2008

Identification and characterization of the E(var)3-5 gene in *Drosophila melanogaster*

Pranoti Sahasrabhojane
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Identification and characterization of the *E(var)3-5* gene in
Drosophila melanogaster

Pranoti Sahasrabhojane

Thesis submitted to the
Eberly College of Arts and Sciences
at West Virginia University
in partial fulfillment of the requirements
for the degree of

Master of Science
in
Cellular and Molecular Biology

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Morgantown, West Virginia
2008

Keywords: PEV, *E(var)3-5*, euchromatin

Abstract

Identification and characterization of the *E(var)3-5* gene in
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The goals of my research project were to clone the *E(var)3-5* gene and characterize the lethality of *E(var)3-5* mutations. The *E(var)3-5* gene was identified by dominant mutations that enhanced PEV of w^{m4} , thus implicating the gene product in promoting a euchromatic chromatin structure (Dorn *et al.* 1993). These mutations were found to be recessive lethal as well and the lethal phenotype was used to map the *E(var)3-5* gene (Dorn *et al.* 1993; this work). I characterized the lethality of *E(var)3-5* mutations and found that lethality in *E(var)3-5* hemizygotes is manifested at the pupal stage. I mapped the lethality to a region defined by *Df(3R)Exel7310*. I generated five FLP-FRT mediated deletions within the region defined by *Df(3R)Exel7310* however, my complementation analysis revealed that *E(var)3-5* mutants were not allelic to any of the genes in that region. However I identified a mutation in one of the genes, *CG17360* in all *E(var)3-5* mutants examined. The *CG17360* mutants exhibit an unexpanded wing phenotype.

Acknowledgements

My introduction to the world of *Drosophila melanogaster* began when I joined Dr. Karen S. Weiler's lab to complete my Masters degree. I must take this opportunity to thank her sincerely for being my advisor and giving me excellent guidance throughout my Masters career. I have learned some valuable lessons being with her that I will cherish for the rest of my life.

I would also like to thank my committee members, Dr. Ashok Bidwai and Dr. Clifton Bishop for all the helpful suggestions and comments throughout my Masters career. Their friendly attitude made my Masters journey very easy.

The biology department at life sciences where I pursued my Masters degree was always a home away from home. I would like to thank the departmental staff and faculty members who were extremely helpful.

I wish to sincerely thank my friends Marissa, Suman, Manish, Madhu, Madhumati, Brahma, Shalaka, Ekta and Sushant, Sandhya, Anasua, Debolina. I would also like to thank my friends from India, Shireen and Vidyadhar, Anvaya, Sarika and Sharvari for just being there for me.

I would like to thank my mother and father, my brother, my grandmothers and grandfathers who have been and always be my life support. My mother-in-law and my father-in-law, Vaibhav dada, Deepa vahini, Adwait and Parth, I sincerely thank you for all the support.

Last but not the least, I acknowledge and thank my husband, Umesh Karandikar. It was not easy staying away from him to pursue my Masters, but his love, support and encouragement, made everything very easy. He has always been a source of inspiration to me and I am very proud to be his wife.

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Chapter 1: Introduction

Emil Heitz in 1928 first made the cytological distinction between the two nuclear compartments, euchromatin and heterochromatin (in Heitz 1928; reviewed by Weiler and Wakimoto 1995; Grewal and Elgin 2002). He carefully analyzed the pattern of chromosomes in moss cells during the cell cycle and observed that some regions of the chromosomes remained condensed during the interphase unlike other chromosomal regions. These compact chromosomal regions that failed to decondense during the interphase and telophase of the mitotic cycle were identified as heterochromatin. Heitz defined euchromatin as that part of the genome that condensed during the mitotic phase and decondensed during the interphase. Brown (1966) further characterized heterochromatin as facultative and constitutive heterochromatin. Facultative heterochromatin refers to euchromatic sequences that are condensed for many cell generations but can be decondensed. The known examples of facultative heterochromatin are the inactive X chromosome in female mammals and the repressed paternal set of chromosome in male cockroach bugs (Brown 1966; reviewed by Dimitri *et al.* 2005; Corradini *et al.* 2007)

Biochemical differences between euchromatin and heterochromatin

The two nuclear domains, euchromatin and heterochromatin, can be differentiated from each other due to differences in histone modifications. The nucleosomes that constitute the basic unit of chromatin consist of 146 bp of DNA wrapped around a histone octamer. The histone octamer consists of two subunits each of H2A, H2B, H3 and H4 (reviewed by Kornberg and Lorch 1999). The histones are subjected to a variety of post-translational modification such as acetylation, methylation and phosphorylation (Garcia-Ramirez *et al.* 1995; Strahl *et al.* 1999; Nowak and Corces 2000). It has been suggested that the pattern of histone modification on euchromatin and heterochromatin can govern the packaging of chromatin as open and closed states (Strahl and Allis 2000; reviewed by Jenuwein and Allis 2001).

Acetylation of lysine residues on histone H4 is most studied. In general, hyperacetylation of histones is linked to an increase in transcription whereas hypoacetylation has been linked to repressive chromatin (Hebbes *et al.* 1988; reviewed by Grunstein 1997; Turner 2000; Turner and O'Neill 1995; Litt *et al.* 2001). Munks *et al.* (1991) have shown that H4 is acetylated on lysine at positions 5, 8, 12 and 16 in drosophila cell lines S2 and Kc using antibodies that detected all forms of acetylated lysine. In the *Drosophila melanogaster* polytene chromosomes, euchromatic bands and interbands are associated with H4 acetylated at positions 5 and 8 whereas the heterochromatic regions are marked with acetylated H4K12. The X chromosome in *D. melanogaster* males was shown to be enriched in H4K16 (Turner *et al.* 1992; Bone *et al.* 1994).

Methylation of lysine residues on histones H3 and H4 has been intensively researched. On histone H3 lysine can be methylated at positions 4, 9, 27 and 36. On histone H4 lysine can be methylated at position 20. The lysine residues on H3 and H4 can be mono, di and tri-methylated (reviewed by Cheung and Lau 2005; Lachner *et al.* 2003). In *D. melanogaster*, methylated H3K4 is a major euchromatic mark and has been shown to be associated with interband regions (Ebert *et al.* 2006). Schubelar *et al.* (2004) also provided experimental evidence indicating that promoters of active genes are enriched in methylated H3K4 (Schubelar *et al.* 2004). However in a recent investigation by Yasuhara and Wakimoto (2008) on histone modification pattern, it was reported that 5' region of *Moca-cyp*, *CG1646* and *CG5514* are not enriched in H3K4me2, but rather are enriched in H3K9 modification (Yasuhara and Wakimoto. 2008). Several enzymes such as ASH1, TRX and TRR that possess H3K4 methyltransferase activity have been identified (Beisel *et al.* 2002; Sedkov *et al.* 2003; Byrd and Shearn 2003)

In *Drosophila melanogaster*, mono and dimethylation of lysine 9 and methylation of K27 on H3, as well as trimethylation of H4 at K20, are considered as major heterochromatic marks (Ebert *et al.* 2006). However methylated H3K27 and trimethylated H4K20 are also detected in some euchromatin regions. *Su(var)3-9* has been identified as the predominant H3K9 methyltransferase in *D.*

melanogaster (Schotta *et al.* 2002). Similarly Suv4-20 has been identified as an H4K20 methyltransferase (Schotta *et al.* 2004). The other H3K9 methyltransferase identified in *D. melanogaster* are dSETDB1 (Tzeng *et al.* 2007) and dG9a (Mis *et al.* 2006). The dSETDB1 enzyme has been shown to methylate H3K9 on euchromatin and chromosome 4 (Seum *et al.* 2007). dG9a has been identified as a multi-catalytic methyltransferase since it methylates H3 on lysine 9 and 27 and H4 (Stabell *et al.* 2006). Recently it has been demonstrated that the transcribed regions of heterochromatic genes are associated with dimethylated H3K9 (Yasuhara and Wakimoto 2008).

Phosphorylation on H3 at serine 10 has been associated with transcription elongation in fruit flies (Ivaldi *et al.* 2007). The JIL-1 kinase has been shown to be responsible for phosphorylating H3 at S10 (Wang *et al.* 2001). On the euchromatin, JIL-1 kinase has been shown to localize on multiple sites on interband region (Yin *et al.* 1999). Loss of JIL-1 kinase has been shown to cause ectopic distribution of heterochromatic marker H3K9me2 and HP1, especially on the X-chromosome. JIL-1 kinase has been implicated in maintaining euchromatic regions. The JIL-1 kinase phosphorylates H3 at S10, preventing methylation of neighboring H3K9 residue by the Su(var)3-9 methyltransferase. As a result HP1 cannot bind to H3K9 consequently preventing heterochromatin formation (Zhang *et al.* 2006).

Sequence composition differs between euchromatin and heterochromatin

The *Drosophila melanogaster* genome measures approximately 180 Mb in size. The euchromatic sequences comprise about two-thirds of the genome whereas one-third of the genome is heterochromatic (Adams *et al.* 2000; reviewed by Celniker and Rubin 2003). The *D. melanogaster* genome annotation predicts about 13,379 protein-coding genes in 116.8 Mb of euchromatic sequence. The number of protein coding exons is estimated to be around 54,934 with an average of 4.6 exons per gene. The euchromatic genome annotation has identified complex gene models that are categorized as nested gene, overlapping genes, alternatively transcribed genes and dicistronic gene. About 1,038

euchromatic genes are categorized as nested genes, which are defined as being within the introns of other genes. 15% (2,054) of euchromatic genes are described as overlapping genes. About 2,279 are categorized as alternatively transcribed genes. Dicistronic genes were defined as genes that produce mRNAs containing two separate and non-overlapping coding regions. About 31 dicistronic genes were identified (Misra *et al.* 2002; reviewed by Celniker and Rubin 2003). An analysis of 24 Mb of heterochromatin was presented in release 5.1 of the *D. melanogaster* heterochromatin (Smith *et al.* 2007). This annotation predicted 613 protein-coding genes. 186 single-exon genes and 427 multiple-exon genes together comprise the 613 predicted genes. Nested, overlapping or dicistronic genes have not been identified so far in the fruitfly heterochromatin. Heterochromatic gene introns are usually five times longer than euchromatic gene introns. The increased length of the introns is attributed to the sequence composition that consists of fragmented transposable elements. Euchromatin has a higher gene density (12.6 gene per 100 kb) than heterochromatin (1.8 to 4.4 gene per 100 kb). Pseudogenes map to both euchromatin and heterochromatin but the density is three times greater in heterochromatin than in euchromatin (Smith *et al.* 2007).

Transposable elements (TEs) are an integral part of the *D. melanogaster* genome and are present in both euchromatin and heterochromatin (Adams *et al.* 2000). TEs are categorized under two classes based on their mechanism for transposition. Class I elements, also known as retrotransposons, require an RNA intermediate for transposition. Retrotransposons are further categorized into LTR (long terminal repeat) transposons and LINE/SINE (long and short interspersed element). Class II elements, also known as transposons, insert themselves into the genome through a DNA intermediate. The DNA transposons possess terminal inverted repeats and encode their own transposase. A systematic analysis of transposable elements in euchromatin was performed by Kaminker *et al.* (2002). About 96 families of transposable elements have been identified so far in fruitfly euchromatin. The LTR transposons comprise the majority among TEs in euchromatin. About 30-50 different TE families are scattered in the *D.*

melanogaster heterochromatin. Of the 24 Mb of heterochromatin that has been curated, about 16 Mb primarily consists of LTR transposons and LINEs (Smith *et al.* 2007). The transposable elements however are incomplete sequences. The majority of heterochromatic and euchromatic transposons are found interspersed within other elements (Kaminker *et al.* 2002; Smith *et al.* 2007).

Pimpinelli *et al.* (1995) analyzed the distribution pattern of 11 different TEs using fluorescent *in situ* hybridization in the heterochromatin of mitotic chromosomes. Their study indicated that transposable elements have a tendency to cluster in a heterochromatic region. Clustering of transposable elements is not affected by the heterochromatic DNA sequence. Analysis of wild-type strains from geographically distant populations showed that TE clusters were conserved among the populations. The authors thus suggested that transposable elements are inherent constituents of heterochromatin (Pimpinelli *et al.* 1995)

About two-third of the heterochromatic DNA of the *D. melanogaster* genome consists of satellite DNA, which is underrepresented in the curated regions due to its recalcitrance to cloning (reviewed by Celniker and Rubin 2003). The repetitive DNA elements known as satellite DNAs were first identified using the technique of cesium chloride density gradient centrifugation. Satellite DNAs showed a different buoyant density than the bulk of the DNA in the cesium-chloride centrifugation, hence the name (Yunis and Yasmineh 1971). Four predominant satellite DNAs occur in *D. melanogaster* heterochromatin. Three of these satellite DNAs, namely the 1.672g/cm², 1.686 g/cm² and 1.705 g/cm² satellites, are known as simple satellites and consist of tandem repeats of 5-10 bp in length. The fourth satellite DNA with a buoyant-density of 1.688 g/cm² is a complex satellite consisting of tandem repeats of 359 bp (reviewed by Gatti and Pimpinelli 1992). The composition of satellite DNAs has been extensively analyzed by cloning the 1.672, 1.686 and 1.705 satellite DNAs (Lohe and Brutlag 1986). Within these clones, the authors identified 11 new repeats consisting of repeat units of 5, 7 and 10 bp, which were different from the already documented predominant repeats. A mitotic chromosome map depicting the distribution of major and minor satellite DNAs on heterochromatin was created by Lohe *et al.*

(1993). The Y and 2nd chromosomes have the highest density of satellite DNAs. The AATAT repeats that are the predominant repeats of the 1.672 satellite are found at 4 sites on the Y chromosome. The 5 bp AAGAG and 7 bp AAGAGAG repeats of the 1.705 satellite are found primarily on the 2nd and Y chromosomes with a little distribution on the X and 3rd. The predominant AAGAC repeats of the 1.686 satellite are found at four different sites on the Y chromosome and a single site on the 2nd chromosome. The X chromosome heterochromatin is primarily composed of 1.688 satellite. Nine simple satellite sequences make up 80% of the Y chromosome and 5 simple satellites make up 70% of the 2nd chromosome (Lohe *et al.* 1993).

Position effect variegation (PEV)

The phenomenon of position effect variegation (PEV) was first discovered by Muller in 1930 but was genetically characterized by Schultz in 1936. Muller (1930) irradiated flies with X-rays in an attempt to isolate mutants with chromosome abnormalities. In one such experiment he discovered flies that variegated for eye color. He termed the phenomenon as “eversporting displacement”, and speculated it involved a chromosomal abnormality such as an inversion, duplication, translocation or deletion (Muller 1930). Jack Schultz analyzed the salivary glands of these mutants from Muller’s experiment (Schultz 1936). In each case he found that variegation was due to a chromosomal rearrangement, and speculated that inert chromosomal material (heterochromatin) plays a role in variegation. Schultz (1936) suggested that the extent of variegation was proportional to the amount of inert chromosomal material at the rearrangement breakpoint. We currently appreciate that position effect variegation occurs due to displacement of genes into a different chromosomal environment and is a general phenomenon (reviewed by Weiler and Wakimoto 1995; Grewal and Elgin 2002). A normally expressed euchromatic gene undergoes inactivation when placed artificially by chromosomal rearrangement next to heterochromatin. The classic example of PEV is the inactivation of the *white* (*w*) gene in the chromosomal rearrangement *In(1)w^{m4}*.

An inversion displaces the *w* gene near to a block of centric heterochromatin and as a result the *w* gene is inactivated. However, this inactivation is a variable event. In some cells the *w* gene is repressed whereas in other cells it undergoes regular transcription. A white patch within the eye is a result of inactivation of the *w* gene. A red patch within the eye is observed if the *w* gene is transcribed. This event of PEV in *Drosophila melanogaster* gives rise to flies that have mottled or variegated eyes.

PEV involving heterochromatic genes has also been demonstrated. Schultz first discovered rearrangements involving the heterochromatic *light (lt)* gene (Schultz and Dobzhansky 1934). Anita Hessler (1958) carried out a detailed investigation involving the position effect of the *lt* gene. Hessler isolated 35 rearrangements that variegated for the expression of the *lt* gene. The rearrangements displaced the heterochromatic *lt* gene near a block of euchromatin. Salivary gland analysis of these rearrangements displayed some peculiar characteristics. In all the rearrangements one breakpoint was located near the *lt* gene. The second breakpoint however was located in the distal euchromatin of either the X, 2nd or the 3rd chromosomes. Rearrangements with breakpoints in the Y or the 4th chromosome were not recovered. Chromosome rearrangements with the 2nd breakpoint located in the proximal euchromatin or centromeric heterochromatin were not isolated. Hessler thus suggested that heterochromatic gene variegation is different from euchromatic gene variegation (Hessler 1958). Subsequent studies by Wakimoto and Hearn (1990) on *lt* gene variegation confirmed Hessler's observation. The heterochromatic gene, *rolled (rl)* also displays variegation as a result of its displacement from a heterochromatic environment (Eberl *et al.* 1993). These observations argued that heterochromatic genes required a heterochromatic environment for proper expression. Studies that were conducted on *lt* variegating rearrangements and *rl* variegating rearrangements also showed that the expression of neighboring 2L and 2R heterochromatic genes, respectively, was affected (Wakimoto and Hearn 1990; Eberl *et al.* 1993)

Factors affecting PEV

A variety of factors such as temperature, dosage of the Y chromosome and genetic modifiers influence or modify position effect variegation (reviewed by Girtan and Johansen 2008). The Y dosage effect is most easily observed in male flies. In XY males bearing a *w*-variegated inversion, a variegated eye phenotype is seen. In XO males, however, the absence of the Y chromosome leads to enhancement of PEV. In XYY males, one extra copy of the Y chromosome leads to a suppression of PEV (Gowen and Gay 1934).

Position effect variegation is also altered by temperature. Hartmann-Goldstein (1967) extensively studied the effect of temperature on position effect variegation. In general, a decrease in temperature enhances the silencing of a gene subjected to PEV. For example, variegation of the *w* and *Notch* genes due to their translocation near the 4th chromosome centromere was analyzed at 25°C, 19°C and 14°C. Variegation of the *w* gene was detected as variability in pigmentation of Malpighian tubule cells whereas variegation of the *Notch* gene was observed as a wing abnormality. The results indicated that the variegation was most severe at 14°C.

The histone gene complex also modifies PEV. The histone gene complex maps to the 2nd chromosomal arm at cytological position 39D2-3 to E1-2, and codes for all five histones. To test the effect of histone dosage on PEV, Moore *et al.* (1979) generated five deficiencies encompassing the histone gene complex. *Df(2L)65* and *Df(2L)161* deleted the entire histone gene complex. *Df(2L)84* had one-breakpoint in the histone gene such that only a part of complex was deleted, and *Df(2L)1* and *Df(2L)12* deleted regions adjacent to histone gene complex. The effects of these deficiencies were tested on two variegating rearrangements *In(1)w^{m4}* and *B^{sv}Y*. It was observed that deficiencies *Df(2L)84*, *Df(2L)65* and *Df(2L)161* suppressed the variegation of *white* gene in *In(1)w^{m4}* and *B^{sv}* phenotype (Moore *et al.* 1979).

PEV and chromatin structure

Cytological studies have indicated that euchromatic chromosomal sequences affected by PEV are visualized as densely packaged regions in polytene chromosomes and that the extent of the dense packaging was observed to be sensitive to lower temperature, analogous to the sensitivity of gene expression to lower temperature (for example Hartmann-Goldstein 1967). Zhimulev *et al.* (1986) studied the chromosomal rearrangement $T(1:2)dor^{var7}$, for which the chromosomal region 1A-2B7-8 that includes the *ecdysone* (*ecs*) locus is translocated to the centromeric heterochromatin of the 2nd chromosome. In the salivary glands of $T(1:2)dor^{var7}$ larvae, the chromosome morphology at the 2B region appeared dense, indicating the spread of heterochromatin. *In situ* hybridization experiments showed that heterochromatinization of the 2B region made it less accessible to DNA probes derived from the 2B region. The heterochromatinized 2B region was also shown to be late-replicating. These results led to the proposal that PEV was associated with altered chromatin structure (Zhimulev *et al.* 1986). The *w* locus has also been studied to observe effects of PEV on chromosome morphology. Hayashi *et al.* (1990) conducted experiments to observe changes in chromatin structure associated with $In(1)w^{m4}$. The authors studied the chromosome morphology of the 3C-E region in polytene chromosome squashes. They observed that in the $In(1)w^{m4}$ strain, the 3C-E region appeared euchromatic in only 19% of nuclei when compared to a wild-type strain. An *in situ* hybridization experiment using the *w* gene probe to test the accessibility of the *w* locus revealed that $In(1)w^{m4}$ showed decreased accessibility as compared to an unrearranged chromosome. These experiments supported the hypothesis that PEV was associated with an altered chromosome structure (Hayashi *et al.* 1990).

Molecular methods to assess changes in accessibility of chromatin associated with variegation have yielded mixed results. A DNase I sensitivity test of the *w* gene in the $In(1)w^{m4}$ strain to analyze changes at the chromatin level in the presence and absence of mutations that modify PEV was performed. Surprisingly the pattern of DNase I digestion of the *w* gene in the $In(1)w^{m4}$ strain

was not significantly different than the *ln(1)w^{m4}* strain with modifiers (Hayashi *et al.* 1990). Chromosome structure changes associated with position effect variegation were also investigated by Wallrath and Elgin (1995). A special *P* element containing the *w* gene and the *hsp26* gene was mobilized throughout the genome and lines that were variegating for the expression of the *w* gene were isolated. The insertions in these lines were localized to pericentric heterochromatin and telomeric chromatin. A restriction enzyme assay was performed on these lines to measure the accessibility of the *hsp26* promoter. Reduced accessibility to restriction enzymes was observed for the transgenes inserted into pericentric heterochromatin. The reduced accessibility of restriction enzymes was attributed to altered chromosome structure. In addition, micrococcal nuclease digestion of variegating lines revealed a more ordered structural array of nucleosomes over the *hsp26* promoter than for the non-variegating lines. These studies provided further evidence that position effect variegation causes alterations in the chromosome structure (Wallrath and Elgin 1995)

Biochemical factors that affect histones also affect position effect variegation. The effect of non-acetylated and hyperacetylated histones on PEV was studied by Mottus *et al.* (1980). *ln(1)w^{m4}* larvae were treated with varying concentrations of butyrate and n-propionate, which inhibit histone deacetylase activity and consequently increases the level of hyperacetylated histones. It has been demonstrated that active chromatin is associated with hyperacetylated histones (Davie and Candido 1978). Mottus *et al.* (1980) observed that hyperacetylated histones limited the spread of heterochromatin, consequently preventing the inactivation of *w* gene leading to suppression of PEV (Mottus *et al.* 1980).

Models of position effect variegation

Different models have been proposed to explain the mechanism underlying position effect variegation. Tartof *et al.* (1984) proposed the boundary model to explain the linear propagation of heterochromatin into euchromatic

sequences ultimately leading to a variegated phenotype. In their experiments the authors addressed basic questions pertaining to PEV such as the specificity of heterochromatic sequences that induce PEV, the nature of sequence at the heterochromatin-euchromatin junction, and the involvement of sequences other than heterochromatic sequences to cause variegation. To clarify these questions three strains variegating for the expression of the *white* gene, w^{m4} , w^{mMc} and w^{m51b} , were studied. The strains appeared to represent simple inversions where the *white* locus was placed next to heterochromatin. Molecular analysis of sequences at the heterochromatin-euchromatin junction of the three variegating rearrangements revealed transposon-like sequences. This suggested that transposon-like sequences might be responsible for inducing PEV. The variegating strains were irradiated by X-rays to obtain revertants. Analysis of revertants would clarify if the transposon-like sequences at the heterochromatin-euchromatin junction would lead to a variegated phenotype. Restriction enzyme digestion and *in situ* hybridization experiments revealed that the revertant chromosomes still contained some heterochromatin-derived sequences adjoining the *w* locus. These results indicated that the transposon-like sequences were not responsible by themselves to induce a variegated phenotype. Based on their results the authors proposed the boundary model to explain heterochromatin induced gene silencing (Figure 1.1). According to this model a heterochromatic domain is formed between two initiator sites (i). A heterochromatin domain can also be formed between the initiator site (i) and the terminator site (t). Heterochromatinization of the DNA sequence starts at this initiator site and terminates at the t site. A variegated phenotype appears due to a breakpoint between the two i sites or between the i the t sites. Such initiation and termination sites are dispersed within a heterochromatic block (Tartof *et al.* 1984). However, Experimental evidence proving the existence of initiation sites (i) and termination sites (t) is lacking (Sabl and Henikoff 1996)

Figure 1.1 Boundary model for heterochromatin formation

The X chromosome is shown with the box representing heterochromatin, the line representing euchromatin and the circle indicating the centromere. The black block represents two initiation sites (i) for heterochromatin formation. A In an unarranged chromosome, the heterochromatic domain is formed between the i sites. B In the variegating inversion chromosome, the w^+ gene undergoes silencing when displaced next to heterochromatin due to its spread. Figure adapted from Tartof *et al.* (1989).

Another model by Karpen and Spradling (1990) proposed the possibility that DNA loss might be responsible for PEV. This theory was based on the molecular analysis of a mini-chromosome, *Dp(1;f)1187*. The mini-chromosome was derived from the *In(1)sc⁸* chromosome through deletions of DNA sequence both proximal and distal to the *scute* (*sc*) locus. As a result, only 9 genes including the *yellow* (*y*) gene remained adjacent to the heterochromatic breakpoint. The *y* gene displayed PEV that was suppressed on addition of a Y chromosome. Southern blot analysis was performed on mini-chromosome DNA isolated from salivary glands to assay for any associated changes near the heterochromatin-euchromatin junction. The result indicated that heterochromatin near the breakpoint was underrepresented. The results also revealed that euchromatic sequences located 1.9 kb away from the heterochromatin-

euchromatin junction were underrepresented. The authors determined that euchromatic sequences were highly underrepresented in salivary-gland nuclei (39 fold underrepresented on average). A trend was observed in which sequences located as far as 54 and 103 kb away were underrepresented only 8 to 2.4 fold. The addition of a Y chromosome reduced the severity of under-representation. The authors proposed that under-representation of euchromatic sequences near the heterochromatic breakpoint was due to elimination of these sequences due to the excision of transposons in heterochromatin, thus giving rise to the variegated phenotype (Karpen and Spradling 1990).

Wakimoto and Hearn (1990) proposed a nuclear compartmentalization model to explain the PEV of heterochromatic genes. It was suggested that a nuclear compartment would be formed due to association between heterochromatic regions. This nuclear compartment would have the desired concentration of proteins for the proper expression of heterochromatic genes. A change in the concentration of these heterochromatic proteins would lead to a variegated phenotype (Wakimoto and Hearn 1990).

The compartment model was extended to explain PEV of euchromatic genes as well. The somatic pairing model proposed by Dorer and Henikoff (1994) effectively explains a mechanism of heterochromatin formation and PEV-induced gene silencing. This model was derived from PEV studies of a *P[lacW]* transgene harboring the mini-*white* gene. In presence of a *P* transposase, the *P[lacW]* transgene was transposed to obtain fly lines that showed a variegated eye phenotype. These investigators determined that the fly lines that variegated for mini-*white* expression differed in the copy number and orientation of the *P[lacW]* transgene. In general flies containing three to four tandem repeats showed a higher degree of variegation than flies containing one to two repeats. The authors suggested that silencing of the mini-*white* gene occurs as a result of pairing between the transgene repeats. The authors hypothesized that the pairing of transgene repeats leads to the formation of a hairpin-like structure that sequesters the mini-*white* gene from regulatory elements required for its transcription, resulting in silencing. When the transgenes remain unpaired the

mini-*white* gene is transcribed. Variegation occurs when pairing of transgene repeats occurs in some cells but not others. The authors interpreted that heterochromatin formation occurs as a result of pairing between transgene repeats that are in close vicinity of each other. They proposed that the pairing of repeats attracted heterochromatin-specific proteins, giving rise to a heterochromatic chromocenter. Similarly, heterochromatin formation might be due to association of natural transposon sequences. Heterochromatin formation by these natural transposons would be independent of specific transposon sequences but would be dependent upon the length and number of transposons (Dorer and Henikoff 1994).

The euchromatic *brown* (bw^+) gene is a well-studied example for PEV of euchromatic gene. The somatic pairing model was used by Sabl and Henikoff (1996) to explain heterochromatin formation and PEV-induced gene silencing using $P[bw^+]$ transposons containing the *brown* gene. X-ray mutagenesis of flies containing the $P[bw^+]$ transposon yielded fly lines that exhibited variegated eye phenotype. Molecular analyses of these variegating fly lines indicated that they differed in copy number and orientation of the $P[bw^+]$ transposons. Fly lines that contained a high copy number of transposon repeat exhibited the lowest expression of the bw^+ gene. On the other hand fly lines that contained one or two of the transposons repeat showed increased expression of the bw^+ gene. Interestingly, fly lines that contained tandem repeats of the $P[bw^+]$ transposons in the left orientation showed minimum expression of the *bw* gene than fly lines containing tandem repeats of $P[bw^+]$ transposons in right orientation. The authors proposed that tandem repeats pair with each other and form complex structures that associate with heterochromatin, in turn resulting in inactivation of the bw^+ gene (Sabl and Henikoff 1996)

Csank and Henikoff (1996) investigated PEV involving a *brown*^{Dominant} (bw^D) allele. The bw^D has a block of heterochromatin inserted at the *bw* locus that trans-inactivates the other wild-type bw^+ copy resulting in a variegated eye phenotype. Csank and Henikoff (1996) demonstrated that the extent of inactivation of the bw^+ gene is influenced by its proximity to heterochromatin.

Chromosomal rearrangements that increased the distance between bw^D and centric heterochromatin were obtained. Fluorescent *in-situ* hybridization studies of interphase nuclei containing the rearranged chromosome demonstrated that increasing the distance between bw^D and centric heterochromatin lead to decreased inactivation of the bw^+ copy and *vice versa*. Similarly mutation in PEV suppressing genes (discussed below) lead to decreased inactivation of the bw^+ allele. Csink and Henikoff thus suggested that the extent of PEV of the bw^+ gene is dependent on its proximity to heterochromatin and heterochromatic proteins are also involved in this process. (Csink and Henikoff 1996)

Modifiers of position effect variegation

Moore *et al.* (1979) discovered that deficiency of the histone gene complex causes suppression of PEV. This discovery lead to the idea that the identification of other proteins involved in chromatin architecture might provide insight into the mechanism of PEV. This discovery provoked researchers to investigate proteins involved in PEV to better understand the process of heterochromatinization or chromosome condensation and chromosome assembly (Reuter *et al.* 1982; Sinclair *et al.* 1983).

Dominant modifier mutations were isolated in several different genetic screens based on their ability to modify the w^{m4} rearrangement (Reuter and Wolff 1981; Reuter *et al.* 1982; Sinclair *et al.* 1983; Reuter *et al.* 1987; Sinclair *et al.* 1989). Genes that when mutated lead to the suppression of PEV were named *Su(var)* genes. Mutation of suppressor genes limits the spread of heterochromatin. Suppressor genes are thus implicated in encoding proteins essential for heterochromatin formation (Reuter and Wolff 1981; Sinclair *et al.* 1983). Genes that when mutated lead to the enhancement of PEV were named *E(var)* genes. Enhancer genes are thus implicated in encoding proteins essential for euchromatin formation. Enhancer genes can function in different ways to promote euchromatin formation. Enhancer gene can encode proteins that directly inhibit the spread of heterochromatin or indirectly they can inhibit the activity of a repressor for a suppressor gene (Sinclair *et al.* 1989). About 150 PEV modifying

genes are estimated to be present in the genome of *D. melanogaster* (reviewed by Schotta *et al.* 2003).

Modifiers genes that exert their effect on gene silencing when present only in a single dose are known as haplo-dependent modifiers. For example, a haplo-dependent suppressor gene causes suppression of PEV when present in a single dose. Likewise, haplo-enhancers cause enhancement of PEV when present in a single dose. A few modifier genes exert opposite effects on gene silencing when duplicated and are known as triplo-dependent modifiers. For example a modifier gene identified as a haplo-suppressor with a triplo-enhancer effect will cause suppression of PEV when present in a single dose but will enhance PEV when present in three doses (reviewed by Schotta *et al.* 2003).

Su(var) genes

Molecular characterization of some suppressor genes has shown that these proteins are involved in heterochromatin formation. Heterochromatin protein 1 (HP1), was identified as the first non-histone chromosomal protein associated with centric heterochromatin (James and Elgin 1986). The *Su(var)2-5* gene is a haplo-suppressor with a triplo-enhancer effect (Eissenberg *et al.* 1992). The HP1 protein is characterized by the presence of an amino-terminal chromo domain and a carboxy terminal chromo-shadow domain (Aasland and Stewart, 1995; Paro and Hogness, 1991). The chromo domain targets HP1 to heterochromatic locations. Immunofluorescent studies using antibodies for the HP1 protein have shown that HP1 is associated with the 4th chromosome and at telomeres on 2R, 3R and X chromosomes (James *et al.* 1989). Later studies have revealed that HP1 also associates with some euchromatic sites (Fanti *et al.* 2003) and HP1 has been implicated in upregulating the *Hsp70* gene (Piacentini *et al.* 2003). HP1 has been shown to bind methylated H3K9 through the chromo domain, an important step in heterochromatin formation (Bannister *et al.* 2001).

Su(var)3-9 has been identified as a haplo-suppressor and triplo-dependent enhancer of PEV (Schotta *et al.* 2002). As noted earlier, *Su(var)3-9* encodes a histone methyltransferase that methylates histone H3 at lysine 9 in

the centric heterochromatin (Rea *et al.* 2000). The Su(var)3-9 protein has been shown to interact with HP1 (Schotta *et al.* 2002). Localization of HP1 to heterochromatin is dependent on Su(var)3-9 and *vice versa*. This indicates that Su(var)3-9 plays an important role in regulating heterochromatin formation

Su(var)3-7 is also a haplo-suppressor with a triplo-enhancer effect, and encodes a protein containing seven zinc fingers. It is associated with heterochromatic DNA, and has particular affinity for the AATAT and 353bp repeats (Cleard and Spierer 2001). The Su(var)3-7 protein has been shown to interact with the Su(var)3-9 and HP1 proteins (Cleard *et al.* 1997).

Su(var)3-3 has been identified as the drosophila homolog of the human LSD1 amine oxidase (Rudolph *et al.* 2007). Su(var)3-3 causes demethylation of H3K4me1 and H3K4me2. Removal of the H3K4me1 and H3K4me2 methylation marks is an essential step for Su(var)3-9 dependent H3K9 methylation and subsequent heterochromatin formation.

Su(var)3-6, which is allelic to *PP1* gene (protein-phosphatase) at 87B, encodes the catalytic subunit of PP1 (Baksa *et al.* 1993). The Su(var)3-6 protein has been postulated to regulate the phosphorylation state of proteins involved in heterochromatin formation (Dombradi and Cohen 1992).

E(var) genes

The protein products of *E(var)* genes have been implicated in euchromatin formation. However, Weiler and Wakimoto (2002) have suggested that transcriptional factors can also behave as enhancer proteins recruited by subsets of euchromatic genes for their normal expression. This was suggested by the results of molecular characterization of several *E(var)* genes. For example *E(var)3-93E*, the first molecularly characterized enhancer gene, encodes a transcriptional activator identified as *dE2F* (Seum *et al.* 1996). It is also a cell-cycle regulator and its function is analogous to the mammalian *E2F*. Since dE2F is a transcriptional activator, Seum *et al.* (1996) proposed that dE2F would play a role in chromatin decondensation process by acting as a positive regulator of an enhancer gene.

E(var)3-93D (also identified as *Mod(mdg4)* (Dorn *et al.* 1993a; Gerasimova *et al.* 1995) encodes a protein that harbours the BTB/POZ domain also found in other transcriptional regulators. The *E(var)3-93D* protein has been implicated in euchromatin formation by binding to the Suppressor of Hairy wing (Su(Hw) protein (Gerasimova *et al.* 1995). The Su(Hw) protein has been shown to bind the gypsy insulator sequence (Spana *et al.* 1988). Insulators are regulatory elements that act as barriers to inhibit the spread of heterochromatin and also can disrupt important enhancer-promoter interactions (reviewed by Brasset and Vaury 2005).

The haplo-enhancer gene *Trithorax-like* has been shown to encode the GAGA transcription factor (Farkas *et al.* 1994). The N-terminal tamtrack motif of GAGA factor promotes association with other euchromatic proteins and thus maintains an euchromatic chromatin state. Recently it was proposed that expression of the *w* gene is maintained by GAGA factor and FACT (Nakayama *et al.* 2007). The GAGA factor and FACT mediate the recruitment of the histone variant H3.3 which has been implicated in inhibiting heterochromatin formation.

Enhancer genes that encode proteins other than transcription factors have also been identified. The *Hel* gene or (*hel*) is a haplo-dependent enhancer with a triplo-dependent suppressor effect (Eberl *et al.* 1997). It encodes a protein HEL that is similar to the ATP-dependent RNA helicase. Cytological studies indicate that the HEL protein localizes to the nucleus and is present on chromosomes throughout the interphase of the cell cycle (Eberl *et al.* 1997). The association of HEL protein with salivary-gland chromosomes and enhancement of PEV suggested that it might be involved in regulating a euchromatic structure. The authors proposed that HEL protein could bind RNA instead of DNA and regulate chromatin structure. The *HEL protein* might inhibit the association of mRNA at the transcription start site thus keeping it accessible to transcription. Alternatively the HEL protein could bind an RNA component of the chromosome and prevent heterochromatin formation.

Upb, which encodes the enzyme ubiquitin protease, was also identified as *E(var)1* (Henchoz *et al.* 1996). The *Upb* mutants display a haplo-enhancer effect

as well as a triplo- suppressor effect on PEV. Henchoz *et al* proposed that *Upb* helps in maintaining a euchromatic chromatin state. The Upb protein would maintain the level of ubiquitinated enhancer proteins. As a result the enhancer proteins would not be subject to degradation and would maintain a euchromatic chromatin state (Henchoz *et al.* 1996).

The recently identified *E(var)3-9* gene encodes a protein containing zinc-fingers, thus suggesting that it could be a nucleic-acid binding protein (Weiler 2007). *E(var)3-9* was shown to enhance PEV of euchromatic genes but suppress PEV of heterochromatic genes. These experiments thus implicated a role of wild-type *E(var)3-9* product in euchromatin regulation.

The field of PEV has been a subject of intense research over the years and still continues to provide valuable insights. Molecular characterization of suppressor genes has revealed their involvement in heterochromatin formation process. On the other hand molecular characterization of enhancer genes has revealed that these genes encode euchromatic proteins ranging from transcription factors to chromatin remodeling factors to enzymes (Table 1.1). Dorn *et al* (1993b) conducted a mutagenesis screen that led to the isolation of lot of enhancer mutations, of which only a few have been molecularly characterized to date. The *E(var)3-5* mutants were also isolated in the same screen. I pursued the identification and molecular characterization of the *E(var)3-5* mutants, as a small step towards understanding the process of chromosome decondensation.

Table 1.1 Enhancer genes of PEV

Gene name	Function	PEV phenotype	Cloning reference
<i>Brahma</i>	Chromatin remodeling	Haplo-enhancer	Tamkun <i>et al.</i> 1992
<i>E(var)3-93D {mod(mdg4)}</i>	Apoptosis, transcription factor, insulator function.	Haplo-enhancer	Dorn <i>et al.</i> 1993a
<i>Trithorax-like</i>	Transcription factor	Haplo-enhancer	Farkas <i>et al.</i> 1994
<i>Zeste</i>	Transcription factor	Recessive enhancer	Judd 1995
<i>E2F</i>	Transcriptional activator	Haplo-enhancer and triplo suppressor	Seum <i>et al.</i> 1996
<i>RPD3</i>	Histone deacetylase	Haplo-enhancer	De Rubertis <i>et al.</i> 1996
<i>Ubp</i>	Ubiquitin specific protease	Haplo-enhancer and triplo suppressor	Henchoz <i>et al.</i> 1996
<i>Hel</i>	ATP dependent RNA helicase	Haplo-enhancer and triplo suppressor	Eberl <i>et al.</i> 1997
<i>Asx</i>	Transcription factor	Haplo-enhancer	Sinclair <i>et al.</i> 1998
<i>BEAF-32</i>	Insulator-binding protein	Haplo-enhancer and triplo-suppressor	Gilbert <i>et al.</i> 2006
<i>E(var)3-9</i>	Unknown	Haplo-enhancer	Weiler 2007
<i>Lamin</i>	Nuclear lamin	Haplo-enhancer	Bao <i>et al.</i> 2007

Overview of thesis

The goals of my research project were to characterize mutations in and clone the *E(var)3-5* gene. Seven *E(var)3-5* mutants were gifted to our lab by Dr.

Gunter Reuter. Genetic analysis using meiotic mapping of the lethal phenotype in our lab showed that *E(var)3-5* is at map position 3-50.56 +/- 0.28 map units, which corresponds to cytological location 86DE (Weiler unpublished). Complementation tests of *E(var)3-5* mutants with the deficiency stocks indicated that *E(var)3-5* is present in the region from 86E2-4 to 87B1-5

My first goal was to characterize the lethality of *E(var)3-5* mutations. This study would enable me to understand the functions of the *E(var)3-5* gene product. I used the approach of analyzing the lethality produced by homozygous *E(var)3-5* embryos and deficiency/*E(var)3-5* embryos. If the lethality occurred during embryogenesis, as anticipated from preliminary experiments, I would analyze the terminal phenotype of unhatched embryos. These unhatched embryos would be observed carefully for any abnormalities in the development pattern. If third instar larvae were recovered, I intended to observe polytene chromosomes and neuroblast chromosomes. Polytene chromosomes would be observed to study defects in the banding pattern of the chromosomes, or abnormality in levels of polyteny. Neuroblast chromosomes would be observed to identify defects in mitosis. Chapter three describes my work to characterize the lethality of *E(var)3-5* mutants.

My second goal was to identify the genetic locus on the third chromosome that corresponds to *E(var)3-5*. To identify which gene might be a potential candidate for *E(var)3-5*, I pursued the approach of complementation tests analysis using deficiency stocks (deficiency mapping). Given the size of available deficiencies, it was anticipated that I would generate deletions within the region defined by a noncomplementing deficiency using FRT-bearing transgenes and *FLP* recombinase. These deletions would be used to better map *E(var)3-5*. Additionally, complementation tests would be carried out with lethals in region of interest to eliminate or include the possibility of that particular gene being *E(var)3-5*. After the *E(var)3-5* gene was identified, transgenes would be generated to analyze if the transformants rescued lethality. My work to identify the *E(var)3-5* gene is described in chapter two. Unfortunately I concluded that the lethality of the original mutants was not the result of mutations in a single gene.

As the *CG17360* locus was primarily responsible for the lethality and caused surviving flies to have an unexpanded wing phenotype, I characterized this gene. I also studied the relationship between *CG17360* and the E(var) phenotype.

Chapter 2: Identification of the *E(var)3-5* gene.

Introduction

Dorn *et al.* (1993b) carried out large-scale experiments to isolate PEV modifying genes that behaved as enhancers. The isolation of PEV enhancing gene would facilitate the understanding of, the process of chromosome decondensation or euchromatin formation. Classic mutagens such as X-rays, EMS were used. In addition the $\pi 2$ *P*-element and modified *P*-elements such as *Icarus-neo*, *pUCHsneory*⁺, *P[IArB]* were also used. The enhancer mutations were selected based on their ability to enhance the variegation of *white*^{mottled4} (*w*^{m4}) allele. A large number of enhancer mutations were isolated and it was estimated that around 50-60 enhancer loci exist on the second and third chromosome. Mutant alleles of *E(var)3-5* were isolated as a dominant enhancers of variegation of *w*^{m4} mutation. In the $\pi 2$ *P*-element mutagenesis screen, four recessive lethal alleles of *E(var)3-5* were isolated on third chromosome. Similarly in the *Icarus-neo* mutagenesis screen six recessive lethal alleles of *E(var)3-5* were isolated. However the *E(var)3-5* mutations did not revert in the presence of a transposase and none of the *E(var)3-5* alleles retained an *Icarus-neo* homology as detected by Southern blot analysis of mutant lines. The same was true for *E(var)3-4* alleles, which were also isolated in the *Icarus-neo* mutagenesis. Dorn *et al.* (1993b) thus suggested that the mutations might be spontaneous in nature. The *E(var)3-5* alleles were mapped on the right arm of chromosome three (Dorn *et al.* 1993b)

Seven *E(var)3-5* alleles were gifted generously to our lab by Dr. Gunter Reuter. Previous meiotic mapping data showed that the lethality of *E(var)3-5* mutants was located at map position 3-50.56 $\pm$ 0.28 map units, which corresponds to cytological location 86DE (Weiler, unpublished). Complementation tests of *E(var)3-5* mutants with deficiency stocks indicated that *Df(3R)cu/TM6B*, *Tb* and *Df(3R)M86D/TM3*, *Ser* complemented, whereas *Df(3R)M-Kx1/TM3*, *Sb Ser* and *Df(3R)T-32 cu sr e/MRS* did not complement

(Weiler, unpublished). These data indicated that *E(var)3-5* was present in region 86E2-4 to 87B1-5.

I planned to map the approximate location of the *E(var)3-5* gene by carrying out complementation tests with several *Exelixis* deficiencies having defined end-points. Once I narrowed the approximate location, I intended to generate smaller deletions using the FLP-FRT system to map the *E(var)3-5* mutation to a smaller region. *P* elements and *PBac* transposons such as *PBac{RB}* and *PBac{WH}* would be used for making deletions. All these transposons contain a FRT site of 199 basepair. The FRT site is 5' (in *XP* and *WH* transposons) and is 3' (in *RB* transposons) of the *white*⁺ (*w*⁺) transgene (Parks *et al.* 2004). Trans-recombination between FRT sites due to the action of *FLP* recombinase results in a genomic deletion or duplication (Figure 2.1A). Depending on the pair of transposons used, a deletion can be identified due to the loss of *w*⁺ marker.

Figure 2.1 *FLP*-FRT mediated deletion generation and detection.

The chromosomes are indicated as black lines. The two transposons are shown as blue and red triangles. A. Recombination between the FRT sites due to *FLP* recombinase results in a hybrid transposon, which is *w*⁻ in the example shown. B. The PCR strategy for identifying the deletions is illustrated, with the primers shown as arrows. Two inward primers can synthesize a unique product for some hybrid elements (above). Other hybrid elements are identified by the presence of the new combination of transposon ends on the chromosome (below).

After obtaining these deletions, I planned to carry out complementation tests of these deletions with *E(var)3-5* mutants. Once I identified the *E(var)3-5* gene based on its failure to complement, I intended to generate one or more transgenes using the appropriate DNA fragments. I planned to determine if the transgene would rescue the lethality and the enhancer phenotype of *E(var)3-5* mutants, which would confirm that the identified gene is *E(var)3-5*.

To study the dosage-dependent enhancer effect of the *E(var)3-5* gene, I planned to generate a duplication that would carry three copies of *E(var)3-5* gene using the *FLP-FRT* system described above. Since the function of a single wild type *E(var)3-5⁺* gene is to promote euchromatin, the effect of three wild-type copies of *E(var)3-5⁺* should be suppression of PEV.

A Northern analysis was planned to detect the expression pattern of *E(var)3-5* transcript during different stages of development and in various tissue types.

Materials and methods

Drosophila stocks and culture conditions: All the strains and crosses were maintained in cornmeal-malt medium established as a standard media recipe by Bloomington Drosophila Stock Center (<http://flystocks.bio.indiana.edu>). The crosses were maintained at 25°C unless otherwise noted. The *E(var)3-5* mutants alleles were isolated by Dorn *et al.* (1993b) and were gifted to our lab by R. Dorn and G. Reuter. The Exelixis deficiencies *Df(3R)Exel7310*, *Df(3R)Exel 6162*, *Df(3R)Exel7312*, *Df(3R)Exel8155* and *Df(3R)Exel7313* and *PBac* insertion lines *PBac{WH}glo^{f02674}*, *PBac{WH}CG6962^{f05936}*, and *PBac{RB}CG17360^{e02295}* were obtained from Bloomington Stock Center. The *P* and *PBac* element stocks *P{XP}I(3)neo38^{d03325}*, *PBac{WH}f01607*, *PBac{RB}CG6950^{e01513}*, *P{XP}CG31368^{d00672}* and *PBac{WH}CG17360^{f05458}* were obtained from the Exelixis Collection at the Harvard Medical School. Information on other mutants used in this study is available at Flybase (<http://flybase.bio.indiana.edu/>).

Deletion generation using the FLP-FRT system: For obtaining deletions, females bearing an X chromosome expressing *FLP* recombinase, *P[ry⁺ hs-FLP] y w*, and two third chromosome *P* or *PBac* element insertions containing FRT sites were heat shocked at 37°C for one hour for the first six days of development to induce expression of the *FLP* recombinase. The adults were then crossed to *iso w¹¹¹⁸ /Y; wg^{Sp-1} /Cyo ; sens^{Ly-1} / TM6B, Tb* males. From the resulting cross five *w⁻* Tb Hu males were recovered for a *w⁻* deletion, and for a *w⁺* deletion at least 50 *w⁺* Tb Hu males were recovered. The males were stocked by crossing to *iso w¹¹¹⁸; iso2; Dr¹ /TM6B, Tb Hu* females. PCR analysis was done on the recovered males after setting up the cross to confirm the deletion. The PCR strategy is illustrated in Figure 2.1B. The deletion was confirmed by detecting the hybrid *P* or *PBac* element or by detecting the two *PBac* element ends, one originating from each original transposon. The same strategy was applied for generating a duplication. Table 2.1 lists the deletions and duplication and the primers used for detecting them. Table 2.2 lists the sequence of the primers. Four isolates were recovered for *Df(3R)D1M1*, *Df(3R)D7M1*, *Dp(3R)D1M1*. Two isolates were recovered for *Df(3R)D5M3* and *Df(3R)CG17360*. One isolate was recovered each for *Df(3R)D2M2* and *Df(3R)D4M1*. The primers were purchased from Invitrogen.

Table 2.1 PCR analyses of putative deletions and duplications

Deletion	Deletion type	Left primer	Right primer	Expected fragment size
<i>Df(3R)D1M1</i>	w^-	XP5' in	RB3' in	1.7 kb
<i>Df(3R)D2M2</i>	w^-	XP5' in	RB3' in	1.7 kb
<i>Df(3R)D4M1</i>	w^-	WH5' in	XP5' in	1.8 kb
<i>Df(3R)D3M1^a</i>	w^-	RB3' in	XP5' in	1.7 kb
<i>Df(3R)D5M3</i>	w^-	WH5' in	XP5' in	1.8 kb
<i>Df(3R)D6M1^a</i>	w^+	RB3' in	WH5' in	7.3 kb
<i>Df(3R)D7M1</i>	w^+	WH5' out	f01607 R	400 bp
		WH3' out	f02674 F	700 bp
<i>Df(3R)CG17360</i>	w^+	WH3' out	f05458 R	190 bp
		RB3' out	CG17360 4265 R	726 bp
<i>Dp(3R)D1M1</i>	w^-	XP5' in	WH5' in	1.8 kb

^aThe deletion was not obtained.

Table 2.2 Sequence of primers used for identification of deletions and duplication

XP5' in ^a	5'-AATGATTCGCAGTGGGAAGGCT-3'
RB3' in ^a	5'-TGCATTTGCCTTTTCGCCTTAT-3'
WH5' in ^a	5'-GACGCATGATTATCTTTTACGTGAC-3'
WH5' out ^b	5'-TCCAAGCGGCGACTGAGATC-3'
WH3' out ^b	5'-CCTCGATATACAGACCGATAAAAC-3'
RB3' out ^b	5'-CCTCGATATACAGACCGATAAAAC-3'
f01607 R ^c	5'-CTTGGATTTGACGGGCTCTTATCG-3'
f02674 F ^c	5'-ATGTGACCAACGAAGGAATGA-3'

^aTransposon-specific primers directing synthesis inward; designed by Parks *et al.* (2004)

^bTransposon-specific primers directing synthesis outward; designed by Parks *et al.* (2004)

^cGenomic primers

Reversion of the *PBac{RB}CG17360^{e02295}* element: In order to excise the *PBac{RB}CG17360^{e02295}* element for obtaining *CG17360* revertants, male flies bearing *PBac{RB}CG17360^{e02295}* element and a second chromosome expressing *PBac* transposase, *CyO P(tub-PBac/T)*, were crossed to *w⁻; TM3, Sb St e/TM6B, Tb Hu e* females. The *PBac{RB}CG17360^{Rev}* revertant males identified as *w⁻ Sb* were recovered and stocked. In *CG17360* revertants the excision of 5' end of element was detected using the primer sets *CG17360 3219F* and *RB5' out* (5'-TCCAAGCGGCGACTGAGATG-3') whereas the excision of 3' end was detected using the primer sets *CG17360 4265R* and *RB3' out*.

In an attempt to obtain revertants in which the *PBac{RB}CG17360^{e02295}* has excised precisely, a *CG17360* revertant identified as *PBac{RB}CG17360^{e02295w⁻}* was obtained in which the *w⁺* transgene was mutated.

Stubble (Sb) variegation: Table 2.3 lists the genotype of strains tested for an effect on *Sb* variegation. Flies bearing an *E(var)3-5* mutation, *CG17360* mutation or deletion mutation balanced with *TM3, Sb* were crossed to *w¹¹¹⁸; Dr^{Mio}/ TM3, P{w⁺GAL4-twi.G} P{w⁺UAS-2xEGFP} Sb Ser* flies. The resulting *Sb⁺ Dr⁻* males were crossed to *T(2:3)Sb^v, In(3R)Mo Sb/ TM3, Ser* virgin females. The progeny to be assayed for variegation of the *Sb* gene were identified as *Ser⁺ Dr⁺*. A total of fourteen bristles per fly were scored for being *Sb* or wild type. The macrochaetes scored were the anterior and posterior sternopleurals (4), the humerals (4), the posterior dorso-centrals (2) and the anterior and posterior scutellars (4).

Table 2.3. Genotype of strains used for *Sb* variegation

<i>T(2:3)Sb^v, In(3R)Mo Sb^l/ TM3, Ser</i>
<i>w¹¹¹⁸ ; E(var)3-5⁰¹/TM3, P{w⁺GAL4-<i>twi</i>.G} P{w⁺UAS-2xEGFP} Sb Ser</i>
<i>w¹¹¹⁸ ; E(var)3-5⁰³/TM3, P{w⁺GAL4-<i>twi</i>.G} P{w⁺UAS-2xEGFP} Sb Ser</i>
<i>w ; Df(3R)D1M1/TM3, Sb st e</i>
<i>w ; Df(3R)D2M2/TM3, Sb st e</i>
<i>w ; Df(3R)D4M1/TM3, Sb st e</i>
<i>w¹¹¹⁸; Df(3R)Exel7310/TM3, P{w⁺GAL4-<i>twi</i>.G} P{w⁺UAS-2xEGFP} Sb Ser</i>
<i>w ; PBac {RB}CG17360^{e02295}/TM3, Sb st e</i>
<i>w ; PBac {RB}CG17360^{Rev}/TM3, Sb st e</i>
<i>w¹¹¹⁸ ; Dr^{Mio}/TM3, P{w⁺GAL4-<i>twi</i>.G} P{w⁺UAS-2xEGFP} Sb Ser</i>

PCR analysis: A series of overlapping primers were produced for the coding region of *CG17360*. The primers were designed using the software Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and were obtained from Invitrogen. According to Release 5.1 of the fly genome the sequence coordinates for *CG17360* are 3R: 7,606,851..7,612,736 (<http://flybase.bio.indiana.edu/reports/FBgn0037949.html>). Nucleotide 7,606,851 was considered as position 1 for the purpose of naming the primers, so that the sequence coordinate for *CG17360* 622F corresponds to 7,607,472. The sequence coordinates for other *CG17360* primers can be obtained similarly. The primer sets were *CG17360* 622F (5'-AACGCTTGAGTAATGTATATGGAAA-3') and *CG17360* 1608R (5'-GAGTATGTAGCCTCCGAGTGG-3'), *CG17360* 1480F (5'-TGTGCTTCTCCTCTTGAACCA-3') and *CG17360* 2457R (5'-AACTTATCATTTTCAGGCTTTTCAAAC-3'), *CG17360* 2327F (5'-TTGACACACAGACTATTTCTTCCA-3') and *CG17360* 3318R (5'-CGCAGCAACAAGCACCAG-3'), *CG17360* 3219F (5'-TGATGGTTGCTGCTGGTGT-3') and *CG17360* 4265R (5'-GAGCCCAATATCGGAGATGC-3'), *CG17360* 4145F (5'-

CCTGCTCGAGTATGACATTTG-3') and CG17360 5340R (5'-ACACAAATAATGGTTTACCGACTT-3'), CG17360 3154F (5'-TGAGCGGTGAAGGAAGGTGT-3') and CG17360 3978R (5'-CGTCCCTGAGCTGGACAGA-3'), CG17360 3734F (5'-ATTCCTCTTGCGATGCAA-3') and CG17360 4578R (5'-CGGTCTGCACTATGACACCTC-3'), CG17360 4475F (5'-AGTTGGAAGTCTGCACACG-3') and CG17360 5349R (5'-CGCCGAGATACACAAATAATGG-5'), CG17360 3941F (5'-GCGATTTGTGCTCTGGTTGTCT -3') and CG17360 4635R (5'-AAATGCCTGCTCGGATGTG -3'). Genomic DNA was isolated from ten first instar homozygous *E(var)3-5* larvae and from single flies of the genotype *E(var)3-5/D2M2* (alleles one through seven) and *CantonS* (Gloor *et al.* 1993). In brief, a single fly or ten larvae was mashed in 50 ul of 1X squishing buffer with proteinase K and incubated at 37°C for 25-30 minutes. Proteinase K was inactivated by heating the samples at 95°C for 1-2 minutes. For a 25 ul PCR reaction a 2ul volume of the DNA prep was used.

Southern analysis: Genomic DNA was isolated from 50-60 flies of the six *E(var)3-5/TM6B* stocks and *Canton S/TM6B* using the protocol "Purification of total DNA from insects using the DNeasy blood and tissue kit" from Qiagen. Purified DNA was digested with *Pst*I and the equivalent of 10 flies loaded in each lane of an agarose gel. Following separation and transfer to a nylon membrane, the blot was hybridized to a DIG-labelled PCR probe synthesized using primers CG17360 3219F and CG17360 4265R according to the manufacturers instructions (Roche).

Eye pigmentation assay: The genotype of strains tested for an effect on w^{m4} variegation is indicated in Table 2.4. Virgin females bearing an *E(var)3-5* mutation or deletion mutation balanced with TM6B were crossed to *ru h th st p^o cu sr Pr e ca/TM3, Sb e* males. The resulting $Pr^+ e^+$ males were crossed to *In(1)w^{m4}* virgin females. The progeny to be assayed for variegation of the *w* gene were identified as $Pr^+ e^+$. All the crosses were established at 25°C. The $Pr^+ e^+$

flies were aged for 5 days at 25°C and stored at -80°C. The red eye pigment was measured according to the method of Ephrussi and Herold (1944). For each genotype, red pigment was extracted using acidified ethanol from 10 samples, each containing 10 heads. The samples were split into two tubes for duplicate processing, and optical absorbance at 480nm was recorded.

Table 2.4 Genotype of strains for w^{m4} variegation

$ln(1)w^{m4}$
$w ; E(var)3-5^{01}/TM6B, Tb$
$w^{1118} ; Df(3R)D1M1/TM6B, Tb$
$w^{1118} ; Df(3R)D2M2/TM6B, Tb$
$w^{1118} ; Df(3R)D4M1/TM6B, Tb$
$w^{1118} ; Df(3R)D5M3/TM6B, Tb$
$w^{1118} ; Df(3R)Exel7310/TM6B, Tb$

Beta-galactosidase staining: The effect of deletion $Df(3R)Exel7310$ and mutations in $CG17360$ was tested on variegation of the $LacZ$ gene.

$ln(3L)BL1/TM6C, Sb Tb$ virgin females were crossed to males bearing an $E(var)3-5$ mutation, $CG17360$ mutation or deletion mutation. The mutant chromosomes were balanced over the $TM6C, Sb Tb$ balancer. Beta-galactosidase staining was performed on Tb^+ larvae from the resulting cross. The salivary glands of Tb^+ larvae were dissected and fixed in 3.7% formaldehyde for 15 to 20 minutes at room temperature. The salivary glands were rinsed three times in PBS solution for 10 minutes each. Samples were incubated in a staining solution (10mM $NaH_2PO_4 \cdot H_2O$, 150mM $NaCl$, 1mM $MgCl_2 \cdot 6H_2O$, 3.1mM $K_4[Fe^{II}(CN)_6]$, 3.1mM $K_3[Fe^{III}(CN)_6]$, 0.3% Triton X 100) containing 0.2% X-gal and incubated at 37°C overnight. After overnight incubation, the samples were rinsed three times in PBS for ten minutes each. The samples were mounted in 10% glycerol, and the number of nuclei stained in each lobe was counted.

Synthesis of $P\{w^+ \text{UAS-}CG17360\}$ construct and sequencing: A *CG17360* DNA fragment was PCR amplified using primers *CG17360* 622F and *CG17360* 5349R, Phusion polymerase (NEB) and genomic DNA isolated from *CantonS* flies as a template. It was cloned into the vector pCR-Blunt (Invitrogen) and was sequenced. The *CG17360* 1480F and *CG17360* 1608R, *CG17360* 2327F and *CG17360* 2457R, *CG17360* 3219F and *CG17360* 3318R, *CG17360* 4145F and *CG17360* 4265R were used as sequencing primers (described in PCR analysis section). After verifying that the sequence of the clone did not have any mutations, it was subcloned into the pP[UAS] vector as a *KpnI* and *NotI* restriction fragment.

Generation of $P\{w^+ \text{UAS-}CG17360\}$ transgenic flies: The $P\{w^+ \text{UAS-}CG17360\}$ construct was injected into w^{1118} embryos by Rainbow Transgenic Flies. Eight insertions were obtained on the 2nd chromosome and eight insertions were obtained on the third chromosome.

Results

Deficiency mapping of *E(var)3-5* lethality: Previous work showing that *Df(3R)M-Kx1/TM3, Sb Ser* and *Df(3R)T-32 cu sr e/MRS* failed to complement the lethality of *E(var)3-5* indicated that it mapped in the genomic region 86E2-4 to 87B1-5. I carried out complementation tests between *E(var)3-5* mutants and Exelixis deficiency mutants to map the lethality to a defined genomic locus. In this experiment *Deficiency/TM6B, Tb Hu* males were crossed to *E(var)3-5/TM3, P\{w^+ GAL4-twi.G\} P\{w^+ UAS-2xEGFP\} Sb Ser* female. A 25% ratio of $Sb^+ Hu^+$ adult *E(var)3-5/deficiency* progeny was expected if the mutants complemented. The complementation test results indicated that *E(var)3-5* mutants failed to complement *Df(3R)Exel7310* mutants (Table 2.5).

Table 2.5 Lethality mapping of *E(var)3-5* using Exel deficiencies

Males	<i>E(var)3-5⁰¹/TM3, Sb</i> females				<i>E(var)3-5⁰⁴/TM3, Sb</i> females			
	Sb Hu ⁺	Sb Hu	Sb ⁺ Hu	Sb ⁺ Hu ⁺	Sb Hu ⁺	Sb Hu	Sb ⁺ Hu	Sb ⁺ Hu ⁺
<i>Df(3R)</i> <i>Exel</i> 7310	(34) 37.7	(33) 36.6%	(23) 25.5%	0	(6) 18.7	(16) 50%	(10) 31.2%	0
<i>Df(3R)</i> <i>Exel</i> 6162	0 ^a	(23) 23.7%	(36) 37.1%	(38) 39.1%	0 ^a	(13) 24.5%	(22) 41.5%	(18) 33.9%
<i>Df(3R)</i> <i>Exel</i> 7312	(27) 20.7%	(30) 23.0%	(35) 27%	(38) 29.2%	(11) 16.1%	(15) 22.0%	(21) 30.8%	(21) 30.8%
<i>Df(3R)</i> <i>Exel</i> 8155	(48) 34.2%	(25) 17.8%	(31) 22.1%	(36) 25.7%	(7) 17.5%	(5) 12.5%	(13) 32.5%	(15) 37.5%
<i>Df(3R)</i> <i>Exel</i> 7313	(45) 25.8%	(40) 22.9%	(41) 23.5%	(48) 27.5%	(36) 31.3%	(12) 10.4%	(33) 28.6%	(34) 26%

The number in the parentheses indicated the number of progeny scored.

^a *Df(3R)6162/TM3, Sb* progeny were not obtained.

The genomic coordinates for each deficiency are as follows:

Df(3R)Exel7310 3R:7584303..7712814.

Df(3R)Exel6162 3R:7713466..8106805.

Df(3R)Exel7312 3R:7803578..7905849.

Df(3R)Exel8155 3R:7819266..7939447.

Df(3R)Exel7313 3R:7933838..8106582.

Generation of targeted deletions: The lethality mapping data using

Df(3R)Exel7310 indicated that *E(var)3-5* maps in the 86F6-87A1 region.

Fourteen predicted genes map to this cytological interval. To identify which of these fourteen gene corresponds to *E(var)3-5*, I generated 5 smaller deletions using *FLP*-recombinase and FRT elements. Table 2.6 indicates the *P* and *PBac* elements used for obtaining the deletions. Figure 2.2 highlights the number of genes deleted in each deficiency. *Df(3R)D2M2* and *Df(3R)D4M1* together delete all of the genes, except *Lk6*. *Df(3R)Exel9019* deletes *Lk6*

(<http://flystocks.bio.indiana.edu>).

Table 2.6 *P* and *PBac* elements used for obtaining deletions

Deletion	Proximal P element	Distal P element
<i>Df(3R)D1M1</i>	<i>P{XP}l(3)neo38^{d03325}</i>	<i>PBac{RB}CG17360^{e02295}</i>
<i>Df(3R)D2M2</i>	<i>P{XP}l(3)neo38^{d03325}</i>	<i>PBac{RB}CG6950^{e01513}</i>
<i>Df(3R)D4M1</i>	<i>PBac{WH}glo^{f02674}</i>	<i>P{XP}CG31368^{d00672}</i>
<i>Df(3R)D5M3</i>	<i>PBac{WH}CG6962^{f05936}</i>	<i>P{XP}CG31368^{d00672}</i>
<i>Df(3R)D7M1</i>	<i>PBac{WH}f01607</i>	<i>PBac{WH}glo^{f02674}</i>
<i>Df(3R)CG17360</i>	<i>PBac{WH}CG17360^{f05458}</i>	<i>PBac{RB}CG17360^{e02295}</i>

The genomic coordinates for each deficiency are listed below

Df(3R)D1M1 3R:7604127..7611052.
Df(3R)D2M2 3R: 7604127..7647966.
Df(3R)D4M1 3R: 7644087..7712923.
Df(3R)D5M3 3R: 7708215..7712923.
Df(3R)D7M1 3R: 7634175..7644087.
Df(3R)CG17360 3R: 7606891..7611052.

Figure 2.2 *FLP-FRT* mediated targeted deletions in *Df(3R)Exel7310* region

<i>Lk6</i>					
<i>l(3)neo38</i>	<i>Df(3R)D1M1</i>	<i>Df(3R)D2M2</i>			
<i>CG17360</i>					
<i>HisC</i>					
<i>Sbf</i>					
<i>CG31116</i>					<i>Df(3R)D7M1</i>
<i>CG6946</i>					
<i>CoVa</i>					
<i>CG6950</i>			<i>Df(3R)D4M1</i>		
<i>CG6959</i>					
<i>Sad</i>					
<i>CG6962</i>					
<i>CG31368</i>				<i>Df(3R)D5M3</i>	
<i>mth15</i>					

The genes defined by the *Df(3R)Exel7310* region are listed in the first column. The genes deleted in each deficiency are indicated.

Complementation test of *E(var)3-5* mutants with targeted deletions: I carried out complementation tests between *E(var)3-5* mutants and deletion mutants to identify the gene that might be allelic to *E(var)3-5*. A 33% ratio of Sb^+ flies was expected if the two mutants complemented each other. The complementation tests were set up in vials. The results are shown in Table 2.7. The complementation test results indicated that *Df(3R)D4M1*, *Df(3R)D5M3* and *Df(3R)D7M1* complement the lethality of *E(var)3-5* mutants. With *Df(3R)D1M1* and *Df(3R)D2M2*, Sb^+ progeny were obtained but the percentage was low (except for *Df(3R)DIM1* line 3), which indicated that *E(var)3-5* mutants fail to complement these deficiencies.

Df(3R)D1M1 and *Df(3R)D2M2* delete two genes, *CG17360* and *l(3)neo38*. To identify which of these genes might be allelic to *E(var)3-5*, I carried out complementation tests of *PBac{RB}CG17360^{e02295}* and *mwh l(3)neo38 red e* mutant females with *E(var)3-5⁰¹* mutant males. The complementation tests were set up in vials. A 33% ratio of Sb^+ flies was expected if the two mutants complemented each other. The results are indicated in Table 2.8. After performing the tests I discovered that the *l(3)neo38* mutants do not have a recessive lethal phenotype and the flies of the stock were Sb^+ . Therefore, *l(3)neo38* is not allelic to *E(var)3-5*. These results pointed out that *CG17360* might be allelic to *E(var)3-5*. However the ratio of Sb^+ progeny (25.6%) was only slightly lower than expected (Table 2.8). I repeated the complementation test between *PBac{RB}CG17360^{e02295}* and *E(var)3-5* mutant alleles and a consistent number of *E(var)3-5/PBac{RB}CG17360^{e02295}* Sb^+ progeny was not obtained each time (Table 2.9).

Table 2.7 Complementation test of *E(var)3-5* mutants and deletion mutants

Males	Females			
	<i>E(var)3-5⁰¹</i>		<i>E(var)3-5⁰³</i>	
	Sb	Sb ⁺	Sb	Sb ⁺
<i>Df(3R)D1M1 isolate 1</i>	64 (91.4%)	6 (8.5%)*	118 (90.7%)	12 (9.2%)*
<i>Df(3R)D1M1 isolate 2</i>	58 (96.6%)	2 (3.3%)*	71 (85.5%)	12 (14.4%)*
<i>Df(3R)D1M1 isolate 3</i>	97 (75.1%)	32 (24.8%)*	117 (88.6%)	15 (11.3%)*
<i>Df(3R)D1M1 isolate 4</i>	79 (98.7%)	1 (1.2%)*	65 (94.2%)	4 (5.7%)*
<i>Df(3R)D2M2</i>	143 (90.5%)	15 (9.4%)*	109 (97.3%)	3(2.6%)*
<i>Df(3R)D4M1</i>	108 (61.3%)	68 (38.6%)	116(55.2%)	94 (44.7%)
<i>Df(3R)D5M3</i>	67 (60.3%)	44 (39.6%)	12(58.7%)	87(41.2%)
<i>Df(3R)D7M1 isolate 4</i>	93 (62.4%)	56 (37.5%)	102 (60.7%)	66 (39.2%)
<i>Df(3R)D7M1 isolate 26</i>	90 (58.4%)	64 (41.5%)	88 (67.1%)	43 (32.8%)

The *E(var)3-5* mutant alleles and deletion alleles were balanced over the *TM3*, *Sb* balancer. This data was derived from one experiment. The tests were set up in vials.

* The flies had unexpanded wings.

Table 2.8 Complementation test of *E(var)3-5⁰¹* mutant allele

Females	Trial	<i>E(var)3-5⁰¹</i> males		
		Sb	Sb+	Total
<i>l(3)neo38</i>	1	36 (41.8%)	50 (58.1%)	86
	2	145 (37.6%)	240 (62.3%)	385
<i>PBac{RB}CG17360^{e02295}</i>	1	122 (74.3%)	42 (25.6%)*	162

The chromosomes in mutant alleles were balanced over the *TM3*, *Sb* balancer. The complementation tests were carried out in vials.

* The flies had unexpanded wings.

Table 2.9 Complementation tests of *PBac{RB}CG17360^{e02295}* mutant alleles

	<i>Trial</i>	<i>E(var)3-5⁰¹</i> males		<i>E(var)3-5⁰³</i> males		<i>PBac{RB}</i> <i>CG17360^{e02295}</i> males	
Females		Sb	* Sb ⁺	Sb	* Sb ⁺	Sb	* Sb ⁺
<i>PBac{RB}</i> <i>CG17360^{e02295}</i>	1	215	11	244	1	-	-
		95.1%	4.8%	99.5%	0.4%		
	2	145	44	204	27	-	-
		76.7%	23.2%	88.3%	11.6%		
<i>E(var)3-5⁰¹</i>	1	-	-	-	-	188	5
						97.4%	2.5%
	2	-	-	-	-	133	36
						78.6%	21.3%
<i>E(var)3-5⁰³</i>	1	-	-	-	-	220	3
						98.6%	1.3%
	2	-	-	-	-	108	22
						(83.0)	16.9%

The chromosomes in mutant alleles were balanced over the *TM3, Sb* balancer. The numbers in the brackets indicate the % of Sb and Sb⁺ progeny.

* The Sb⁺ flies had unexpanded wings.

In addition, *E(var)3-5/Df(3R)D1M1* and *E(var)3-5/Df(3R)D2M2* flies were found to have an unexpanded wing phenotype. *E(var)3-5/ PBac{RB}CG17360^{e02295}* flies also exhibited an unexpanded wing phenotype. In order to verify that this phenotype was linked to the *PBac* insertion in *CG17360*, I generated revertant alleles of *PBac{RB}CG17360^{e02295}* and carried out complementation tests with the *E(var)3-5* mutant alleles. The purpose of this experiment was to determine whether the revertant allele restores the wing phenotype, and to verify that the reduction in viability observed for *PBac{RB}CG17360^{e02295}/E(var)3-5* flies was due to the *CG17360* insertion mutation. The results are shown in Table 2.10. The results indicated that *E(var)3-5⁰¹/ PBac{RB}CG17360^{Rev}* and *E(var)3-5⁰³/ PBac{RB}CG17360^{Rev}* had a

wild-type wing phenotype. Similarly, in $PBac\{RB\}CG17360^{Rev}/PBac\{RB\}CG17360^{e02295}$ and $PBac\{RB\}CG17360^{Rev}/PBac\{RB\}CG17360^{e02295w-}$ flies the wing phenotype was wild-type indicating that one wild-type copy of *CG17360* is essential for obtaining normal wings. The $PBac\{RB\}CG17360^{Rev}/E(var)3-5^-$ flies were observed at the expected frequency (38.9% and 34.3%), showing that mutation of *CG17360* caused the reduction in viability. However, there appeared to be additional mutations on the $PBac\{RB\}CG17360^{e02295}$ chromosome that slightly reduced viability when this chromosome was homozygous (e.g. $PBac\{RB\}CG17360^{Rev}/PBac\{RB\}CG17360^{Rev}$

Table 2.10 Complementation tests of $PBac\{RB\}CG17360$ alleles

	$PBac\{RB\}CG17360^{e02295w-}$		$PBac\{RB\}CG17360^{Rev}$		$PBac\{RB\}CG17360^{e02295}$	
Males	Sb	Sb ⁺	Sb	Sb ⁺	Sb	Sb ⁺
$E(var)3-5^{01}$	110	25*	369	232	379	73*
	81.4%	18.5%	60.9%	38.9%	83.8%	16.1%
$E(var)3-5^{03}$	61	22*	412	216	366	14*
	73.4%	26.5%	65.6%	34.3%	96.2%	3.6%
$PBac\{RB\}CG17360^{Rev}$	185	39	328	116	470	102
	82.5%	17.4%	73.8%	26.1%	82.1%	17.8%
$Df(3R)D1M1$	137	11	394	204	355	33
	92.5%	7.4%	65.8%	34.1%	91.4%	8.5%
$Df(3R)D2M2$	89	12	437	210	235	23
	88.1%	11.8%	68.4%	31.5%	93.5%	6.4%
$PBac\{RB\}CG17360^{e02295w-}$	33	9	327	67	412	19
	78.5%	21.4%	82.9%	17%	95.6%	4.4%

The chromosomes in mutant alleles and the revertant allele were balanced over the *TM3*, *Sb* balancer. The data derived from a single experiment.

* *Sb⁺* flies have unexpanded wings.

The *PBac{RB}CG17360^{e02295}* insertion in *CG17360* may not eliminate the function of *CG17360* and this could be why the *PBac{RB}CG17360^{e02295}* mutants partially complement *E(var)3-5* mutants. I therefore generated a null allele of *CG17360* by deleting coding sequence of *CG17360* using the *FLP-FRT* mediated technique. Two isolates were obtained, *Df(3R)CG17360^{M28}* and *Df(3R)CG17360^{M56}*. I carried out complementation tests of *Df(3R)CG17360^{M56}* mutant alleles. A 33.3% ratio of *Sb*⁺ progeny was expected if the mutants complemented. The complementation tests were performed in vials. Also it was learned that *Df(3R)CG17360^{M56}/TM3*, *Sb st e* flies had an unexpanded wing phenotype. Thus all mutant alleles had to be balanced over the *TM6C*, *Sb Tb* balancer. The complementation test results indicated that a null allele of *CG17360* partially complements *E(var)3-5* (ratio of *Sb*⁺ progeny, 16.3% and 18.2%).(Table 2.11). A low ratio of *Sb*⁺ progeny (12.1%) was obtained with *PBac{RB}CG17360^{e02295}* flies. Also a low ratio of *Sb*⁺ progeny (12%) representing the genotype *Df(3R)D1M1/ Df(3R)CG17360^{M56}* was obtained when two deletion mutants were crossed to each-other.

Table 2.11 Complementation tests of *Df(3R)CG17360^{M56}* mutant allele in vials

Males	<i>Df(3R)CG17360^{M56}</i> females		
	<i>Sb</i>	<i>Sb</i> ⁺	Total
<i>E(var)3-5⁰¹</i>	556 (83.5%)	109 (16.3%)*	665
<i>E(var)3-5⁰³</i>	665 (81.7%)	148 (18.2%)*	813
<i>PBac{RB}CG17360^{Rev}</i>	774 (71.9 %)	300 (27.9%)	1074
<i>Df(3R)D1M1</i>	808 (87.8%)	111 (12.0%)*	919
<i>PBac{RB}CG17360^{e02295}</i>	802 (87.8%)	111 (12.1%)*	913

The *CG17360*, *E(var)3-5* and *CG17360^{Rev}* mutant chromosomes were balanced over the *TM6C*, *Sb Tb* balancer. The number in the parenthesis indicates the % of *Sb* and *Sb*⁺ progeny. The complementation tests were carried out in vials. The *PBac{RB}CG17360^{Rev}* was used as control. The data was derived from a single experiment.

*The flies had unexpanded wing phenotype.

To determine if the variability in results (frequency of Sb^+) might be related to the high-density growth conditions in vials, I repeated complementation test done in bottles. The results for complementation test of $Df(3R)CG17360^{M56}$ mutant allele done in bottles is shown in Table 2.12. The complementation test indicated that $Df(3R)CG17360^{M56}$ mutant allele complement $E(var)3-5$ mutants alleles since the expected ratio of Sb^+ progeny was obtained (Table 2.12). Overall the results of complementation tests done in bottles and vials indicated “differences” in ratio of Sb^+ progeny obtained. Variability in the ratio of Sb^+ progeny was observed with complementation tests in vials. These results suggested that Sb^+ progeny representing genotypes $E(var)3-5^-/CG17360^-$ and $E(var)3-5^-/Df(3R)D1M1$ were sensitive to density conditions in bottles versus vials.

Table 2.12 Complementation test of $Df(3R)CG17360^{M56}$ mutant allele in bottles

	$Df(3R)CG17360^{M56}$ females			
Males	Trial	Sb progeny	Sb^+ progeny	Total
$E(var)3-5^{01}$	1	408 (65.9%)	205 (33.9%)*	613
	2	564 (70.7%)	233 (29.2%)*	797
$E(var)3-5^{03}$	1	298 (64.1%)	166 (35.7%)*	464
	2	346 (62.8%)	204 (37%)*	550
$PBac\{RB\}CG17360^{Rev}$	1	446 (66.8%)	222 (33.2%)	668
	2	774 (71.9%)	300 (27.9%)	1074
$Df(3R)D1M1$	1	544 (75.9%)	172 (24.0%)*	716
	2	565 (78.1%)	158 (21.8%)*	723
$PBac\{RB\}CG17360^{e02295}$	1	489 (77.9%)	138 (22%)*	627
	2	593 (82.1%)	129 (17.8%)*	722

The chromosomes of all the mutant alleles were balanced over the $TM6C$, Sb Tb balancer. The number in the brackets indicates the % of Sb and Sb^+ progeny. The $PBac\{RB\}CG17360^{Rev}$ was used a control. * indicates unexpanded wings.

As these results indicate that the lethality of *E(var)3-5⁻* mutations does not map to *CG17360*, I repeated my complementation tests of *E(var)3-5⁰¹* and *E(var)3-5⁰³* mutant alleles with *Df(3R)Exel7310*, the *FLP-FRT* deletion alleles and the *PBac{RB}CG17360^{e02295}* allele in bottles. This experiment repeated crosses shown in Tables 2.5 and 2.7, but was designed to get an estimate of *Sb⁺* progeny under low density culture conditions. The results for the complementation tests are shown in Tables 2.13 and 2.14. The results showed an increase the recovery of *Sb⁺* progeny for the *Df(3R)D1M1* and *Df(3R)D2M2* crosses as compared to the results obtained when crosses were performed in vials, indicating that culture density affected the results. However, the fraction of *Sb⁺* progeny for *Df(3R)D1M1*, *Df(3R)D2M2* and *PBac{RB}CG17360^{e02295}* was still slightly below that obtained for *Df(3R)D4M1* and *Df(3R)Exel9019*, suggesting that mutation of *CG17360* does decrease viability even under low density culture conditions. Interestingly, the *E(var)3-5⁻* alleles still failed to complement *Df(3R)Exel7310*. This result is curious because the other deficiencies together remove the entire region deleted by *Df(3R)Exel7310*.

Table 2.13 Complementation test of *E(var)3-5⁰¹* mutant allele

Males	<i>E(var)3-5⁰¹</i> females		
	<i>Sb</i> progeny	<i>Sb⁺</i> progeny	Total
<i>Df(3R)D1M1</i>	226 (71.7%)	89 (28.2%) *	315
<i>Df(3R)D2M2</i>	282 (67.5%)	135 (32.3%) *	417
<i>Df(3R)D4M1</i>	243 (59.4%)	166 (40.5%)	409
<i>Df(3R)Exel9019</i>	504 (60.9%)	323 (39%)	827
<i>Df(3R)Exel7310</i>	298 (98%)	6 (2%) *	304
<i>PBac{RB}CG17360^{e02295}</i>	234 (71.4%)	93 (28.4%)*	327

The chromosomes of all the mutant alleles were balanced over the *TM3* balancer. The data was derived from single experiment. *Df(3R)Exel9019* deletes *Lk6*.

* The *Sb⁺* flies had unexpanded wings.

Table 2.14 Complementation test of *E(var)3-5⁰³* mutant allele

Males	<i>E(var)3-5⁰³</i> females		
	Sb progeny	Sb ⁺ progeny	Total
<i>Df(3R)D1M1</i>	613 (77%)	182 (22.8%)*	795
<i>Df(3R)D2M2</i>	749 (81.3%)	171 (18.5%)*	920
<i>Df(3R)D4M1</i>	199 (65.8%)	103 (34.1%)	302
<i>Df(3R)Exel9019</i>	653 (65.1%)	349 (34.8%)	1002
<i>Df(3R)Exel7310</i>	498 (99%)	5 (1%)*	503
<i>PBac{RB}CG17360^{e02295}</i>	585 (75.2%)	192 (24.7%)*	777

The chromosomes of all the mutant alleles were balanced over the *TM3* balancer. The data was derived from a single experiment.

* The Sb⁺ flies had unexpended wings

Complementation test of the *E(var)3-5⁰⁷* mutant allele: The *E(var)3-5⁰⁷* mutant allele was isolated in a $\pi 2$ mutagenesis screen. I carried out a complementation test of the *E(var)3-5⁰⁷* mutant allele with *Df(3R)Exel7310* and *Df(3R)D1M1* mutants. A 33% ratio of Sb⁺ progeny was expected if the mutants complemented. The results are indicated in Table 2.15. The complementation test results indicated that the lethality of the *E(var)3-5⁰⁷* mutant did not complement the lethality of the *Df(3R)Exel7310* mutant allele. However the *E(var)3-5⁰⁷* mutant partially complemented *Df(3R)D1M1*, similar to the results observed for *E(var)3-5⁰¹* allele.

Table 2.15 Complementation test of *E(var)3-5⁰⁷* mutant allele

Males	<i>E(var)3-5⁰⁷</i> females		Total progeny scored
	Sb	Sb ⁺	
<i>Df(3R)Exel7310</i>	43 (%)	0 (%)	43
<i>Df(3R)D1M1</i>	62 (%)	22 (%)*	84

The mutant chromosomes were balanced over the *TM3, Sb balancer*. The data was derived from single trial. The complementation tests were set up in vials.

* Sb⁺ flies had unexpanded wing phenotype.

Complementation test of the *E(var)3-4⁰⁹* mutant allele: Six of the *E(var)3-5* alleles were isolated in the *Icarus-neo* mutagenesis. The *E(var)3-4⁰⁹* allele was also identified in the *Icarus-neo* screen. I carried out complementation tests between *E(var)3-5⁰¹* females and *E(var)3-4⁰⁹* males to determine if complementation occurs between these mutant alleles. A 25% ratio was expected if the mutants complemented each other. The results are indicated in Table 2.16. Since a 25% ratio of Sb⁺ Cy⁺ was not obtained, the complementation test results indicated that the lethality of the *E(var)3-5⁰¹* mutant did not complement the lethality of the *E(var)3-4⁰⁹* mutant allele. In addition, the unexpanded wing phenotype of the few *E(var)3-5⁰¹/E(var)3-4⁰⁹* progeny suggested that the chromosomes share mutations in *CG17360*.

Table 2.16 Complementation test of *E(var)3-4⁰⁹* mutant allele

Males	<i>E(var)3-5⁰¹</i> females				
	Sb ⁺ Cy ⁺	Sb ⁺ Cy	Sb Cy ⁺	Sb Cy	Total
<i>It^{G10}; E(var)3-4⁰⁹/TSTL Cy Tb Hu e</i>	4(1.1%)*	118 (34.7%)	167 (49.1%)	51 (15%)	340

The mutant *E(var)3-5⁰¹* chromosome was balanced over the *TM3, P{w⁺GAL4-twi.G}, P{w⁺UAS-2xEGFP}, Sb Ser balancer*. The Sb⁺ Cy⁺ progeny represent the genotype *E(var)3-5⁰¹/E(var)3-4⁰⁹*. The complementation tests were done in vials.

*Flies had unexpanded wings.

PCR analysis of *CG17360* in *E(var)3-5* mutant alleles: Complementation test analysis between *E(var)3-5* mutants and *CG17360* mutants yielded *E(var)3-5*/*CG17360* progeny that exhibited an unexpanded wing phenotype. These results indicated that *E(var)3-5* mutants might harbor mutations in the *CG17360* gene. PCR analysis of the *CG17360* gene was performed for *E(var)3-5* alleles. DNA obtained from homozygous *E(var)3-5* mutants or *Df(3R)D2M2/E(var)3-5* flies was used to amplify the coding region of *CG17360* using the primer sets described in Materials and Methods. DNA obtained from *CantonS* flies was used as a positive control. The PCR results are listed in Table 2.17 and illustrated in Figure 2.3. The results indicated that initially a 1064 bp region and a 844 bp region could not be amplified by primers sets 3219F-4265R and 3734F-4578R respectively. This suggested that mutations exist in the genomic region defined by 3219F and 4578R. Further narrowing down the region of mutation, indicated that a 433 bp and a 324 bp region could not be amplified by primer sets 4145F-4578R and 3941F-4265R respectively. As the primer combination 4145F-4265R was not a good combination, analyses could not be performed using this primer set. However, the other results suggest that the block to PCR amplification maps to the 4145-4265 region. Overall the result indicated that *CG17360* harbors mutation in the region defined by primers 3941F and 4265R.

Table 2.17 PCR analysis of *CG17360* in *E(var)3-5* alleles

Primer set used	<i>E(var)3-5</i> allele(s) used	Expected Size	Observation
622F-1608R	<i>E(var)3-5</i> ⁰¹	986 bp	986 bp
1480F-2457R	<i>E(var)3-5</i> ⁰¹	977 bp	faint band of 977 bp
2327F-3318R	<i>E(var)3-5</i> ⁰¹	991bp	991bp band
3154F-3978R	Alleles 01-06	825bp	825bp band
3219F-4265R	Alleles 01-06	1046bp	DNA band not observed
3734F-4578R	Alleles 01-06	844bp	DNA band not observed
3941F-4265R	Alleles 01-06	324bp	DNA band not observed
2327F-4265R	<i>E(var)3-5</i> ⁰¹	1938bp	DNA band not observed
4145F-4578R	Alleles 01-06	433bp	DNA band not observed
4475F-5349R	<i>E(var)3-5</i> ⁰¹	875bp	875bp DNA band observed

The expected DNA bands were observed using *Canton S* DNA.

Figure 2.3 PCR analysis of *CG17360* in *E(var)3-5* allele

The *CG17360* transcription unit is shown as black arrow in 5' to 3' direction. The forward and reverse arrowheads represent the forward and reverse primers respectively. Black lines indicate that a PCR product was formed. The broken lines indicated that PCR product was not formed. The primer combinations indicated in the figure are as follows: 1 = 622F-1608R; 2 = 1480F- 2457R; 3 = 2327F-3318R; 4 = 3154F-3978R; 5 = 4475F-5349R; 6= 2327F-4265R; a = 3219F-4265R; b = 3734F-4578R; c = 4145F-4578R; d = 3941F-4265R.

PCR analysis of *CG17360* in the *E(var)3-5⁰⁷* mutant strain: The *E(var)3-5⁰⁷* allele was isolated in a $\pi 2$ *P*-element mutagenesis screen, a different screen than that used for the isolation of the six *E(var)3-5* alleles. PCR analysis of the *CG17360* coding gene sequence region was done for the *E(var)3-5⁰⁷* strain to determine the presence of mutations. Genomic DNA was isolated from *E(var)3-5⁰⁷/Df(3R)D1M1* flies and was subjected to PCR analysis. The results are indicated in Table 2.18. Similar results like that observed for six *E(var)3-5* alleles were observed for *E(var)3-5⁰⁷* indicating that the genomic region between 3941F-4265R harbors mutations.

Table 2.18 PCR analysis of *CG17360* in *E(var)3-5⁰⁷* mutant allele

Primers sets used	Expected DNA band	Observed results
622F-1608 R	986 bp	986 bp DNA band observed
1480F-2457R	977 bp	faint band of 977 bp observed
2327F-3318R	991bp	991bp DNA band observed
3154F-3978R	825bp	825bp DNA band observed
3219F-4265R	1046bp	Expected DNA band not observed
3734F-4578R^a	844bp	Expected DNA band not observed
3734F-4265R	531bp	Expected DNA band not observed
3941F-4265R	324bp	Expected DNA band not observed
4475F-5349R	875bp	875bp DNA band observed
622F-5349R^a	4727bp	Expected DNA band not observed
3734F-5349R^b	1615bp	Expected DNA band not observed

The expected DNA bands were observed using *Canton S* DNA that was used as positive control.

^a PCR was done using DNAzyme polymerase.

^b PCR was done using Phusion Taq Polymerase

PCR analysis of CG17360 in the *E(var)3-4⁰⁹* mutant: PCR analysis was done for the *E(var)3-4⁰⁹* mutant allele to determine if its failure to complement *E(var)3-5⁰¹* was due to the same mutation in CG17360. DNA was isolated from *E(var)3-4⁰⁹/Df(3R)D1M1* flies and was analyzed by PCR using the primer sets 3734F-4578R. DNA isolated from *Canton S* flies was used as a positive control. The results revealed that the primers could not amplify the expected 844bp DNA band in *E(var)3-4⁰⁹/Df(3R)D1M1* DNA as opposed to *Canton S* DNA. The same primer set could not amplify the 844bp region in the *E(var)3-5* alleles. This indicated that the *E(var)3-4⁻* mutant also harbored a mutation in the CG17360 gene in the same region.

Southern analysis of *E(var)3-5* alleles: A Southern blot experiment was performed on six *E(var)3-5* alleles to analyze the nature of the mutations detected by PCR, and is shown in Figure 2.4B. *Pst*I digested *E(var)3-5* and *Canton S* DNA was probed with a 1064bp DNA fragment. The probe recognized 1.0 kb and 1.9 kb DNA fragments in control *Canton S* DNA (Figure 2.4A). It was hypothesized that the mutation preventing PCR amplification of region 3941F-4265R might be an insertion. The mutation would disrupt a *Pst*I fragment in the *E(var)3-5* alleles as a result the expected 1.0 kb and 1.9 kb DNA bands would not be observed. The Southern analysis results are shown in Figure 2.4B. As expected, two DNA bands of 1.0 kb and 1.9 kb are recognized by the probe in the control *Canton S* DNA. However in all the six *E(var)3-5* alleles, DNA bands of 1.0 kb and 1.9 kb were also obtained and no unique bands were observed. This suggested that all the six *E(var)3-5* alleles carry similar mutations. Also a 4 kb DNA band is observed in all the alleles including *Canton S* DNA, which was unexpected.

Figure 2.4 Southern analysis of *E(var)3-5* alleles

The *PstI* restriction enzyme map of *CG17360* is illustrated in A. The central *PstI* site is at the genomic coordinate 3R: 7,610,846. The genomic region defined by 3219F and 4265R could not be amplified by PCR for the *E(var)3-5* mutants. The probe was generated using primers 3219F and 4265R. The results of Southern analysis using the probe indicated in A are shown in B. Lane 1: *CantonS* DNA, used as positive control. Lanes 2-7: *E(var)3-5* alleles 01-06. The wild type and *E(var)3-5* alleles were balanced using the *TM3* balancer.

Mapping the enhancer effect of the *E(var)3-5* mutation: The effect of deletions *Df(3R)D1M1*, *Df(3R)D2M2*, *Df(3R)D4M1*, *Df(3R)D5M1* and *Df(3R)Exel7310* was tested on variegation of the w^{m4} variegating allele to determine whether the enhancer phenotype of *E(var)3-5* mutants maps in the 86F6-87A1 region. *ln(w^{m4})* females were crossed with males bearing an *E(var)3-5* mutation or deficiency mutation. *Df(3R)D7M1* mutants and *PBac{RB}CG17360^{e02295}* mutants were not used in the assay since these mutants have a red eye phenotype and the w^{m4} variegation assay can only be done on mutants having a white eye phenotype. The results for the w^{m4} variegation assay are shown in Table 2.19. Visual observation of *ln(w^{m4})/w* ;

Df(3R)D1M1/+ revealed that these flies exhibited enhanced w^{m4} variegation. However the pigment assay revealed that *Df(3R)D1M1* did not enhance w^{m4} variegation. Also in the first trial $ln(w^{m4})/w$; *Df(3R)D2M2/+* showed lower absorbance values than rest of deficiencies. However the results were not repeatable in the second trial and any conclusive results could not be established from the w^{m4} variegation assay.

Table 2.19 Effect of deficiency mutants on $ln(1)w^{m4}$ variegation

Genotype of females	Average absorbance at 480nm ^b	
	Trial 1	Trial 2
<i>ln(1)w^{m4}/w; E(var)3-5⁰¹/+^a</i>	0.010±0.004	0.008±0.002 ^c
<i>ln(1)w^{m4}/w; Df(3R)Exel7310/+</i>	0.060±0.017	0.060±0.017
<i>ln(1)w^{m4}/w; Df(3R)D1M1/+</i>	0.050±0.014	0.040±0.014
<i>ln(1)w^{m4}/w; Df(3R)D2M2/+</i>	0.030±0.007	0.060±0.031
<i>ln(1)w^{m4}/w; Df(3R)D4M1/+</i>	0.070±0.028	0.060±0.016
<i>ln(1)w^{m4}/w; Df(3R)D5M3/+</i>	0.040±0.006	0.050±0.014

^aFlies bearing the *E(var)3-5⁰¹* mutation were used as control.

^bRed eye pigment was extracted from one sample containing 10 heads and the average absorbance from 10 samples per genotype is shown.

^cAverage absorbance from six samples, each sample contained 10 heads. ± Standard-deviation

The effect of deletions *Df(3R)D1M1*, *Df(3R)D2M2*, *Df(3R)D4M1* and *Df(3R)Exel7310* was tested on variegation of the *Sb* gene to determine whether the enhancer phenotype of *E(var)3-5* mutants maps in the 86F6-87A1 region. The *T(2:3)Sb^v* stock that translocates the *Stubble (Sb)* gene containing a *Sb¹* mutation near second chromosome heterochromatin was used. As a result of the translocation, heterochromatin-mediated inactivation of the *Sb¹* allele results in a wild-type bristle, whereas normal expression results in a short bristle. The *T(2:3)Sb^v* flies thus have a combination of wild-type and *Sb* bristles. A mutation in an enhancer gene results in a decrease in the number of *Sb* bristles whereas a

mutation in a suppressor gene increases the number of *Sb* bristles (Sinclair *et al.* 1983). In this experiment $T(2:3)Sb^V$ females were crossed to males bearing an $E(var)3-5$ mutation or a deletion mutation. Fourteen macrocheates in $T(2:3)Sb^V/E(var)3-5$ and $T(2:3)Sb^V/deletion$ flies were scored for being *Sb* or wild-type. The average number of *Sb* bristles for each genotype is listed in Table 2.20. The $PBac\{RB\}CG17360^{Rev}$ allele was used as an internal control since it represented a wild-type 3rd chromosome isogenic with the deficiency chromosomes. No parental stock is available for the $E(var)3-5$ mutants. The results indicated that in females, $E(var)3-5^{01}$ and $E(var)3-5^{03}$ significantly enhanced *Sb* variegation. $Df(3R)D4M1$ significantly enhanced *Sb* variegation suggesting that an enhancer phenotype maps in the region deleted by $Df(3R)D4M1$. In males, as expected $E(var)3-5^{01}$ and $E(var)3-5^{03}$ significantly enhanced *Sb* variegation. However, enhancement in *Sb* variegation in $Df(3R)D4M1$ was not significantly different in males. I also determined the effect of mutations in the *CG17360* gene on *Sb* variegation. The average number of *Sb* bristles for each mutant is shown in Table 2.21. $PBac\{RB\}CG17360^{Rev}$ allele was used as a control. *CG17360* mutants did not enhance *Sb* variegation.

Table 2.20 Effect of deficiency mutants on *Sb* variegation

Genotype	Females counted	Average number of <i>Sb</i> bristles in females	Males counted	Average number of <i>Sb</i> bristles in males
<i>PBac{RB}CG17360^{Rev}/T(2:3)Sb^V</i>	42	10.4	44	9.9
<i>E(var)3-5⁰¹/T(2:3)Sb^V</i>	41	3**	39	4.2**
<i>E(var)3-5⁰³/T(2:3)Sb^V</i>	33	2.8**	20	3.5**
<i>Df(3R)Exel7310/ T(2:3)Sb^V</i>	36	10.2	41	10.6
<i>Df(3R)D1M1/ T(2:3)Sb^V</i>	66	10.3	67	9.7
<i>Df(3R)D2M2/ T(2:3)Sb^V</i>	35	10.2	48	9.7
<i>Df(3R)D4M1/T(2:3)Sb^V</i>	39	8**	35	9.4

Statistical analysis was performed separately for males and females, using one way ANOVA. Those genotypes statistically different ($P < 0.001$) from the control *PBac{RB}CG17360^{Rev}/ T(2:3)Sb^V* strain are indicated by **. The results for deficiency *Df(3R)D4M1* differed in males and females. Data were derived from a single trial.

Table 2.21 Effect of *CG17360* mutant alleles on *Sb* variegation

Genotype	Trial 1		Trial 2	
	Females counted	Average number of <i>Sb</i> bristles	Females counted	Average number of <i>Sb</i> bristles
<i>PBac{RB}CG17360^{Rev}/T(2:3)Sb^V</i>	20	9.5	31	9.2
<i>E(var)3-5⁰¹/T(2:3)Sb^V</i>	29	3.3**	24	4.3**
<i>E(var)3-5⁰³/T(2:3)Sb^V</i>	14	4**	30	3.8**
<i>PBac{RB}CG17360^{e02295}/T(2:3)Sb^V</i>	17	10.5	41	10.4
<i>Df(3R)D1M1/T(2:3)Sb^V</i>	42	10.1	30	10.1

Statistical analysis was performed, using one way ANOVA. Those genotypes statistically different ($P < 0.001$) from the control *PBac{RB}CG17360^{Rev}/ T(2:3)Sb^V* strain are indicated by **.

The effect of the *Df(3R)Exel7310* deletion and *CG17360* mutants was tested on variegation of the *LacZ* gene to detect the presence of enhancer in the region defined by *Df(3R)Exel7310*. *In(3L)BL1* females containing a single insertion of *P[w⁺ hs-lacZ]* transgene positioned near heterochromatin due to inversion were crossed to males containing a mutation in *E(var)3-5* or *CG17360*, or *Df(3R)Exel7310* or *PBac{RB}CG17360^{Rev}* control chromosome. Salivary glands of mutant larvae of appropriate genotype were dissected and stained to analyze the expression of the *LacZ* transgene. Results are shown in Table 2.22 and 2.23. The results indicated that like *E(var)3-5⁰¹*, *Df(3R)Exel7310* mutants enhanced *LacZ* variegation indicating the presence of an enhancer in the region deleted by this deficiency. The *PBac{RB}CG17360^{e02295}* and *PBac{RB}CG17360^{M56}* mutants however did not enhance *LacZ* variegation. In one experiment the sex of *Df(3R)Exel7310* and *E(var)3-5* larvae was noted, but a lot of inconsistency in staining of nuclei was observed (data not shown).

Table 2.22 Effect of *Df(3R)Exel7310* mutants on *LacZ* variegation

Genotype	Trial	Number of lobes counted	Average nuclei stained ($\pm$ standard deviation)
<i>In(3L)BL1/Df(3R)Exel7310</i>	1	22	10.1 $\pm$ 6.8
	2	13	9.3 $\pm$ 5.6
<i>In(3L)BL1/E(var)3-5⁰¹</i>	1	16	9.5 $\pm$ 3.5
	2	15	8.1 $\pm$ 5

Statistical analysis was done using one-way ANOVA. *Df(3R)Exel7310* and *E(var)3-5⁰¹* were not significantly different from each other ($P>0.05$). Data derived from two trials. Sex of larvae was not determined.

Table 2.23 Effect of *CG17360* mutants on *LacZ* variegation

Genotype	Trial	Number of lobes counted	Average nuclei stained ($\pm$ standard deviation)
<i>In(3L)BL1/E(var)3-5⁰¹</i>	1	20	11.2 $\pm$ 5.5
	2	14	10.4 $\pm$ 7.7
<i>In(3L)BL1/E(var)3-5⁰³</i>	1	31	8.0 $\pm$ 5.3
	2	13	7.7 $\pm$ 6.3
<i>In(3L)BL1/PBac{RB}CG17360^{Rev}</i>	1	25	14.6 $\pm$ 6.2
	2	17	19.4 $\pm$ 7.8
<i>In(3L)BL1/PBac{RB}CG17360^{e02295}</i>	1	41	19.2 $\pm$ 8.0
	2	20	24.2 $\pm$ 7.1
<i>In(3L)BL1/Df(3R)CG17360^{M56}</i>	1	36	20.3 $\pm$ 7.3
	2	15	19.6 $\pm$ 6.4

The *PBac{RB}CG17360^{Rev}* chromosome was used a control. Statistical analysis was done using one-way ANOVA. The *E(var)3-5⁰¹* and *E(var)3-5⁰³* enhanced *LacZ* variegation ($P < 0.001$) compared to *PBac{RB}CG17360^{e02295}* and *Df(3R)CG17360^{M56}* mutants. Data derived from two trials. Sex of the larvae was not determined.

Sequencing a *CG17360* clone: A DNA fragment containing the *CG17360* gene was obtained from *Canton S* genomic DNA that was PCR amplified using primers *CG17360* 622F and *CG17360* 5349R, and cloned into vector pCR-Blunt (Invitrogen). Sequencing of *CG17360* clones revealed a single nucleotide polymorphism in the region defined by *CG17360* 3219F and *CG17360* 4265R. The change however did not alter the coding sequence of *CG17360*.

Over-expression analysis of *CG17360*: The *CG17360* gene was cloned into the pUAST transformation vector in order to create a rescue construct for analysis of *CG17360*. Prior to determining, whether transgene could complement *CG17360* mutations or influence PEV, control experiments were

performed to examine the effects of ectopic *CG17360* expression. I used different GAL4 drivers to determine whether over-expression of *CG17360* affects viability or results in any abnormal phenotype in flies. For this experiment *yw*; *P{w⁺; Act5C-Gal4}25FOI/CyO* and *w*; *P{w⁺ Hsp70-Gal4}/CyO* virgin females were crossed to *P{w⁺ UAS-CG17360}/CyO* males bearing the *CG17360* transgene (lines # 123, 41B, 66B). A ratio of 33% *Cy⁺* progeny representing the genotype *P{w⁺; Act5C-Gal4}25FOI/P{w⁺ UAS-CG17360}* or *P{w⁺ Hsp70-Gal4}/P{w⁺ UAS-CG17360}* was expected if over-expression did not affect viability. The results are indicated in Table 2.24. The results indicated that over-expression of *CG17360* did not affect viability since the expected ratio of the *Cy⁺* flies was observed and any abnormal phenotype in these flies was not detected.

Table 2.24 Analysis of over-expression of *CG17360*

Males	<i>yw</i> ; <i>P{w⁺; Act5C-Gal4}25FOI/ CyO</i> females		<i>w</i> ; <i>P{w⁺ Hsp70-Gal4}/CyO</i> females	
	<i>Cy⁺</i>	<i>Cy</i>	<i>Cy⁺</i>	<i>Cy</i>
<i>P{w⁺ UAS-CG17360}/ CyO</i> #123	43 (34.9%)	80 (65%)	45 (30.2%)	94 (67.6%)
<i>P{w⁺ UAS-CG17360}/ CyO</i> #41B	57 (40.7%)	83 (59.2%)	39 (35.4%)	71(64.5%)
<i>P{w⁺ UAS-CG17360 }#66B</i> (homozygous)	44 (43.1%)	58 (56.8%)	78 (54.5%)	65 (45.4%)

Rescue of the unexpanded wing phenotype by the *UAS-CG17360*

transgene: Previous experiments have shown that *CG17360⁻* mutants and *E(var)3-5/CG17360⁻* mutants have unexpanded wings. This experiment was performed to determine whether *CG17360* transgene rescued the wing phenotype. A positive result would mean that the transgene was expressed and functional. In this experiment females bearing the *Act5C-Gal4* transgene on the second chromosome and bearing a *CG17360* mutation on the third chromosome were mated with males containing the *UAS-CG17360* transgene on the second

chromosome and bearing an *E(var)3-5* mutation on the third chromosome. The results are shown in Table 2.25. It was expected that the *Cy Sb*⁺ flies would have closed wings since they represented the genotype *P{w⁺UAS-CG17360⁺}/CyO; E(var)3-5⁰¹/PBac{RB}CG17360^{e02295w-}* or *P{w⁺;Act5C-Gal4}25FOI/CyO; E(var)3-5⁰¹/PBac{RB}CG17360^{e02295w-}*. However these flies had open wings. This indicated an error in making a stock required for this experiment and was not pursued further.

Table 2.25 Rescue of wing phenotype by the *UAS-CG17360* transgene

Males	<i>w;P{w⁺; Act5C-Gal4}25FOI/CyO; PBac{RB}CG17360^{e02295w-}/TM6C, Sb Tb</i> females			
	<i>Cy</i> ⁺ <i>Sb</i> ⁺	<i>Cy</i> <i>Sb</i>	<i>Cy</i> ⁺ <i>Sb</i>	<i>Cy</i> <i>Sb</i> ⁺
<i>P{w⁺ UAS-CG17360}/CyO; E(var)3-5⁰¹/TM6C, Sb Tb #41B</i>	37	90	62	45
<i>P{w⁺ UAS-CG17360}/CyO; E(var)3-5⁰¹/TM6C, Sb Tb #66B</i>	4	91	-	40
<i>P{w⁺ UAS-CG17360}/CyO; E(var)3-5⁰¹/TM6C, Sb Tb #28A</i>	19	76	29	41

Discussion

A dominant enhancer mutation identified as *E(var)3-5* was discovered along with other *E(var)* mutations by Dorn *et al.* (1993) in a mutagenesis screen for isolating potential enhancer genes. I pursued experiments to identify the *E(var)3-5* gene. Initial complementation test performed using Exelixis deficiencies indicated that *E(var)3-5* maps in the region defined by *Df(3R)Exel7310*. Further complementation analysis using FLP-FRT mediated deletions of the region defined by *Df(3R)Exel7310* indicated that *E(var)3-5* is not allelic to the genes of the region defined by *Df(3R)Exel7310*. PCR analysis of *E(var)3-5* alleles indicated the presence of mutations in *CG17360* gene. PEV assays were done to detect the enhancer effect in 86F6-87A1 region and in

CG17360 mutants. The results obtained using different assay are discussed below.

Complementation tests of *E(var)3-5* mutant alleles with *Df(3R)D1M1* and *Df(3R)D2M2* and CG17360 mutants performed in vials *versus* bottles indicated differences. Initial results predicted that *E(var)3-5* might be allelic to CG17360 since a low ratio of the *E(var)3-5/PBac{RB}CG17360^{e02295}* was obtained. However this ratio was not consistent when the complementation tests were repeated in vials and complementation tests done in bottles gave close to the expected ratio. These results indicated that the *E(var)3-5/PBac{RB}CG17360^{e02295}* progeny were sensitive to larval density conditions in vials and bottles. In a study conducted by Jiro (1962) on larval population density *versus* eclosion rate, it was observed that vials containing a high larval density showed a low eclosion percentage of flies. In my complementation test studies the inconsistency in the ratio of *E(var)3-5/PBac{RB}CG17360^{e02295}* progeny can be attributed to larval population density (Jiro 1962).

Complementation tests of *E(var)3-5* with five FLP-FRT mediated deletions indicated that *E(var)3-5* is not allelic to any of the genes in the region defined by *Df(3R)Exel7310*. These results indicated that observed lethality of *E(var)3-5* mutants with *Df(3R)Exel7310* mutants is due to a cumulative effect of mutations in genes in the region defined by *Df(3R)Exel7310*.

The *E(var)3-5/Df(3R)D1M1*, *E(var)3-5/Df(3R)D2M2* and *E(var)3-5/PBac{RB}CG17360^{e02295}* flies exhibited an unexpanded wing phenotype. Complementation tests between *E(var)3-5* alleles and the *PBac{RB}CG17360^{Rev}* allele revealed that *E(var)3-5/PBac{RB}CG17360^{Rev}* progeny had a wild-type wing phenotype. These results indicated that *E(var)3-5* alleles harbor mutations in CG17360 and as a result the *E(var)3-5/PBac{RB}CG17360^{e02295}* flies exhibit an unexpanded wing phenotype. PCR analysis of the CG17360 gene in *E(var)3-5* mutant alleles was done to confirm the presence of any associated mutations. PCR analysis results revealed that a particular primer set, 3941F-4265R, was unable to amplify a 324 base pair region in CG17360 in *E(var)3-5* alleles. This indicated that CG17360 harbors mutations in between the primer binding sites for

primers 3941F and 4265R. The *E(var)3-5⁰⁷* also harbored a mutation in this region.

The *E(var)3-4* allele also harbored a mutation in *CG17360*. One possible explanation is that the *E(var)* alleles might have been confused during stock maintenance. Another possibility is that the parental strains used for obtaining these mutants originally harbored mutations in *CG17360* since *E(var)3-5* mutants isolated from different mutagenesis screens harbored the same mutation. Complementation test between the *E(var)3-5⁰¹* allele and the *E(var)3-4⁰⁹* allele revealed non-complementation between them. The *E(var)3-5⁰¹/E(var)3-4⁰⁹* flies represented 1% of the progeny as opposed to 25%. The *CG17360* mutation in both the *E(var)* chromosomes might lead to underrepresentation of the progeny, resulting in a low ratio. However, the presence of other mutations on the *E(var)* chromosomes is also likely given the low recovery.

The nature of the mutation in the *E(var)3-5* mutant alleles could not be established using PCR alone. I performed a Southern blot analysis on six *E(var)3-5* alleles to assess the nature of mutation associated with *CG17360*. The six alleles obtained from the *Icarus-neo* mutagenesis were used. A probe that detected 1.0 kb and 1.9 kb DNA fragments in the control *Canton S* DNA was used. It was hypothesized that if the mutation were an insertion, a *Pst*I restriction fragment would be disrupted in the *E(var)3-5* alleles, and as a result the probe would not detect the desired 1.0 and 1.9 kb fragments. The results revealed that the 1.0 and 1.9 kb fragments could be detected in all the alleles including the control. It can be suggested that the mutation is an inversion of the sequences between the *Pst*I sites, thereby detecting the expected DNA bands in mutant alleles. An unexpected third DNA band of 4 kb was also detected in all the alleles including the control *Canton S* DNA. The band might be due to partial digestion of genomic DNA obtained from *Canton S* and *E(var)3-5* alleles, or a polymorphism present on the *TM6B* chromosome.

I conducted experiments to determine if the enhancer phenotype of *E(var)3-5* mutants maps in the region defined by *Df(3R)Exel7310*. A *w^{m4}* variegation assay was done to detect the presence of the enhancer phenotype.

Inconclusive results were obtained in two separate trials. The w^{m4} assay should be repeated again and should be performed more meticulously. The effect of deficiencies was tested on variegation of *Stubble* (*Sb*) gene. *Df(3R)D4M1* significantly enhanced *Sb* variegation in females, indicating the presence of an enhancer in the region deleted by this deficiency. However *Df(3R)Exel7310* that encompasses genes deleted by *Df(3R)D4M1* did not enhance *Sb* variegation. These results indicated the presence of other suppressor genes in this region that would negate the effect of an enhancer gene. As a result, *Df(3R)Exel7310* flies would fail to enhance *Sb* variegation. *Df(3R)D4M1* males however did not enhance *Sb* variegation. The disparity observed in the results can be attributed to the heterochromatic Y chromosome of males. However the experiment needs to be repeated again to verify these results.

Since the *E(var)3-5* alleles harbor mutations in *CG17360*, I assessed the effect of mutation in *CG17360* on *Sb* variegation. Enhancement of *Sb* variegation was not observed for the *PBac{RB}CG17360^{e02295}* mutant allele. However the results obtained point towards suppression of *Sb* variegation by the *PBac{RB}CG17360^{e02295}* mutant allele.

The effect of the *Df(3R)Exel7310* deletion and mutations in *CG17360* were tested on variegation of the *LacZ* gene. The results obtained indicated that *CG17360* mutants do not enhance *LacZ* variegation, similar to results obtained for *Sb* variegation. However the results also indicated that *Df(3R)Exel7310* mutants enhance *LacZ* variegation. This is unexpected because *Df(3R)Exel7310* failed to enhance *Sb* variegation. The *LacZ* variegation using *Df(3R)Exel7310* mutants should be repeated to verify the results.

Chapter 3: Lethal phase analysis of *E(var)3-5* mutants

Introduction

The aim of this study was to characterize the lethal phenotype and study the effects of the *E(var)3-5* mutation as a means to reveal the function of *E(var)3-5*. The proposed role of enhancer proteins is to maintain a euchromatic chromatin state (Sinclair *et al.* 1989). Disruption of a euchromatin chromatin state due to mutations in enhancer genes has revealed defects during embryogenesis. For example, lack of a functional *Bramha* (*brm*) gene product results in late embryonic lethality in homozygous *brm* embryos. Defects in maternally contributed *brm* gene product resulted in early embryonic lethality detectable at the gastrulation phase (Brizuela *et al.* 1994). Embryonic defects due to mutations in *Trithorax-like* (*Trl*) gene have also been recorded (Bhat *et al.* 1996). The *Trl* locus encodes the GAGA transcription factor (Farkas *et al.* 1994). The above data implicates the importance of *E(var)* proteins during development.

The dominant enhancer mutation *E(var)3-5* was identified by Dorn *et al.* (1993b). Six *E(var)3-5* alleles were isolated in an *Icarus-neo P* element mutagenesis screen whereas four *E(var)3-5* alleles were isolated in a $\pi 2 P$ element mutagenesis screen. All of the *E(var)3-5* alleles have been reported to be recessive lethal (Dorn *et al.* 1993b).

Previous complementation tests analysis between the seven *E(var)3-5* alleles did not yield any heteroallelic *E(var)3-5* adults (Weiler *et al.* unpublished data). Also, lethal analysis of heterozygous *E(var)3-5⁰¹/E(var)3-5⁰⁴* embryos revealed variability in timing of lethality and segmentation was observed in most of the embryos. However, those studies did not utilize the embryonic marker GFP that would unequivocally permit the clear distinction of homozygous *E(var)3-5* embryos from homozygous balancer embryos.

My study utilized the balancer chromosome that expresses the embryonic marker EGFP (enhanced green fluorescent protein). EGFP was shown to be expressed at stage eight during the embryo development (Halfon *et al.* 2002). Stage eight is the germ band extension stage that occurs approximately three

hours 45 min after egg laying. Thus the use of EGFP permits early detection and distinction of the different genotypes of embryos. I planned to analyze the lethality in homozygous *E(var)3-5* embryos and hemizygous *E(var)3-5* embryos. The homozygous *E(var)3-5* embryos and hemizygous *E(var)3-5* embryos could be recognized as yellow embryos due to yellow embryo fluorescence due to the lack of EGFP marker. On the other hand embryos that contained a single copy of GFP would have been distinguished due green embryo fluorescence. I planned to analyze developmental defects, if any, in yellow embryos using phase contrast microscopy (Wieschaus and Nusslein-Wolhard 1998). If the lethality did not occur at an embryonic stage but ensued at later stages such as 3rd instar stage it would be advantageous to analyze salivary gland chromosomes. Since the proposed function of the *E(var)* class of genes is to promote euchromatin formation, observation of salivary gland chromosomes might be helpful to identify any defects in chromosome structure such as banding pattern abnormalities.

Materials and methods

Drosophila stocks and culture conditions: All the strains and crosses were maintained in cornmeal-malt medium described as a standard media recipe by Bloomington Drosophila Stock Center (<http://flystocks.bio.indiana.edu>). The crosses were established at 25°C unless otherwise noted. The *E(var)3-5* mutant alleles were isolated by Dorn *et al.* (1993) and were gifted to our lab by R. Dorn and G. Reuter. The synthesis of *Df(3R)D1M1* is described in chapter two and all other stocks were obtained from the Bloomington Stock center and are described in <http://flystocks.bio.indiana.edu> (Table 3.1)

Table 3.1 Genotype of strains used for lethality analysis

$w^{m4}; E(var)3-5^{01}/TM3, Sb Ser$
$w^{m4}; E(var)3-5^{02}/TM3, Sb Ser$
$w^{m4}; E(var)3-5^{03}/TM3, Sb Ser$
$w^{m4}; E(var)3-5^{04}/TM3, Sb Ser$
$w^{m4}; E(var)3-5^{05}/TM3, Sb Ser$
$w^{m4}; E(var)3-5^{06}/TM3, Sb Ser$
$w^{1118}; Df(3R)Exel7310/TM6B, Tb$
$W; PBac\{RB\}CG17360^{e02295}/TM3, Sb st e$
$w^{1118}; Df(3R)D1M1/TM3, Sb st e$
$w^{1118}; Dr^{Mio}/TM3, P\{w^+ GAL4-twi.G\} P\{w^+ UAS-2xEGFP\} Sb Ser$

Analysis of lethal phase:

Each of the $E(var)3-5^-$ chromosomes (alleles one through six) was balanced with the $TM3, P\{w^+ GAL4-twi.G\} P\{w^+ UAS-2xEGFP\} Sb Ser$ chromosome that expresses the EGFP during embryonic development. Homozygous $E(var)3-5^-$ embryos were obtained from $E(var)3-5^-/TM3, P\{w^+ GAL4-twi.G\} P\{w^+ UAS-2xEGFP\} Sb Ser$ males and females. The $E(var)3-5^-/E(var)3-5^-$ homozygous embryos exhibited a yellow embryo fluorescence due to lack of EGFP: a ratio of 25% was expected. The sibling $E(var)3-5^-/TM3, P\{w^+ GAL4-twi.G\} P\{w^+ UAS-2xEGFP\} Sb Ser$ embryos were identified as green embryos and exhibited a green embryo fluorescence due to one copy of EGFP; a ratio of 50% was expected. The sibling bright-green embryos representing the genotype $TM3, P\{w^+ GAL4-twi.G\} P\{w^+ UAS-2xEGFP\} Sb Ser/TM3, P\{w^+ GAL4-twi.G\} P\{w^+ UAS-2xEGFP\} Sb Ser$ expressed two copies of EGFP and exhibited a bright green fluorescence. A ratio of 25% was expected for these embryos.

Each of the $E(var)3-5^-$ chromosomes (alleles one through six) and $Df(3R)Exel7310$ chromosome was balanced with the $TM3, P\{w^+ GAL4-twi.G\} P\{w^+ UAS-2xEGFP\} Sb Ser$ chromosome that expresses the EGFP during embryonic development. Hemizygous $E(var)3-5^-/Df(3R)Exel7310$ embryos were

obtained from *E(var)3-5/TM3, P{w⁺GAL4-twi.G} P{w⁺UAS-2xEGFP} Sb Ser* males and *Df(3R)Exel7310/TM3, P{w⁺GAL4-twi.G} P{w⁺UAS-2xEGFP} Sb Ser* virgin females. The *E(var)3-5/Df(3R)Exel7310* embryos exhibited a yellow embryo fluorescence due to lack of EGFP: a ratio of 25% was expected. The sibling *E(var)3-5/TM3, P{w⁺GAL4-twi.G} P{w⁺UAS-2xEGFP} Sb Ser* and *Df(3R)Exel7310/TM3, P{w⁺GAL4-twi.G} P{w⁺UAS-2xEGFP} Sb Ser* embryos were identified as green embryos and exhibited a green embryo fluorescence due to one copy of EGFP; a ratio of 50% was expected. The sibling bright-green embryos representing the genotype *TM3, P{w⁺GAL4-twi.G} P{w⁺UAS-2xEGFP} Sb Ser/TM3, P{w⁺GAL4-twi.G} P{w⁺UAS-2xEGFP} Sb Ser* expressed two copies of EGFP and exhibited a bright green fluorescence. A ratio of 25% was expected for these embryos.

The *PBac{RB}CG17360^{e02295}* and the *Df(3R)D1M1* chromosome was balanced with the *TM3, P{w⁺GAL4-twi.G} P{w⁺UAS-2xEGFP} Sb Ser* chromosome that expresses the EGFP during embryonic development. Hemizygous *PBac{RB}CG17360^{e02295}/Df(3R)D1M1* embryos were obtained from *Df(3R)D1M1/TM3, P{w⁺GAL4-twi.G} P{w⁺UAS-2xEGFP} Sb Ser* virgin females and *PBac{RB}CG17360^{e02295}/TM3, P{w⁺GAL4-twi.G} P{w⁺UAS-2xEGFP} Sb Ser* males. The *PBac{RB}CG17360^{e02295}/Df(3R)D1M1* embryos exhibited a yellow embryo fluorescence due to lack of EGFP: a ratio of 25% was expected. The sibling *Df(3R)D1M1/TM3, P{w⁺GAL4-twi.G}, P{w⁺UAS-2xEGFP}, Sb Ser* and *PBac{RB}CG17360^{e02295}/TM3, P{w⁺GAL4-twi.G}, P{w⁺UAS-2xEGFP}, Sb Ser* embryos were identified as green embryos and exhibited a green embryo fluorescence due to one copy of EGFP; a ratio of 50% was expected. The sibling bright-green embryos representing the genotype *TM3, P{w⁺GAL4-twi.G} P{w⁺UAS-2xEGFP} Sb Ser/TM3, P{w⁺GAL4-twi.G} P{w⁺UAS-2xEGFP} Sb Ser* expressed two copies of EGFP and exhibited a bright green fluorescence. A ratio of 25% was expected for these embryos.

For each experiment, about 100-150 females were combined with 100-150 males in bottles supplemented with yeast paste. The cultures were incubated a minimum of two days prior to embryo collection. Eggs were collected

on apple-grape juice agar plates, which were replaced every hour. Once it was established that females were laying a constant rate, the freshly laid embryos were collected and lined up neatly on apple-grape juice agar plates. The embryos were incubated at 25°C and monitored over a period of two days. The plates were examined three times during the first day and once or twice on the second day. A Leica MS5 stereomicroscope fitted with an epifluorescent illuminator and a GFP (470nm) filter was used. Larvae that hatched from yellow embryos were transferred to vials. 15-20 larvae were transferred per vial. The vials were placed at 25°C. The larvae were observed each day for viability. The dead larvae were picked from the vials and their mouthhooks were isolated to determine the stage of lethality. The number of teeth in the larval mouthhooks were observed to identify the larval instar stage (Roberts and Standen 1998)

Results

Homozygous *E(var)3-5* mutants exhibit lethality during the embryonic 1st and 2nd larval instar stages: The hatching rates of homozygous *E(var)3-5* embryos of six *E(var)3-5* alleles were analyzed. In brief, the *E(var)3-5* females were crossed to *E(var)3-5* males, both having the EGFP balancer chromosome, to obtain three different progeny classes as indicated in Figure 3.1. The homozygous yellow embryos contained two mutant copies of *E(var)3-5* and were assayed for hatching and viability. The heterozygous green embryos of the genotype *E(var)3-5/TM3, P{w⁺GAL4-twi.G} P{w⁺UAS-2xEGFP} Sb Ser* were heterozygous for a wild-type copy of *E(var)3-5* and were used as an internal control for comparing the hatching rate of embryos. Three to four trials were completed for studying the lethality associated with each *E(var)3-5* alleles, with a minimum of 150 embryos per trial. The results are indicated in Table 3.2. The hatching rate in homozygous *E(var)3-5* embryos was determined to be more than 65%. Statistical analysis using one-way ANOVA ($P>0.05$) indicated no significant differences in the hatching rates of homozygous *E(var)3-5* embryos having different mutant alleles. The hatching rate of heterozygous embryos that contained one wild-type copy of *E(var)3-5* was more than 90% (Table 3.2).

Although a 25% ratio for homozygous *E(var)3-5* embryos was expected, a slightly higher ratio was observed. Unfertilized embryos might contribute to the higher ratio of homozygous *E(var)3-5* embryos.

Figure 3.1 Genetic scheme for lethal phase analysis

Table 3.2 Embryonic lethality of homozygous *E(var)3-5*⁻ mutants

Allele	Total	Yellow embryos ^a		Green embryos ^b		Bright green embryos ^c	
		Total (n)	Hatched (n)	Total (n)	Hatched (n)	Total (n)	Hatched (n)
<i>E(var)3-5</i> ⁰¹	948	30.1% (286)	69.9 % (200)	46.2% (438)	92.6 % (406)	23.6 % (224)	0
<i>E(var)3-5</i> ⁰²	1081	28.2% (305)	81.6 % (249)	48.5% (525)	93.9 % (493)	23.2 % (251)	0
<i>E(var)3-5</i> ⁰³	936	28.9% (271)	67.8 % (184)	47.6% (446)	96.1 % (429)	24.0 % (225)	0
<i>E(var)3-5</i> ⁰⁴	1095	26.8% (294)	70.0 % (206)	47.3% (519)	93.0 % (483)	25.7 % (282)	0
<i>E(var)3-5</i> ⁰⁵	1001	27.8% (279)	68.4 % (191)	47.7% (478)	92.0 % (440)	24.3 % (244)	0
<i>E(var)3-5</i> ⁰⁶	980	29.6% (291)	73.8% (215)	46.2% (453)	94.0% (426)	24.0% (236)	0

^a The yellow embryos represent the homozygous *E(var)3-5* embryos (alleles one through six).

^b The green embryos represent the genotype *E(var)3-5/TM3, P{w⁺GAL4*twi*.G} P{w⁺UAS-2xEGFP} Sb Ser*.

^c The bright green embryos represent the genotype *TM3, P{w⁺GAL4*twi*.G} P{w⁺UAS-2xEGFP} Sb Ser/TM3, P{w⁺GAL4*twi*.G} P{w⁺UAS-2xEGFP} Sb Ser*

First instar larvae homozygous for *E(var)3-5* mutant alleles 02, 03 and 04 were analyzed for viability. The homozygous *E(var)3-5* larvae exhibited lethality during the first or second instar stages. A few third instar homozygous *E(var)3-5*⁰⁴ were also obtained. However, there was bacterial contamination in the vials containing the larvae, and lethality results for all of the larvae could not be obtained.

***E(var)3-5/Df(3R)Exel7310* embryos exhibit lethality at the pupal stage:** As described in chapter two, *Df(3R)Exel7310* failed to complement *E(var)3-5* mutant alleles, suggesting that *E(var)3-5* is present in the region deleted by this deficiency. The hatching rates of hemizygous *Df(3R)Exel7310/E(var)3-5*

embryos was determined. In brief, the *Df(3R)Exel7310/TM3, P{w⁺GAL4-twi.G} P{w⁺UAS-2xEGFP}Sb Ser* females were crossed to *E(var)3-5⁻/TM3, P{w⁺GAL4-twi.G} P{w⁺UAS-2xEGFP}Sb Ser* males to obtain three different progeny classes as indicated in Figure 3.1. In yellow hemizygous *Df(3R)Exel7310/E(var)3-5⁻* embryos the lethality would be due to a single mutant copy of *E(var)3-5⁻* or due to mutations in any of the genes in that region. The heterozygous green embryos of the genotype *E(var)3-5 or Df(3R)Exel7310/TM3, P{w⁺GAL4-twi.G} P{w⁺UAS-2xEGFP}Sb Ser* were heterozygous for a wild-type copy of *E(var)3-5* and were used as an internal control for comparing the hatching rate of embryos. Three to four trials were completed to analyze the lethality associated with each heterozygous allele, with a minimum of 150 embryos per trial.

Four of the *E(var)3-5* mutant alleles exhibited a hatching rate about 73% in the hemizygous state. *E(var)3-5⁰⁴* and *E(var)3-5⁰⁶* had a lower rate. However, statistical analysis using one-way ANOVA indicated no significant difference in the hatching rates of hemizygous embryos ($P > 0.05$). The hatching rate of heterozygous *E(var)3-5 or Df(3R)Exel7310/TM3, P{w⁺GAL4-twi.G} P{w⁺UAS-2xEGFP}Sb Ser* embryos that contained one wild-type copy of *E(var)3-5* was more than 90% (Table 3.3). The percentage of yellow embryos recovered from each cross was slightly higher than expected. Unfertilized yellow embryos might contribute to the observed higher ratio of homozygous yellow embryos.

Table 3.3 Embryonic lethality of hemizygous *E(var)3-5/Df(3R)Exel7310* embryos

Allele	Total	Yellow embryos ^a		Green embryos ^b		Bright green embryos ^c	
		Total (n)	Hatched (n)	total (n)	Hatched (n)	Total (n)	Hatched (n)
<i>E(var)3-5⁰¹</i>	707	31.6% (224)	73.2% (164)	43.7% (309)	94.4% (292)	23.1% (164)	0
<i>E(var)3-5⁰²</i>	633	28.9% (183)	72.6% (133)	48.1% (305)	95.0% (290)	22.9% (145)	0
<i>E(var)3-5⁰³</i>	874	27.3% (239)	72.3% (173)	46.7% (409)	93.3% (383)	25.8% (226)	0
<i>E(var)3-5⁰⁴</i>	766	32.2% (247)	58.7% (145)	45.6% (350)	95.4% (334)	22.0% (169)	0
<i>E(var)3-5⁰⁵</i>	875	27.0% (237)	72.9% (173)	45.6% (399)	95.7% (382)	27.3% (239)	0
<i>E(var)3-5⁰⁶</i>	787	32.0% (252)	65.0% (164)	45.8% (361)	93.2% (340)	22.% (174)	0

^a The yellow embryos represent the hemizygous *E(var)3-5/Df(3R)Exel7310* embryos (alleles one through six).

^b The green embryos represent the genotype *E(var)3-5* or *Df(3R)Exel7310/TM3, P{w⁺GAL4twi.G} P{w⁺UAS-2xEGFP} Sb Ser*.

^c The bright green embryos represent the genotype *TM3, P{w⁺GAL4twi.G} P{w⁺UAS-2xEGFP} Sb Ser/TM3, P{w⁺GAL4twi.G} P{w⁺UAS-2xEGFP} Sb Ser*.

The lethality of *Df(3R)Exel 7310/E(var)3-5* mutant larvae was analyzed. For one trial, the hemizygous larvae were placed in vials and analyzed for viability. The larval mouthhooks of any dead larvae were isolated to determine the larval instar stage. Lethality in these hemizygous larvae was primarily manifested during the pupal stage (Table 3.4). The few adults flies that eclosed had unexpanded wings.

Table 3.4 Viability of *Df(3R)Exel7310/E(var)3-5* larvae

Genotype	Lethal phase						
	Total	Unknown ^a (n)	1 st instar n (%)	2 nd instar n (%)	3 rd instar n (%)	Pupae n (%)	Adult ^b n (%)
<i>Df(3R)Exel7310</i> <i>E(var)3-5</i> ⁰¹	76	6	13 (19%)	0 (0%)	1 (1%)	55 (79%)	1 (1%)
<i>Df(3R)Exel7310</i> <i>E(var)3-5</i> ⁰²	133	31	23 (23%)	6 (6%)	1 (1%)	71 (70%)	1 (1%)
<i>Df(3R)Exel7310</i> <i>E(var)3-5</i> ⁰³	86	10	14 (18%)	0 (0%)	3 (4%)	59 (78%)	0 (0%)
<i>Df(3R)Exel7310</i> <i>E(var)3-5</i> ⁰⁴	127	5	17 (14%)	22 (18%)	2 (1%)	80 (65%)	1 (0.8%)
<i>Df(3R)Exel7310</i> <i>E(var)3-5</i> ⁰⁵	99	7	5 (5%)	12 (13%)	2 (2%)	78 (84%)	0 (0%)
<i>Df(3R)Exel7310</i> <i>E(var)3-5</i> ⁰⁶	99	11	17 (19%)	10 (11%)	2 (2%)	59 (67%)	0 (0%)

^a Larvae were designated as unknown when the larvae could not be recovered from vials. These larvae are not included in the percentages calculated for hatched individuals.

^b The wings of the eclosed flies did not expand.

***PBac{RB}CG17360^{e02295}/Df(3R)D1M1* mutants exhibit lethality during the pupal stage:** Initial complementation tests of *E(var)3-5* mutants with *CG17360* mutants done in vials indicated the possibility that *E(var)3-5* might be allelic to *CG17360*. This observation was based on the fact that the observed ratio of *E(var)3-5/CG17360* progeny was low when a complementation test was performed. I therefore analyzed the lethal phase of *CG17360* hemizygous embryos, to elucidate the function of the *CG17360* gene product during embryogenesis. The lethality in *PBac{RB}CG17360^{e02295}/Df(3R)D1M1* would be due to one mutant copy of *CG17360*. The heterozygous green embryos were used as an internal control for comparing the hatching rates of embryos. The data derived from a minimum of three trials. The results for lethality are summarized in Table 3.5. About 80% of the yellow larvae hatched suggesting

that the *CG17360* gene product is not essential for embryogenesis or that maternally provided gene product is sufficient. The yellow embryos that hatched were analyzed for viability. The lethality analysis of *PBac{RB}CG17360^{e02295}/Df(3R)D1M1* larvae revealed that in 46% of larvae, lethality is manifested at the pupal stage. About 10% of larvae survived until the adulthood phase. The adult flies that eclosed exhibited an unexpanded wing phenotype (Table 3.6)

Table 3.5 Embryonic lethality analysis of *PBac{RB}CG17360^{e02295}/Df(3R)D1M1* embryos

Total embryos	Yellow embryos ^a		Green embryos ^b		Bright green embryos ^c	
	Total (n)	Hatched (n)	Total (n)	Hatched (n)	Total (n)	Hatched (n)
1124	30.1% (340)	80.3% (273)	46.9% (526)	95.7% (504)	22.7% (258)	0

^a The yellow embryos represent the hemizygous *PBac{RB}CG17360^{e02295}/Df(3R)D1M1* embryos (alleles one through six).

^b The green embryos represent the genotype *PBac{RB}CG17360^{e02295}* or *Df(3R)D1M1 /TM3, P{w⁺GAL4twi.G}, P{w⁺UAS-2xEGFP}, Sb Ser.*

^c The bright green embryos represent the genotype *TM3, P{w⁺GAL4twi.G}, P{w⁺UAS-2xEGFP}, Sb Ser/TM3, P{w⁺GAL4twi.G}, P{w⁺UAS-2xEGFP}, Sb Ser.*

Table 3.6 Lethality of *PBac{RB}CG17360^{e02295}/Df(3R)D1M1* larvae

Total scored	Lethality					
	Unknown ^a (n)	1 st instar n (%)	2 nd instar n (%)	3 rd instar n (%)	Pupae n (%)	Adult ^c n (%)
273	37	30 (12.7%)	63 ^b (26.7%)	9 (3.8%)	109 (46.2%)	25 (10.6%)

^a The unknown larvae were not accounted for when calculating the percentages. The larvae were designated unknown when they could not be recovered from vials

^b One larva died while molting, as revealed by double mouthhooks

^c All of the adults exhibited unexpanded wings

Discussion

Position effect variegation involves changes in gene expression as a consequence of chromosomal rearrangements. *E(var)3-5* was isolated as a dominant enhancer mutation in a genetic screen for PEV modifiers (Dorn *et al.* 1993b). The *E(var)3-5* alleles were reported to be recessive lethal.

The embryonic lethality of homozygous *E(var)3-5* embryos was analyzed. The results indicated that 60-70% of the homozygous *E(var)3-5* embryos hatched into larvae. Most died as 1st or 2nd instar larvae. The hatching rate in the hemizygous *E(var)3-5/Df(3R)Exel7310* embryos was recorded to be about 65-70%. The lethality of hemizygous *E(var)3-5* larvae was analyzed. Most hemizygous *E(var)3-5* larvae died as pupae.

About 20-30% of the homozygous *E(var)3-5* and hemizygous *E(var)3-5/Df(3R)Exel7310* embryos did not hatch. Experiments involving the unhatched embryos were not pursued further due to their small number. However it is possible that the unhatched embryos were unfertilized. Another possibility is that maternal gene product supported the embryonic development of most embryos but that the *E(var)3-5* gene product was depleted in the unhatched embryos resulting in embryonic lethality.

The lethality produced in the hemizygous *E(var)3-5/Df(3R)Exel7310* mutant larvae would be due to one mutant copy of *E(var)3-5* protein (hypomorph/deficiency). On the other hand lethality produced in the homozygous *E(var)3-5* larvae would be due to two mutant copies of *E(var)3-5* protein (hypomorph/hypomorph). Thus studying the lethal phase of hemizygous *E(var)3-5* mutants was advantageous compared to homozygous mutants. The lethality in these hemizygous *E(var)3-5/Df(3R)Exel7310* larvae is manifested during the pupal stage whereas the lethality in homozygous *E(var)3-5* larvae is manifested in the early larval instar stages. These results indicate the presence of other random mutations in the homozygous *E(var)3-5* mutants. Since the lethality in *E(var)3-5/Df(3R)Exel7310* larvae is manifested primarily at the pupal stage, the *E(var)3-5* gene product might be essential for pupal development.

The embryonic lethality of *PBac{RB}CG17360^{e02295}/Df(3R)D1M1* embryos was analyzed. 80% hatching rate was observed in these embryos. About 20% of the embryos remained unhatched. As mentioned earlier these embryos might be unfertilized, or may require the maternally contributed gene product for further development. The significant lethality during pupal development and the observation that those individuals surviving until adulthood had unexpanded wings indicates the requirement of the *CG17360* protein during the pupal stage and for wing development.

Chapter 4: Discussion

Position effect variegation (PEV) is observed as alterations in a gene's expression pattern due its misplacement into a new chromosomal environment. For example, a euchromatic gene exhibits variegated expression when displaced near heterochromatin due to a translocation or inversion. Heterochromatic genes also exhibit variegated expression due to their placement away from heterochromatic sequences. The eukaryotic chromosome is organized into two domains, euchromatin and heterochromatin. PEV is thus used as a tool to unfold mysteries underlying the process of heterochromatinization and chromosome structure (reviewed by Weiler and Wakimoto 1995). Dominant mutations of the genetic modifier genes of PEV that enhance or suppress the process of heterochromatinization have been isolated (reviewed by Schotta *et al.* 2003). The protein products of *E(var)* genes are predicted to be important for establishing a euchromatic chromatin state, whereas *Su(var)* gene products are implicated in maintaining a heterochromatic chromatin state (Reuter and Wolff 1981; Sinclair *et al.* 1983)

E(var)3-5 was identified via ten dominant mutant alleles that enhanced PEV of *In(1)w^{m4}* by Dorn *et al.* (1993b). The mutant alleles of *E(var)3-5* were reported to be recessive lethal. Six recessive lethal *E(var)3-5* alleles were isolated in an *Icarus-neo* mutagenesis screen and four alleles were isolated in the $\pi 2$ mutagenesis screen. Seven *E(var)3-5* alleles, six of which originated from the *Icarus-neo* mutagenesis and the seventh allele that originated from $\pi 2$ mutagenesis were provided generously to our lab by Dr. Gunter Reuter.

My project was the identification of the *E(var)3-5* gene and characterization of *E(var)3-5* mutations. My first goal was to characterize the lethality of *E(var)3-5* mutations. Studies on enhancer genes such as *mod(mdg4)* (Buchner *et al.* 2000), *Trl* (Farkas *et al.* 1994), *Asx* (Sinclair *et al.* 1998), and *E2F* (Seum *et al.* 1996) have shown that the protein products of these genes are essential for embryogenesis. The lethality analysis of *E(var)3-5* mutations was undertaken to understand the functions of the *E(var)3-5* gene product. In these experiments I analyzed the hatching rate and lethality of homozygous *E(var)3-5*

embryos (alleles 01 through 06) and hemizygous *Df(3R)Exel7310/E(var)3-5* embryos. Most homozygous *E(var)3-5* embryos had a hatching rate around 70% and the lethality of homozygous *E(var)3-5* larvae was manifested during the first or second larval instar stages. An equivalent hatching rate of ~70% was observed for the hemizygous *Df(3R)Exel7310/E(var)3-5* embryos.

The lethality in *Df(3R)Exel7310/E(var)3-5* larvae is manifested at the pupal stage.

Since the *E(var)3-5* alleles are recessive lethal and homozygous *E(var)3-5* adults are not observed it was expected that the embryos might have a very low hatching rate. However a hatching rate of ~70% for the hemizygous and homozygous embryos indicated that zygotic contribution of *E(var)3-5* product is not essential during embryogenesis or that maternal contribution was sufficient.

Analyzing lethality in hemizygous *Df(3R)Exel7310/E(var)3-5* embryos was advantageous compared to analyzing the lethality in the homozygous *E(var)3-5* embryos. The lethality in the hemizygous embryos would be due to one mutant copy of *E(var)3-5* (hypomorph/deficiency) compared to lethality of homozygous embryos where the lethality would be due to two mutants copies of *E(var)3-5* (hypomorph/hypomorph). Thus the lethality in *Df(3R)Exel7310/E(var)3-5* embryos should be more pronounced due to a lower amount of *E(var)3-5* protein as compared to homozygous *E(var)3-5* embryos. Also in the hemizygous embryos, there would be less associated mutations due to a deficiency chromosome than homozygous embryos where the associated mutations would be present on both the chromosomes. The lethality in homozygous *E(var)3-5* larvae was manifested during the first or second larval instar stages whereas the lethality in hemizygous embryos is manifested during the pupal stage. This indicated that earlier lethality in homozygous *E(var)3-5* larvae might be due to other associated mutations.

A large number (70%) of hemizygous *Df(3R)Exel7310/E(var)3-5* larvae die at the pupal stage. This indicates that *E(var)3-5* protein product is essential for the pupal stage. Lethality in these larvae is also observed at the first and second larval instar stage and about 25% unhatched hemizygous embryos are

also observed. It is possible that there is a requirement of maternally contributed *E(var)3-5* protein during the embryonic stage and 1st or 2nd larval stages. However appropriate experiments should be performed to verify that maternally contributed product is essential. Secondly, the unhatched hemizygous embryos should be analyzed to determine whether they are fertilized.

My second goal was to identify the genetic locus on the third chromosome that corresponded to *E(var)3-5*. The *E(var)3-5* mutation was localized to the third chromosome by Dorn *et al* (1993). Meiotic mapping data showed that *E(var)3-5* was located at map position 3-50.56+/- 0.28 which corresponds to cytological location 86DE (Weiler unpublished). Lethality mapping studies of *E(var)3-5* done in our lab indicated that deficiencies *Df(3R)M-Kx1/TM3,Sb Ser* and *Df(3R)T-32, cu sir e/MRS* failed to complement *E(var)3-5*. This suggested that *E(var)3-5* was present in the region from 86E2-4 to 87B1-5. I continued the approach of deficiency mapping the *E(var)3-5* lethality. Complementation test results with deficiency *Df(3R)Exel7310* revealed non-complementation with *E(var)3-5* mutants. These data suggested that the lethality of *E(var)3-5* mutants maps to the region defined by *Df(3R)Exel7310*.

I generated a series of five *FLP-FRT* mediated deletions in the region defined by *Df(3R)Exel7310* and carried out complementation tests with *E(var)3-5* mutant alleles. Initial complementation tests done in vials suggested that *Df(3R)D1M1* and *Df(3R)D2M2* mutant alleles failed to complement *E(var)3-5* mutations. *Df(3R)D4M1*, *Df(3R)D5M1* and *Df(3R)D7M1* complemented the lethality of *E(var)3-5* mutants. Further complementation tests were done with *l(3)neo38* and *CG17360* mutants, the two genes deleted by both *Df(3R)D1M1* and *Df(3R)D2M2*. The results revealed that *CG17360* might be allelic to *E(var)3-5* since expected ratio of *E(var)3-5/CG17360*⁻ progeny was not observed. However the ratio of *E(var)3-5/CG17360*⁻ progeny was not consistent when the complementation tests were repeated in vials. The ratio varied from 1% to 23%. Further complementation tests done in bottles yielded a higher ratio of *E(var)3-5/CG17360*⁻ progeny. These results indicated that the *E(var)3-5/CG17360*⁻ progeny were sensitive to density condition in bottles versus vials. In vials, if the

conditions were even a little crowded, the *E(var)3-5/CG17360* progeny were underrepresented. One reason might be that *E(var)3-5/CG17360* larvae were not healthy and faced competition from sibling progeny. In addition, the adult *E(var)3-5/CG17360* flies have unexpanded wings and after eclosion, can be trapped in the food. Consequently, I may have missed counting many adults that were lost in the food. Complementation tests of *E(var)3-5* mutants with the *Df(3R)D1M1*, *Df(3R)D2M2* and *Df(3R)D4M1* mutants done in bottles revealed that the deletions partially or fully complemented the lethality of *E(var)3-5* mutations. The complementation test results were intriguing since *Df(3R)Exel7310* encompasses all of these smaller deletions, and clearly failed to complement *E(var)3-5* when the crosses were carried out in either bottles or vials. Very few *E(var)3-5/Df(3R)Exel7310* adult were obtained. These results suggest that lethality of *E(var)3-5* might be a cumulative effect of mutations in genes defined by the *Df(3R)Exel7310* region.

As indicated in chapter three (Table 3.4), the lethality in *E(var)3-5/Df(3R)Exel7310* was manifested primarily at the pupal stage and few adults were obtained. These results agree with the complementation test results where few *E(var)3-5/Df(3R)Exel7310* adults were obtained.

One interesting observation was that *Df(3R)D1M1/E(var)3-5⁰¹*, *Df(3R)D2M2/E(var)3-5⁰¹* and *E(var)3-5⁰¹/CG17360* flies had an unexpanded wing phenotype. The *PBac{RB}CG17360^{M56}* homozygous mutants and the *PBac{RB}CG17360^{M56}/PBac{RB}CG17360^{e02295}* flies also exhibited unexpanded wings. A revertant allele of *CG17360* was generated and complementation tests with *E(var)3-5* mutant alleles were done. The results revealed that *E(var)3-5/PBac{RB}CG17360^{Rev}* progeny had normal wings. This suggested that loss of *CG17360* function resulted in an unexpanded wing phenotype and that *E(var)3-5* mutant strains had mutations in *CG17360*. *E(var)3-5⁰⁷/Df(3R)D1M1* flies also had an unexpanded wing phenotype. Since the *E(var)3-5⁰¹* and *E(var)3-5⁰⁷* alleles were isolated in different mutagenesis screens, it is possible that parental strain used for obtaining the *E(var)3-5* mutations originally harbored mutations in

CG17360. The parental strains could not be obtained from Dr. Gunter Reuter to verify these observations.

The *E(var)3-5* mutant alleles were investigated by PCR analysis to determine whether mutations exist in the *CG17360* gene. The *E(var)3-5* alleles (one through six) and *E(var)3-5⁰⁷* allele were tested. Initial results revealed that a 1064bp and 844bp region defined by primer sets 3219F and 4265R, 3734F and 4578R could not be amplified indicating the presence of mutation. Further PCR analysis, using primer sets that amplified smaller regions, indicated that a 340bp sequence defined by primers 3941F and 4265R could not be amplified, indicating the presence of a mutation. PCR analysis was done on a *E(var)3-4⁰⁹* mutant allele using primer sets 3734F-4578R. A 844bp region defined by these primer sets could not be amplified thereby indicating the presence of a mutation. The *E(var)3-5* alleles (01-06) and *E(var)3-4⁰⁹* allele were isolated in the *Icarus-neo* mutagenesis. The *E(var)3-5⁰⁷* was obtained in a different mutagenesis screen. These results further suggest the fact that parental strains used for isolating these enhancer mutations originally harbored mutations in *CG17360* gene. However it is also possible that the *E(var)* strains could have been confused during stock maintenance.

Complementation tests between *E(var)3-4⁰⁹* and *E(var)3-5⁰¹* mutants in vials yielded *E(var)3-4⁰⁹/E(var)3-5⁰¹* progeny. A low ratio was obtained, which was unexpected since *E(var)3-5* and the *E(var)3-4* mutants should complement each other. A probable explanation for this result is that the *E(var)3-4⁰⁹/E(var)3-5⁰¹* progeny are underrepresented because both chromosomes harbor a mutation in *CG17360*. The complementation tests should be repeated in bottles to verify the results. Another possibility is that during the process of stock maintenance the *E(var)3-4* stock was confused with the *E(var)3-5* stock.

Southern analysis in six *Icarus-neo* mutagenized *E(var)3-5* alleles was done to determine the nature of mutation. *Pst*I digested *Canton S* DNA and *E(var)3-5* DNA was probed with a 1064 bp DNA fragment. The probe recognized 1.0 kb and 1.9 kb fragments in the control *Canton S* DNA. It was expected that if the mutation is a insertion the *Pst*I restriction fragment in the *E(var)3-5* alleles

would be disrupted and as a result the probe would recognize fragments other than 1.0 and 1.9 kb. But surprisingly, 1.0 kb and 1.9 kb fragments were detected in all of the *E(var)3-5* alleles. A probable explanation for these observations is that all the *E(var)3-5* alleles contain an inversion. This inversion is between *Pst*I sites, to give the 1.0 and 1.9 kb DNA bands.

I tested the effect of *E(var)3-5⁰¹*, *Df(3R)Exel7310* and four FLP-FRT mediated deletions on PEV of *In(1)w^{m4}* and *T(2:3)Sb^v*. The *w^{m4}* variegation experiment was performed twice. The *E(var)3-5⁰¹* allele enhanced *w^{m4}* variegation in both the trials but data obtained from *Df(3R)D1M1* and *Df(3R)D2M2* was not consistent each time the experiment was repeated. This experiment needs to be done more meticulously to get conclusive data.

The *E(var)3-5⁰¹* and *E(var)3-5⁰³* alleles enhanced *Sb* variegation. The results also determined that *Df(3R)D4M1* females significantly enhanced *Sb* variegation. One interpretation of this result is that that *E(var)3-5* phenotype maps in the region deleted by this deficiency. In *Df(3R)D4M1* males, a significant enhancement in *Sb* variegation was not observed compared to other deficiencies. In *Df(3R)D4M1* males, the heterochromatic Y chromosome leads to a suppression of *Sb^v* gene as a consequence of which more Stubble bristles are observed. However the experiment needs to be repeated again to verify the results.

I also tested the effect of mutations in *CG17360* on *Sb* variegation. The *PBac{RB}CG17360^{e02295}* and the *Df(3R)CG17360^{M56}* alleles were used. The results indicated that *CG17360* mutants did not enhance *Sb* variegation. Instead, a suppressive effect was observed. The effect of mutations in the *CG17360* gene on *LacZ* variegation were also indicative of a suppressive effect. I also tested the effect of *Df(3R)Exel7310* on variegation of *LacZ* gene. *Df(3R)Exel7310* enhanced *LacZ* variegation. This result was intriguing since *Df(3R)Exel7310* did not enhance *Sb* variegation. The *LacZ* variegation assay needs to be repeated again to verify if the results are repeatable.

Is the mutation in *CG17360* responsible for the *E(var)* phenotype of *E(var)3-5*? Based on the *Sb* variegation results obtained for *CG17360* mutants

alone, it is possible that mutations in *CG17360* are not responsible for the *E(var)* phenotype. However, future experiments involving *CG17360* transgene should be done to determine whether the transgene rescues the *E(var)* phenotype. If the *CG17360* transgene fails to rescue the *E(var)* phenotype of *E(var)3-5*, this suggests that the *E(var)* phenotype maps to a different region. If the mapping of *E(var)3-5* gene is continued, the mapping should be done based on enhancer phenotype rather than the lethality phenotype.

Does *CG17360* mediate heterochromatin formation or is *CG17360* a suppressor of PEV based on *Sb* variegation results? Based on the yeast-two hybrid interactions by Giot *et al.* (2003), *CG17360* interacts with *Pp1-87B*, which has been identified as a suppressor of PEV (Giot *et al.* 2003). *CG17360* might interact with this suppressor and as a result suppress *Sb* variegation. In addition, studies conducted by undergraduate student Catherine Koontz on meiotic chromosome segregation on *CG17360* mutants revealed that *CG17360* mutants severely disrupt X-achiasmate disjunction in *D. melanogaster* females. Her results indicated that the *CG17360* gene product might be essential for heterochromatin formation. The effect of recessive mutations in *CG17360* should also be tested using PEV assays to detect enhancer phenotype if any.

In the genetic screen conducted by Dorn *et al.* (1993b) to isolate enhancer mutations, several other *E(var)* mutations were isolated in the π 2 mutagenesis screen and *Icarus-neo* mutagenesis. It would certainly be interesting to analyze these *E(var)* mutants for mutations in *CG17360*.

Over the years molecular characterization of *E(var)* and *Su(var)* genes has enhanced our knowledge about process of heterochromatin formation and heterochromatin mediated gene silencing. Therefore further experimentation on the *E(var)3-5* mutants should definitely be continued.

Appendix I

Introduction

Previously done mapping studies by graduate student Zhen Li, using *P*-element mediated male recombination, indicated that *CG3281* might be *E(var)3-5*. Mapping data using the *P* element *P{ry+}R307 ry{42}* thought to be inserted near *Hsp70Aa*, indicated that *E(var)3-5* was distal to it. Mapping data using the *P{Epgy2}aur^{EY03490}* insertion indicated that *E(var)3-5* was proximal to *aur*. A total of fourteen recombinants for *P{ry+}R307 ry{42}* and nine recombinants for *P{Epgy2}aur^{EY03490}* were obtained. Three genes are localized between these two *P* elements: *Hsp70Aa*, *CG3281*, and *aurora*. The *Hsp70Aa* gene encodes for a heat shock protein, and no phenotype was expected for deletion of this gene since it is a repeated gene. This indicated that *E(var)3-5* mutants were not allelic to *Hsp70Aa*. Complementation tests of *E(var)3-5* mutants with *aurora*^{87Ac-3} mutant allele indicated that *E(var)3-5* was not allelic to *aurora* gene (Weiler unpublished). This led to the hypothesis that *CG3281* was *E(var)3-5*. I sequenced *CG3281* in the *E(var)3-5* alleles. I also carried out a *P*-element mediated male recombination experiment to extend Zhen Li's results.

Materials and methods

Strains and culture conditions: All of the strains and crosses were maintained in cornmeal-malt medium established as a standard media recipe by Bloomington Drosophila Stock Center (<http://flystocks.bio.indiana.edu>). The crosses were maintained at 25°C unless otherwise noted. The *E(var)3-5* mutants alleles were isolated by Dorn *et al.* (1993) and were gifted to our lab by R. Dorn and G. Reuter. Information of other mutants used in this study is available at Flybase (<http://flystocks.bio.indiana.edu>)

Sequencing *CG3281* in *E(var)3-5* alleles: A series of overlapping primers was produced for the coding region of *CG3281*. The primers were designed using the software Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and were obtained from Invitrogen company. The primer sets were *CG3281* 69F (5'-

ATCGGGCATCTCTGAACATC-3') and CG3281 629R (5'-GGTCTCCATACTTCTCCTCCATTT-3'), CG3281 523F (5'-GGATGTCGTGGAGCTTATAGACCAA-3') and CG3281 1022R (5'-TTGCGTCGGGATGGAATG-3'), CG3281 868F (5'-TTCCCAAGCCCACAAACTG-3') and CG3281 1453R (5'-ACCGCACACAAAGCTCTCG-3'), CG3281 1360F (5'-TGTGCAGTCCAACGACCTTA-3') and CG3281 1884R (5'-TCTAATATACATGGGTTCATTCTACATTCT-3'). DNA was obtained from ten homozygous first instar larvae from all six *E(var)3-5* alleles using the protocol of Gloor *et al.* (1993). The PCR products were purified using Exo-SAP-IT (USB) and sequenced from both ends at the ISU MRCF.

P-element mediated male recombination: A *P*-element mediated male recombination experiment was carried out to determine if the *E(var)3-5* gene is proximal or distal to the *P* insertion *P{wHy}Hsp70Aa^{DG16104}* insertion. In the first set of crosses *st Sb^{sbd-1} e ro ca* virgin females were crossed to *yw ; +/Cy0, H{w⁺ PΔ 2-3} ; st E(var)3-5⁰⁴ ca/ P{wHy}Hsp70Aa^{DG16104}* males. From the resulting cross two phenotypic classes of recombinants were obtained. The phenotypic classes were *st ca⁺* and *st⁺ ca* recombinants. Out of 1567 males scored, 13 recombinants were obtained. Complementation tests between *w¹¹¹⁸; E(var)3-5⁰¹/TM3, P{w⁺GAL4-twi.G} P{w⁺UAS-2xEGFP} Sb Ser* females and *st ca⁺* males were done. From the resulting cross if *Sb⁺ Tb⁺ Hu⁺* progeny were obtained, it was determined that *E(var)3-5* was distal to the *P* insertion. Similarly, complementation tests between *E(var)3-5/TM3, P{w⁺GAL4-twi.G} P{w⁺UAS-2xEGFP} Sb Ser* females and *st⁺ ca* males were done. From the resulting cross if *Sb⁺ Tb⁺ Hu⁺* progeny were obtained, it was determined that *E(var)3-5* was proximal to the insertion.

Results

Sequence analysis of the CG3281 gene: Since previous results indicated that CG3281 was *E(var)3-5*, I sequenced this gene in *E(var)3-5* alleles to determine

whether CG3281 contains any mutations. The sequencing results revealed only a single nucleotide change in coding sequence which was consistent with all of the *E(var)3-5* alleles. These results suggested that CG3281 is not *E(var)3-5*.

P-element mediated male recombination: The purpose of this experiment was to determine whether *E(var)3-5* is proximal or distal to the *P{wHy}Hsp70Aa^{DG16104}* insertion at 87A2. *P element-mediated male recombination was performed by expressing P transposase in st E(var)3-5⁰⁴ ca/ P{wHy}Hsp70Aa^{DG16104} males, and selecting progeny that exhibited recombinant phenotypes. Each recombinant chromosome was stocked. In this experiment E(var)3-5⁰¹/TM3, P{w⁺GAL4-twi.G} P{w⁺UAS-2xEGFP} Sb Ser females were crossed to the st ca⁺ and st⁺ ca recombinants, for complementation analysis. The results are shown in Tables A1, A2, A3 and A4. The results indicated that with st ca⁺ recombinants, *E(var)3-5⁰¹ / st ca⁺* progeny were not obtained and with st⁺ ca recombinants *E(var)3-5⁰¹ / st⁺ ca* progeny were obtained. This indicated that the st ca⁺ chromosomes had the *E(var)3-5⁰⁴ mutation*. It was thus established that *E(var)3-5* gene maps proximal to the *P{Hy}Hsp70Aa^{DG16104}* insertion.*

Table A1 Complementation test of *E(var)3-5* females with st ca⁺ recombinant males

st ca ⁺ recombinant males	<i>E(var)3-5⁰¹/TM3, P{w⁺GAL4-twi.G}, P{w⁺UAS-2xEGFP}, Sb Ser</i> females	
	Sb	Sb ⁺ ^a
D2	50	0
A1*	34	0
D4	57	0

Recombinant chromosomes were balanced over the *TM3, Sb st e* balancer.

^a The absence of Sb⁺ progeny is expected if *E(var)3-5* is proximal to the *P* insertion

Table A2 Complementation test of *E(var)3-5* females with st ca⁺ recombinant males

st ca ⁺ recombinant males	<i>E(var)3-5⁰¹/ TM3, P{w⁺GAL4-twi.G} P{w⁺UAS-2xEGFP}Sb Ser</i> females			
	Sb ⁺ Tb Hu e ⁺	Sb e ⁺	Sb e	Sb ⁺ Tb ⁺ Hu ⁺ e ⁺ ^a
D5	6	5	1	0
F1*	9	0	14	0
D2	10	11	7	0
A1*	26	15	9	0
D1*	25	17	12	0

Recombinant chromosomes were balanced over the *TM6B, Tb Hu e* balancer.

^a The absence of Sb⁺ Tb⁺ Hu⁺ e⁺ progeny is expected if *E(var)3-5* is proximal to the *P* insertion

Table A3 Complementation test of *E(var)3-5* females with st⁺ca recombinant males

st ⁺ ca recombinant males	<i>E(var)3-5⁰¹/ TM3, P{w⁺GAL4-twi.G} P{w⁺UAS-2xEGFP}Sb Ser</i> females	
	Sb	Sb+ ^a
E1	20	10
F1	13	29
D8	20	10

Recombinant chromosomes were balanced over the *TM3, Sb st e* balancer.

^a The presence of Sb⁺ progeny is expected if *E(var)3-5* is proximal to the *P* insertion

Table A4 Complementation test of *E(var)3-5* females with *st⁺ca* recombinant males

st ⁺ ca recombinant male	<i>E(var)3-5⁰¹ / TM3, P{w⁺GAL4-twi.G}, P{w⁺UAS-2xEGFP}, Sb Ser</i> females			
	Sb ⁺ Tb Hu e ⁺	Sb e	Sb e	Sb ⁺ Tb ⁺ Hu ⁺ e ⁺ ^a
E1	15	11	4	11

Recombinant chromosomes were balanced over the *TM6B, Tb Hu e* balancer.

^aThe presence of Sb⁺ Tb⁺ Hu⁺ e⁺ progeny is expected if *E(var)3-5* is proximal to the *P* insertion.

Conclusion

The sequencing results revealed a single nucleotide polymorphism in *CG3281* establishing that *CG3281* is not allelic to *E(var)3-5*. Furthermore, the *P*-element mediated male recombination data established that the lethality of *E(var)3-5* maps proximal to the insertion at 87A. *CG3281* is distal to the *P{wHy}Hsp70Aa^{DG16104}* insertion. This further confirms that *CG3281* is not *E(var)3-5*.

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