nucleotide molecules that target RNA for post-transcriptional gene silencing or genes for heterochromatic silencing. However, other RNA species exist that can cause changes in chromatin structure. Such RNA includes the *roX1* and *roX2* genes that encode an RNA molecule, which does not encode a protein, and is necessary for hyperactivating the X chromosome in *Drosophila* (KELLEY *et al.* 1999). The *Xist* gene in humans works similarly in sex chromosome compensation in females and is necessary for down-regulating one X-chromosome (PARK and KURODA 2001). In both cases, RNA modifies chromatin structure in a functionally constrained region within the nucleus. An RNA molecule produced from *KP* elements could act similarly at *ci* because of the unique chromatin structure found at this locus.

To determine if there is an interaction between the RNAi pathway and PDS, I tested mutations in genes involved in the RNAi pathway, such as *piwi* and *AGO1* for the ability to modify w^+ expression from the *P{lacW}ci* alleles, *T1*, and *BX2*. These tests looked for a dominant interaction. Only the *T1* and *AGO1* combination enhanced variegation. This interaction between *T1* and *AGO1* also resulted in reduced viability and a Minute-like phenotype. To date, this is the first interaction between *AGO1* and a variegated locus described in *Drosophila (THE FLYBASE CONSORTIUM 2003).*

4.3.3 Is there a defense system against TE?

Next to *P* elements, the next best studied TE is the *I* element and its regulation in the germ line parallels *P* element regulation. Crossing

males form Inducer (*I*) strains, which have *I* elements, to females from Reactive (R) strains, which do not have *I* elements, results in hybrid dysgenesis. However, the reciprocal cross does not result in hybrid dysgenesis. Like *P* elements, a maternally inherited cytotype suppresses *I* element expression and transposition. As with *P* elements, this repressive capability builds up through successive generations in the female germ line. Unlike the *P* element, the *I* element is a different type of TE and belongs to the non-LTR or long interspersed like retro-element class. The similarities between *I* and *P* element regulation suggest a common defense mechanism is preventing mobilization.

The ability to repress expression from *I* element constructs depends on both *I* copy number and sequence homology (CHABOISSIER *et al.* 1998; JENSEN *et al.* 1999a; JENSEN *et al.* 1999b). Strains with more insertions expressing sequences from the *I* element acquire repressive capabilities in a fewer number of generations as it passed through the female germ line. This co-suppression requires sequence homology between the *I* elements and does not require any translated product form the *I* element.

Similar to *P* elements, this silencing system also acts through *I*-related elements inserted in heterochromatin (JENSEN *et al.* 2002). Jensen *et al.* (2002) find that expressing an internal fragment from the *I* element can repress expression from *I* elements and transgenic constructs that have the *I* promoter fused to a reporter gene. There is no sequence homology between the *I* element fragment they are expressing and the *I* promoter. However, they argue that the silencing mechanism is acting through another sequence that has sequence homology for both the *I* promoter and transgenic *I* sequence they express in their construct (Figure 4.3.1). After looking for such a sequence, they found that there are *I*-related elements located in pericentromeric heterochromatin that could act as this bridge.

The possibility that another nucleotide sequence can act as a bridge in the silencing reaction arises from Pal-Bhadra's (1999) work. Non-homologous co-suppression occurs between transgenes that do not have homologous sequence. Instead of acting directly between homologous sequences, non-homologous co-suppression acts through a sequence that has homology to both transgenes to mediate the silencing. In this example, the endogenous *alcohol dehydrogenase* (*Adh*) gene acts as a bridge between a *Adh-w* fused gene and a reciprocal *w-Adh* chimera. There is no sequence homology between these chimeric constructs. Removing the endogenous *Adh* gene prevents co-suppression between the *w-Adh* and *Adh-w* constructs. Therefore, the *Adh* gene acts as bridge between transgenic constructs.

Unfortunately, Jensen *et al.* are unable to test the loss of *I*-related elements on *I* element silencing. Based their work, the *I* elements could directly repress each other, only act through the *I* elements in pericentromeric heterochromatin or both. Only by completely removing the *I*-related elements could these possibilities be tested. Thus far, only single *P* elements in heterochromatic locations or heterochromatic *P*

arrays trigger genome wide silencing. I would suggest that it is the same for *I* elements and *I* elements need a sequence in heterochromatin to cause genome wide silencing. Expressing transgenes with *I*-sequence primes or assists silencing at the *I*-related elements in heterochromatin and this assists the silencing mechanism. My approach is different from the author's interpretation, who concluded that silencing could occur directly as well as indirectly through the *I* element in heterochromatin.

The regulatory mechanisms preventing I-R or P-M hybrid dysgenesis occur in the germ line. Therefore these results may not be applicable to PDS at *P{lacW}ci*^{Dplac} that occurs in somatic cells. However, papers published by Pal-Bhadra (PAL BHADRA *et al.* 1998; PAL-BHADRA *et al.* 1997; PAL-BHADRA *et al.* 1999; PAL-BHADRA *et al.* 2002) demonstrate that homologous co-suppression and non-homologous co-suppression occur in somatic cells. This co-suppression relies on either counting the number of gene copies or sensing transcription levels from certain genes. When copy number or transcription levels exceed a certain level their combined effect triggers co-suppression. In this model, all the gene copies contribute equally or their contribution is determined by their transcription rate. Silencing occurs both at the transcriptional level and post-transcriptional level through *polycomb* and RNAi pathways respectively.

4.3.3.1 Cytotype and co-suppression

To summarize Pal-Bhadra's results and apply them to the cytotype models for I and *P* elements, I constructed figure 4.3.1. This diagram studies *I* (A) and *P* (B) elements separately. With I elements, there may be a direct interaction between the different insertions (1). This direct interaction requires no intermediate homologous sequence to mediate the silencing effect. Co-suppression occurs because high expression levels or gene copy number exceeds a certain threshold and this leads to silencing between the insertions. In the indirect model (2), expression or high copy number from various inserts causes changes in the chromatin structure of homologous sequences inserted in heterochromatic regions. For instance, expressing an *I* sequence, such as *open reading frame* 1 (*ORF1*) changes the chromatin structure at the *I*-related sequences inserted in pericentromeric heterochromatin. Heterochromatin formation at the pericentromeric insertion spreads in *trans* to sequences that have homology to the I-related element. Although expression from the *hsp70-ORF1* transgene triggers heterochromatin formation at the *I*-related sequence, this silencing spreads to inserts homologous to either *ORF1* or *ORF2* because the *I*-related element has homology to both sequences.

How high gene copy number or expression leads to this chromatin changes at a certain insertion is not understood. Possibly, the many inserts could cause the production of siRNAs that bind at homologous sequence and seed heterochromatin formation. In addition, how the silencing spreads from this insertion to the other *P* inserts is not understood either. However, repetitive sequences that initiate heterochromatin formation are probably necessary for this to occur. This

means that an insert must be in a heterochromatic region or be tightly linked in *cis* to a region where repressive chromatin usually forms.

With *w-Adh/ Adh-w* co-suppression, a non-transcribed sequence in the *Adh* promoter is necessary for non-homologous co-suppresssion to occur. According to Pal-Bhadra's theory, this DNA segment is possibly a PRE sequence that recruits POLYCOMB (PC) to the *Adh* gene. The presence of *w-Adh* genes causes the PC complex to spread in *cis* and in *trans*, which silences homologous sequences. Although the w^+ gene has homology to both the *w-Adh/ Adh-w*, it does not mediate non-homologous co-suppression between these transgenes. Presumably, w^+ does not mediate this interaction because it lacks this PRE or other heterochromatic like qualities.

The *P* inserts that mimic the P cytotype effects (B) could work based on the co-suppression models described above. However, there are important differences. Firstly, two *P* elements inserts at 1A *(LK-P(1A))*, which are not strongly expressed, reproduce P cytotype-like effects. Therefore the strong co-suppression initiated by these inserts does not require enhancement from other *P* inserts elsewhere in the genome to spread in *trans*. Therefore, many *P* element copies are not necessary to cause P cytotype. Only *P* elements at certain locations cause P cytotype.

However, under special circumstances *P* elements appear to enhance the P cytotype-like effects caused by *P* inserts at certain locations. When there is a single *P-lacZ* construct inserted in the TAS repeats at 1A, the *P-lacZ*(1A) does not suppress hybrid dysgenesis. However, in combination with paternally inherited *P* elements, which do not by-themselves suppress hybrid dysgenesis, the *P-lacZ*(1A) suppresses hybrid dysgenesis. Presumably, the paternally inherited *P* elements enhance co-suppression by acting through the *P-lacZ(1A)* insert. Below, I argue the paternally derived *P* elements enhance heterochromatin formation at *P-lacZ(1A)* and this contributes to the P cytotype-like effects.

Lastly, co-suppression in the *Adh* system and P cytotype involves a different set of chromatin modifiers. Whereas *Pc* is necessary for *w-Adh/ Adh-w* co-suppression, HP1 is necessary for P cytotype effects caused by the *P* inserts at 1A. However, mutations in *Su(var)2-5* do not generally prevent P cytotype produced by *P* strains or the *P* arrays, such as *T1*. These results suggest that heterochromatic modifiers that are responsible for *trans*-silencing depend on the insert's location responsible for the effect. Different inserts, such as *T1*, produce a different heterochromatic complex that acts in *trans* to produce P cytotype. Although mutations in *Su(var)2-5* weakly suppress w^{+mC} variegation from *T1*, mutations in *Su(var)3-7* strongly suppress this variegation. Presumably, mutations in *Su(var)3-7* would strongly interrupt the P cytotype-like effects caused by *T1* if it is part of the same complex in the germ line.

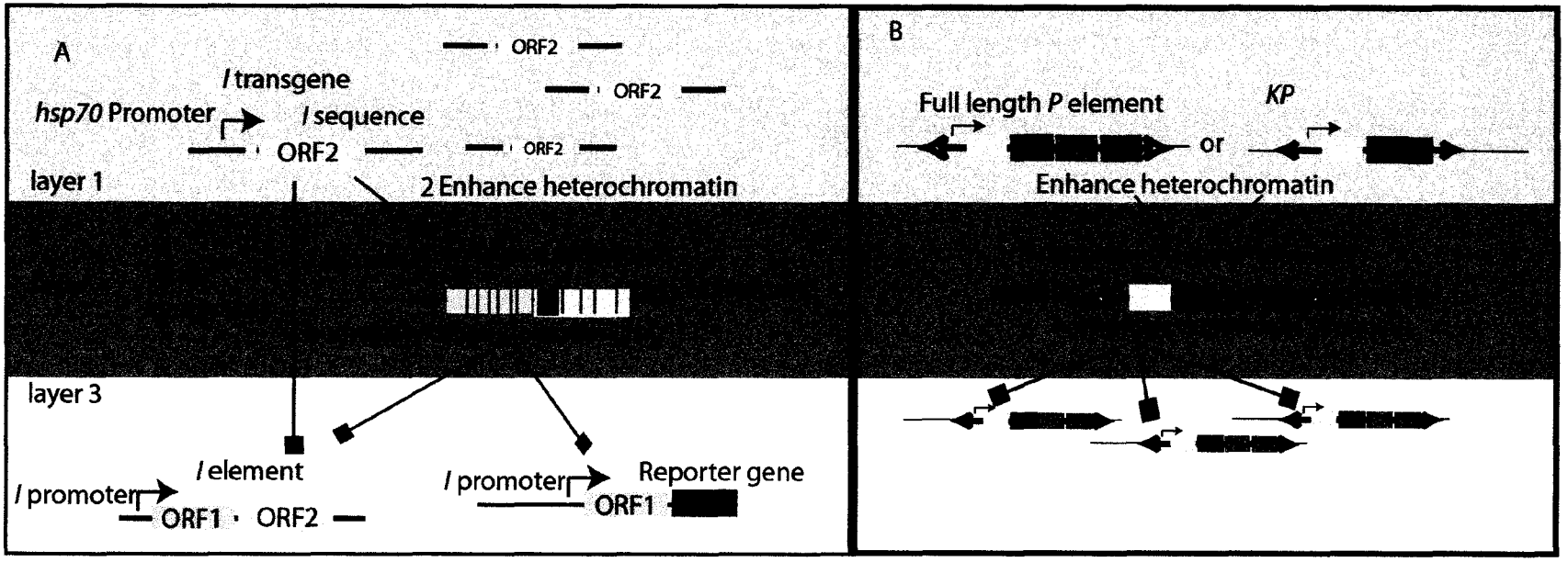


Figure 4.3.1 - Three layers to the nucleotide sequence homology dependent system. Section **A** describes the interactions that occur between *I* elements to produce the cytotype that suppresses *I* element transposition. In layer 1 (light grey), transposable elements act in *trans* to initiate silencing. Transcription from a multiple transgenes with sequence homology to the *I* element results in co-suppression by directly causing silencing from other sequences with similar homology (1). In layer 2 (dark grey), these *I* transgenes also cause non-homologous co-suppression through an *I*-related sequence.This *I*-related sequence is an ancestral *I* element that has a few sequence changes from the modern *I* element (represented by black bars) (2). I postulate that somehow expression from the *I* transgene enhances heterochromatin formation at the *I* related sequence. This enhanced heterochromatic state causes silencing at other genes with homologous sequence to the *I*element. In layer 3 (white), genes with sequence homology to the *I* transgene initiating silencing and to *I*-related sequences are silenced. Section **B** describes the same interactions that could be occurring between *P* elements to generate *P* cytotype. My thesis finds that *P* elements (layer 1) can enhance heterochromatin formation at certain *P* insertions (layer 2) embedded in heterochromatin (represented by the RRRRs). Heterochromatin formation at these certain *P* insertions act in *trans* to silence expression from *P* elements throughout the genome producing P cytotype.

In figure 4.3.1, *T1*, *BX2*, *LK-P(1A)*, and *P{lacW}ci*^{Dplac} fit in the middle row because *P* elements in *trans* enhance heterochromatin silencing at these inserts. In the case of *T1* and *LK-P(1A)* these inserts silence *P-lacZ* expression in the germ line but not in somatic cells. Similarly *P{lacW}ci*^{Dplac} does not affect expression from other w^{+mC} transgenes in somatic cells. However, I have not tested whether *P{lacW}ci*^{Dplac} can suppress hybrid dysgenesis in the germ line.

4.3.4 Is there a link between co-suppression and heterochromatin formation?

Thus far, I have claimed that there is a link between heterochromatin and P cytotype. This claim is based on the experiments that found that mutations in *Su(var)2-5* repress P cytotype effects caused by *LK-P(1A)*. Experiments with the *P{lacW}* repeats further support this claim. The *T1* tandem array is molecularly equivalent to *BX2*, except for a chromosome rearrangement that moves it closer to heterochromatin (DORER and HENIKOFF 1997; RONSSERAY *et al.* 2001). This change in position produces two different phenotypic effects. First, flies with *T1* as the only w^{+} source have predominately white eyes compared to flies with BX2 where pigment accumulates in most of the ommatidia. This indicates the w^{+mC} genes within the *T1* array are consistently more silenced than the *BX2* array. Secondly, the *T1* array produces a P cytotype-like effect, which *trans*-silences other *P* elements within the germ line, while the *BX2* array produces a much weaker effect (RONSSERAY *et al.* 2001). A correlation between these properties suggests that increasing the heterochromatic qualities of a transgene array increases *P* cytotype strength in the germ line. Therefore, factors that increase the heterochromatic qualities at *P* elements arrays should strengthen *P* cytotype.

P elements produce a factor that amplifies the heterochromatic qualities at *P{lacW}ci* and the *P{lacW}* arrays in somatic tissues. These *P* elements could act similarly in the germ line and amplify heterochromatic qualities at *P* arrays and certain *P* inserts. This interaction is depicted between the first and second layer in Figure 4.3.1. *P* inserts at other locations enhance heterochromatic silencing at a *P* insert in heterochromatic sequence, which in turn silences expression from other *P*s throughout the germ line. *P{lacW}ci*^{Dplac} is an example of a *P* inserted in a heterochromatic region where other *P* insertions enhance the heterochromatic silencing. Whether heterochromatic silencing at *P{lacW}ci*^{Dplac} spreads to other *P* inserts in the germ line has not been tested.

4.3.5 How to determine which system modifies the *P* dependent phenotypes?

Both Type I and II elements can cause variegation at *P{lacW}ci*^{Dplac} and other *P* dependent alleles. Removing exon 2 changes the predicted polypeptide structure for the P repressor and correlates with a change in silencing activity thereby suggesting a protein based mechanism for

PDS. To confirm this hypothesis, further research should try to find a correlation between expression from the Type I insert and the ability to modify P dependent phenotypes. Such research will determine whether *P* dependent silencing acts through a mechanism involving co-suppression or protein encoded by the *P* element. A co-suppression model predicts that surpassing a certain *P* expression level will result in silencing and the RNA product does not have to encode a protein product. In contrast, the protein model predicts that there is a continuous direct correlation between *P* expression and silencing at *P{lacW}ci*^{Dplac}. In the protein model, the protein encoded by the RNA is necessary for silencing to occur.

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