# 1 Chromatin Structure and the Regulation of Gene Expression: The Lessons of PEV in *Drosophila*

**Jack R. Girton and Kristen M. Johansen**

Department of Biochemistry, Biophysics, and Molecular Biology,
Iowa State University, Ames, Iowa 50011

## ABSTRACT

Position-effect variegation (PEV) was discovered in 1930 in a study of X-ray-induced chromosomal rearrangements. Rearrangements that place euchromatic genes adjacent to a region of centromeric heterochromatin give a variegated phenotype that results from the inactivation of genes by heterochromatin spreading from the breakpoint. PEV can also result from P element insertions that place euchromatic genes into heterochromatic regions and rearrangements

that position euchromatic chromosomal regions into heterochromatic nuclear compartments. More than 75 years of studies of PEV have revealed that PEV is a complex phenomenon that results from fundamental differences in the structure and function of heterochromatin and euchromatin with respect to gene expression. Molecular analysis of PEV began with the discovery that PEV phenotypes are altered by suppressor and enhancer mutations of a large number of modifier genes whose products are structural components of heterochromatin, enzymes that modify heterochromatic proteins, or are nuclear structural components. Analysis of these gene products has led to our current understanding that formation of heterochromatin involves specific modifications of histones leading to the binding of particular sets of heterochromatic proteins, and that this process may be the mechanism for repressing gene expression in PEV. Other modifier genes produce products whose function is part of an active mechanism of generation of euchromatin that resists heterochromatization. Current studies of PEV are focusing on defining the complex patterns of modifier gene activity and the sequence of events that leads to the dynamic interplay between heterochromatin and euchromatin.    © 2008, Elsevier Inc.

## I. INTRODUCTION: POSITION EFFECT IN *DROSOPHILA*

The discovery of position effects in *Drosophila* began with the efforts of many investigators to generate a coherent picture of the nature of genes and chromosomes and of the relationship between them. Between 1910 and 1930, many genes were discovered in *Drosophila* by the recovery of mutant alleles. Analysis of mutant phenotypes suggested that each gene had a specific function and linkage mapping indicated that each gene had a unique chromosomal location (its locus). Cytological studies of mitotic and salivary gland polytene chromosomes indicated that chromosomes contain two visibly different regions, heterochromatin and euchromatin. Heterochromatin consists of regions that remain condensed and densely stained during interphase, while euchromatin consists of regions that become diffused during interphase and form the visible, banded regions of the polytene chromosomes. Euchromatin contains high concentrations of genes and heterochromatin contains very few genes. Although many believed that differential regulation of gene expression was an underlying feature of eukaryotic development, these investigations did not provide information about how gene activity was regulated. Since chromosomal rearrangements, such as inversions or translocations, that changed gene location but did not eliminate genes often had no visible mutant phenotype, chromosome structure was not believed to have a role in regulating gene function. Chromosomes appeared to simply serve as

vehicles for gene segregation during cell division. Two major findings challenged this view and launched an intensive study of the role of chromosome structure in gene regulation that continues today.

The first finding came in Sturtevant's study of the *Bar* eye mutation (Sturtevant, 1925). *Bar* is a dominant, sex-linked mutation that reduces the number of facets in the compound eye (normally about 800). The *Bar* mutation is a duplication of the *Bar* locus (*B*). Normal females have two copies of the locus, one on each X chromosome (*B/B*), but *Bar*-mutant females have four copies, two on each chromosome (BB/BB). Sturtevant described a new mutation (called *ultraBar* or *double-Bar*) that caused an even greater reduction in the number of facets. The *ultraBar* mutation has three copies of the *Bar* locus on one chromosome (*BBB*) and homozygous *ultraBar* females have a total of six copies (*BBB/BBB*). This suggested that increasing the number of copies of the *Bar* gene causes a decrease in eye size. However, Sturtevant noted that homozygous *Bar* females (*BB/BB*) have an average of $68 \times 12$ facets per eye, while females heterozygous for *ultraBar* (*BBB/B*) have an average of only $45 \times 42$ facets. Since both genotypes contain four copies of the *Bar* locus, this difference could not be due to differences in the number of copies of the locus. Sturtevant concluded that the arrangement of the copies of the *Bar* gene in the chromosome had an influence on *Bar* gene expression. He named this influence "position effect." This suggested that chromosomes are not passive collections of genes, but that they contain an internal structure that has a role in the regulation of gene expression.

The second finding came in 1930, when Muller reported the discovery of a series of X-ray-induced mutations affecting the *white* gene. Previously discovered *white* mutations reduced the amount of eye pigment, changing eye color from the normal uniform red to a uniform orange, yellow, or white (Lindsley and Zimm, 1992; Morgan *et al.*, 1925). Muller's new mutations showed a "mottled" phenotype, with each eye having some white (mutant) and some red (normal) regions (Fig. 1.1). The size, shape, and location of the white regions varied from eye to eye. The mutations causing these mottled phenotypes were chromosomal rearrangements that changed the location of the *white* gene in the chromosome (Muller, 1930). This rearrangement-induced mottled phenotype was named "variegated position effect" or "V-type" to distinguish it from the "stable position effect" or "S-type" position effect of Bar. Muller's discovery of the phenomenon of position-effect variegation (PEV) launched an intense study of the role of chromosome structure in gene expression that has continued for more than 75 years. This interest was partly because of the intriguing novel variegated phenotypes and partly because studies of PEV appeared likely to give important information about how gene expression was controlled by chromosomal organization.

PEV in *Drosophila* presents an interesting example of the historical progression of genetic analysis. During the past 75 years, the discovery of new investigative techniques has invariably led to a multitude of PEV studies using
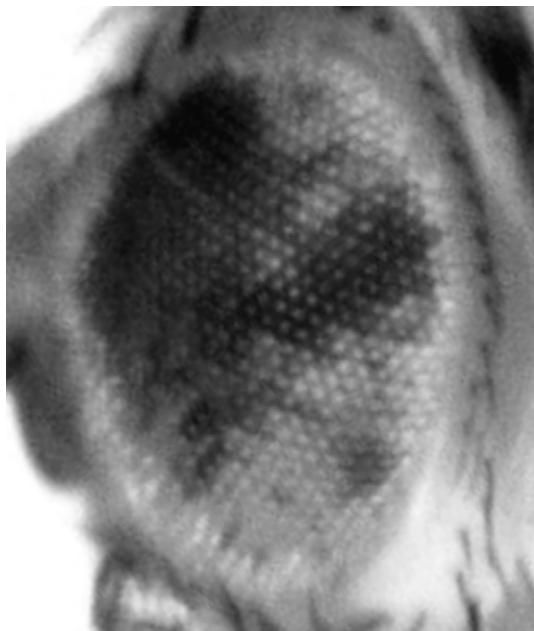
**Figure 1.1.**  Position-effect variegation (PEV) of an inversion chromosome. The mottled pigmenta-
tion observed in the eye of an individual carrying the *In(1)w^{m4}* inversion chromosome
shows regions where the *white* gene has been silenced (white) and regions where the
*white* gene is actively expressed (dark).

these techniques. The sheer number of studies and the volume of information
accumulated about PEV present a formidable task for the reader. There have
been a number of extensive reviews of the experimental literature about PEV
(Farkas *et al.*, 2000; Huisinga *et al.*, 2006; Lewis, 1950; Schulze and Wallrath,
2007; Spofford, 1976; Spradling and Karpen, 1990; Weiler and Wakimoto, 1995;
Zhimulev, 1998). It is not the purpose of this chapter to repeat these works. Our
goal is to summarize what has been learned about certain key aspects of PEV that
occur reliably and repeatedly and which are considered defining features of the
PEV phenomenon. These reflect, in a manner that is not currently understood,
fundamental characteristics of underlying molecular/biochemical processes of
chromatin organization that affect gene regulation. We will attempt to connect
these characteristics of PEV with the results of recent molecular studies of the
formation and molecular structure of chromatin. These results reflect the current
excitement in the field, as it appears that the long examination of PEV may be
approaching the point of finally revealing the role that different forms of

chromatin structure play in the regulation of gene expression, and how that regulation occurs. To cover such a large topic, we will have to be selective in the experiments we discuss and in the original publications we cite.

## II. HISTORICAL BACKGROUND OF THE PEV PHENOTYPE

In 1930, Muller described a series of mutants induced by X-ray treatment. Muller originally discovered the first such mutation as a dominant *Notch* wing mutation. Dominant *Notch* mutations produce nicks or "notches" in the wings of heterozygous (*N*/+) flies. Muller knew *Notch* mutations sometimes resulted from deletions of portions of the X chromosome, so he generated flies heterozygous for the new *Notch* and a chromosome containing a recessive mutant allele of the nearby *white* locus (*N* (?)/+ *w*), expecting to see either a white eye phenotype, if a deletion removing both *Notch* and *white* was present, or a red eye phenotype if it were not. However, what he observed fit neither of these expectations. "To the great surprise of the writer the Notch-winged offspring of this cross had neither white nor normal red eyes, nor even eyes of any uniform intermediate colour. They had mottled eyes, and exhibited various grades and sizes of lighter and darker areas" (Muller, 1930). Further study revealed that the *Notch*-mutant phenotype was also "mottled" and that individuals showing a stronger mutant eye phenotype (lighter eyes) also had a stronger *Notch* phenotype (larger wing notches). This mutation was a translocation that had attached the distal tip of the X chromosome (the normal location of the *Notch* and *white* genes) to the third chromosome. Muller discovered four additional "white-mottled" mutations that did not involve mutations of *Notch* in additional irradiation experiments and all of these also were rearrangements (translocations or inversions) that moved the region of the X chromosome containing the *white* locus. Studying these mutations for more than 50 generations convinced Muller that the white mottled was a permanent mutant phenotype. He concluded that the change in the chromosomal arrangement of the genes on the X chromosome was responsible for the appearance of what he called the "eversporting displacement" phenotype, although he admitted to having no good explanation for how this worked. Muller's paper, with its striking color diagrams showing the white-mottled phenotype, made an enormous impression and stimulated a number of other investigators to begin studying the relationship between chromosomal rearrangements and "mottled" gene expression. Studies of several genes quickly demonstrated that the mottled phenotype was produced only by certain types of chromosomal rearrangements and that the PEV phenotypes given by all rearrangements had a number of consistent characteristics (Demerec, 1941; Dobzhansky, 1936; Lewis, 1950; Muller, 1932).

## A. The PEV phenotype and heterochromatin

The PEV phenotypic effects Muller and other early investigators recovered were all produced by chromosomal rearrangements with one breakpoint close to the variegating gene and the other in a heterochromatic region that brought the affected gene into close proximity to heterochromatin. Examples of such PEV-inducing rearrangements are shown in Fig. 1.2. For example, the $N^{264-52}$ X chromosome contains an inversion with one breakpoint in the centric heterochromatin and the other between *rst* and *w*. This places normal alleles of the *bi*, *ec*, *dm*, *fa*, and *rst* genes close to the centric heterochromatin. This inversion
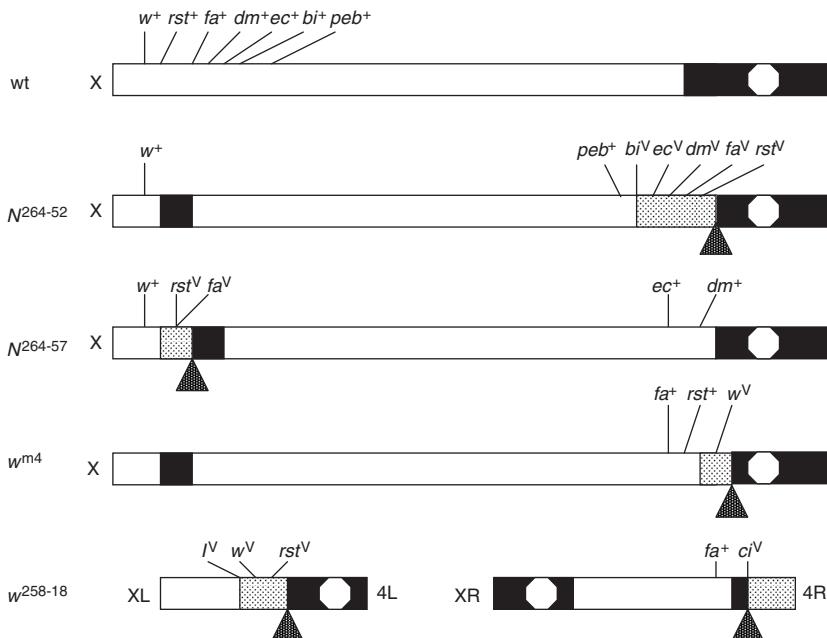


**Figure 1.2.** A diagram showing some of the X chromosome rearrangements discovered by Muller to give position-effect variegation (PEV). The relationship between the chromosomal breakpoint and several genes that are affected is shown. Adapted from Lewis (1950).

generates a variegating loss-of-function phenotype for each of these genes. Females that are heterozygous for the $N^{264-52}$ chromosome and a normal chromosome containing a recessive loss-of-function mutant allele of any of these genes have a variegating mutant phenotype for that gene. The variegation results from the alleles of the genes on the rearranged chromosome being repressed in some cells, and active in other cells. Females heterozygous for the rearranged chromosome and a chromosome with normal alleles of these genes have a normal phenotype, indicating that the PEV effect is recessive and does not affect the alleles on the normal chromosome. Other rearrangements, such as $N^{264-57}$ or $w^{m4}$, have breakpoints in different locations and have different genes that show PEV but the effect is the same. PEV is produced in translocations, such as $w^{258-18}$, that move euchromatic genes on one chromosome adjacent to a heterochromatic region of a different chromosome, or in rearrangements, such as $N^{264-57}$, that move a region of heterochromatin into a euchromatic location (Fig. 1.2). The requirement for PEV is clearly that a euchromatic gene be moved adjacent to a heterochromatic breakpoint (Spofford, 1976; Zhimulev, 1998). This suggested that PEV phenotypes result from heterochromatic chromatin structure "spreading" from the breakpoint into the adjacent euchromatin and inactivating nearby euchromatic genes. The variegated phenotype was proposed to result from the length of spreading along the chromosome being variable.

## B. Heterochromatin and euchromatin

Because heterochromatin plays a central role in any discussion of PEV, it is worthwhile to briefly discuss some key features of heterochromatin and euchromatin. Heitz (1928) discovered that portions of the chromosomes remain highly condensed and heavily stained throughout the cell cycle. He named these regions "heterochromatin" to distinguish them from the regions showing variable staining and condensation, which he called "euchromatin." The boundaries of heterochromatin as defined by such cytological analyses are not precise and may change in different tissues or with different analytical techniques. In *Drosophila,* heterochromatic regions are located near the centromeres (the pericentric heterochromatin), at the telomeres, interspersed throughout the fourth chromosome, throughout the Y chromosome, and at a number of defined "intercalary heterochromatic" sites scattered among the chromosome arms (Zhimulev, 1998; Zhimulev and Belyaeva, 2003). In most *Drosophila* somatic cells, 30–35% of the genome is heterochromatic. Heterochromatin makes up all of the Y chromosome, 40% of the X, 25% of chromosomes 2 and 3, and more than half of the fourth. These heterochromatic regions contain a high percentage of families of repetitive DNA sequences with specific highly and moderately repetitive sequences clustered at particular chromosomal locations. The arrangement of these clusters of sequences is conserved among populations of *Drosophila*

*melanogaster* (Pimpinelli *et al.*, 1995; Weiler and Wakimoto, 1995). In *Drosophila*, the pericentric heterochromatin is divided into two visibly distinct categories, α-heterochromatin and β-heterochromatin. The α-heterochromatin is largely composed of highly repetitive satellite sequences and is located in and around the centromere. It coalesces into a highly condensed, uniform body in the center of the chromocenter in salivary gland nuclei. β-Heterochromatin is more diffuse and expands or "loops out" to form the bulk of the visible chromocenter. β-Chromatin more closely resembles euchromatin. It contains functioning genes interspersed with middle repeated sequences, and a significant percentage consists of sequences derived from transposons (Pimpinelli *et al.*, 1995). More than 40 functioning genes in heterochromatic regions have been identified by mutant phenotypes, including visible phenotypes, lethality, and sterility (Gatti and Pimpinelli, 1992; Weiler and Wakimoto, 1995). *Drosophila* chromosomes contain complex repeated sequences at the telomeres. These include copies of two retrotransposons (HeT-A and TART) at the end of the telomere that have the ability to repair or heal damaged telomeres (Pardue and DeBaryshe, 1999; Wallrath, 2000). Proximal to these sequences, *Drosophila* telomeres contain repeated sequences called telomere-associated sequences (Karpen and Spradling, 1992). These sequences differ in length and sequence from chromosome to chromosome (Walter *et al.*, 1995).

## III. TYPES OF PEV

There are a variety of ways in which a mosaic phenotype can be produced in *Drosophila*, including somatic recombination, somatic chromosomal loss, and somatic P element transposition. In this chapter, we will consider as PEV only those variegated phenotypes that are produced by chromosomal rearrangements that share the common feature that they bring euchromatic genes into close association with heterochromatin without altering the gene itself. These can be grouped into three classes or types based on a number of characteristic features.

### A. Chromosomal rearrangement PEV

The common feature of all chromosomal rearrangements that give PEV is that they either move a euchromatic gene into a pericentric region or move a block of heterochromatic sequences into a euchromatic region. Chromosomal rearrangements involving the X, the second, the third, and the fourth chromosomes give PEV phenotypes (Demerec, 1941; Kaufmann, 1946; Lewis, 1950; Weiler and Wakimoto, 1995; Zhimulev, 1998). The heterochromatic regions that can produce a PEV phenotype are the pericentric regions of all chromosomes, most of

the fourth chromosome, and all of the Y chromosome. Several experiments in which the variegating allele is removed from the rearranged chromosome show the PEV effect is a repression of gene expression and not a permanent change in the allele. Dubinin and Sidorov (1935) used meiotic recombination to remove the variegated allele of the *hairy* gene from a translocation between the third and fourth chromosome. A double crossover in flies heterozygous for this translocation and normal chromosomes moved the variegating allele to the normal chromosome. In the normal chromosome, the allele gave a normal, *hairy*$^+$ phenotype. When this same allele was reinserted into the translocated chromosome by another double crossover event, the allele again showed a variegating phenotype. Similar experiments with other variegating genes gave the same results (Lewis, 1950; Spofford, 1976). Inducing an additional chromosomal rearrangement has also been used to separate the variegating allele from the heterochromatin. Panshin (1938) used X-rays to induce a second translocation event that returned the portion of the X chromosome containing the variegating *white* allele in the $w^{m11}$ translocation to a euchromatic chromosomal location where it again gave a normal phenotype. Several similar experiments demonstrated that variegating alleles of any gene separated from the heterochromatic breakpoint by another chromosomal rearrangement ceases to show the variegating phenotype (Demerec, 1941; Hinton, 1950; Judd, 1955; Lewis, 1950). Another type of evidence that PEV is not the result of a change in gene structure came from studies of gene products using alleles that produce proteins with detectable structural differences. In one such experiment, Bahn (1971) studied individuals heterozygous for T(1;2)OR32 carrying the *Amy2* allele and a normal chromosome carrying the *Amy4,6* allele using gel electrophoresis to detect which electrophoretic variant proteins were produced. In variegating individuals, the level of the Amy2 variant was reduced, and the level of reduction was greater when the strength of the PEV was increased, but the structure of the protein product was not altered. The level of the Amy4,6 enzyme produced by the allele on the normal chromosome was not altered, indicating that the PEV effect acted only on the allele in the rearranged chromosome. In other similar studies, PEV was also found to reduce enzyme levels from alleles of *sc*, *dor*, and *pn* (Gerasimova *et al.*, 1972).

  In a study of PEV reversion, Tartof *et al.* (1984) induced a series of rearrangements of the $w^{m4}$ chromosome. The $w^{m4}$ chromosome has an inversion with one breakpoint about 25 kb from the *white* locus and the other in the centric heterochromatin and produces a white PEV phenotype. Tartof recovered three rearrangements that had a normal phenotype. Each had one breakpoint in the heterochromatin beyond the $w^{m4}$ breakpoint and they all retained some heterochromatic sequences at the original breakpoint. This indicates that the breakpoint itself did not cause the PEV phenotype. Reuter *et al.* (1985) studied 51 reversions of the $w^{m4}$ chromosome and found only 3 true revertants with no

detectable PEV phenotype. The others showed a reduced PEV phenotype, and each of these retained blocks of heterochromatin at the original breakpoint site. These studies indicate that PEV results from the influence of a significant region of heterochromatic sequence adjacent to euchromatic genes, and that it does not simply result from a breakpoint, or from the gene being moved close to a centromere. They also suggest that the PEV effect is correlated with the amount of heterochromatic sequences and that there is a lower limit on the size of the block of heterochromatin that can cause PEV.

## 1. The spreading effect

The strength of PEV (the frequency with which a gene is inactivated) is inversely proportional to distance from the breakpoint (Lewis, 1950). For example, as diagrammed in Fig. 1.2, chromosomes containing the inversion $N^{264-52}$ give a variegated phenotype for five genes (*rst*, *fa*, *dm*, *ec*, *bi*). The strength of the PEV effect on each was measured and genes further from the breakpoint showed less frequent or less strong mutant phenotypes, while those closer to the breakpoint showed more frequent or stronger mutant phenotypes (Demerec, 1940, 1941). This type of observation led to the hypothesis that PEV phenotypes resulted from a physical "spreading" of heterochromatin from the breakpoint into the euchromatic regions of the chromosome (Schultz, 1939). The distance between *Notch* and *white* is 330 kb, and in the original mutation of Muller both are frequently inactivated. The *bi* locus inactivated in the $N^{264-52}$ chromosome is located at least 50 salivary gland chromosome bands away from the breakpoint, a molecular distance of more than 1mb of DNA. The PEV effect of $T(1;4)w^{m258-21}$ can extend over 67 bands (Hartman-Goldstein, 1967), that of $Dp(1;F)R$ over 86 bands (Spofford, 1976; Weiler and Wakimoto, 1995). These observations indicate that the spreading effect is able to cover significant chromosomal distances.

Two early studies suggested that the heterochromatic spreading effect in PEV is linear, that is, does not jump over genes. Demerec and Slizynska (1937) studied the PEV given by the $w^{258-18}$ chromosome of *roughest* (*rst*) and *white* (*w*), two genes whose phenotypes can be scored in the eye. The gene order on the chromosome is: breakpoint, *rst*, *w*. An examination of the PEV phenotype showed the eye facets had one of three phenotypes: smooth and red ($rst^+$, $w^+$), rough and red ($rst^V$ $w^+$), or rough and white ($rst^V$, $w^V$). They observed no facets with a smooth and white ($rst^+$, $w^V$) phenotype and concluded that the variegating effect passed in a linear manner from the breakpoint along the chromosome and always inactivated *roughest* before reaching *white*. Similar results in a study by Schultz (1941) of the PEV phenotype given by $w^{258-21}$ on *split* and *white* reached the same conclusion. In a recent study, Talbert and Henikoff (2000) studied the PEV phenotypes given by X chromosome inversions that had

the arrangement of breakpoint, *w*, and *rst*. They reported cases of $w^+$ cells in a rough ($rst^V$) region of the eye. Does this mean that inactivation has "skipped over" *w* to inactivate *rst*? These results must be interpreted with caution. Talbert and Henikoff (2000) themselves noted that the alleles of *rst* and *w* used in their studies are not necessarily affecting the same cells. The *white* mutation is cell autonomous and its phenotype can be seen in each pigment cell in the eye. However, alleles of *roughest* affect the shape of the facet by causing the inclusion of extra cells in the eye facet. Not all cells in the facet need to be $rst^-$ to give a rough facet phenotype. PEV effects of $w^{m4}$ and other rearrangements on *white* give a fine-scale variegation and individual facets often contain both white and pigmented cells (Girton, personal observation). It is thus possible that a "rough" facet may be mosaic and that the pigmented cells are actually $w^+ rst^+$, and the inactivation did not jump over *w* to inactivate *rst*. A definitive study of this question would need to use markers whose relationship to the breakpoint is known and whose expression can unambiguously be measured in the same cell.

## 2. All genes may show PEV

The first studies of PEV involved genes whose mutation produced a visible phenotype (such as *w*, *fa*, and *rst*). Later studies showed that PEV can be detected for a wide range of genes including those whose mutation produces a quantitative phenotype such as the number of bristles (*hairy*), the level of a detectable enzyme product (tryptophan pyrolase in $v^+$, Tobler *et al.*, 1971), enzymatic variants, (*Amy* and *Pdg*, Bahn, 1971), the amount of kynurenine production in fat body in $T(1;2)ras^v$ and $T(1;Y)y^+$ $Yv^+$ (Rizki, 1961; Tobler *et al.*, 1971), or the rate of ribosomal RNA synthesis in female ovaries ($sc^{S1}$ and $sc^{L8}$, Puckett and Snyder, 1973). Lewis (1950) listed 32 genes shown to variegate. Spofford (1976) concluded that all genes can show a PEV-variegated phenotype in the right rearrangement.

Some genes are normally located in heterochromatic regions of chromosomes. Estimates of how many such genes exist in *Drosophila* range from 40 to 100 or more (Schulze and Wallrath, 2007; Spofford, 1976; Weiler and Wakimoto, 1995). A question that was raised very early in the study of PEV was how a gene whose normal location is heterochromatic would respond in a rearrangement that moved it to a euchromatic location. The first such gene studied was the light gene (*lt*) studied by Schultz and Dobzhansky (1934). The *lt* maps to a region considered to be $\beta$ heterochromatin in chromosome 2 (Hilliker, 1976; Schultz and Dobzhansky, 1934). Rearrangements that move *lt* adjacent to euchromatic regions give a variegated phenotype (Hessler, 1958; Schultz, 1936; Schultz and Dobzhansky, 1934). The conditions under which *lt* PEV inactivation occurs are the opposite of the conditions under which PEV inactivation of euchromatic genes occurs. At least five other heterochromatic genes normally

located near *lt* show similar position effects when placed next to euchromatin (Wakimoto and Hearn, 1990; Weiler and Wakimoto, 1995). Other heterochromatic genes that show this pattern include *cubitus interruptus* (*ci*) (Dubinin and Sidorov, 1935), *peach* (*pe*) in *Drosophila virilis* (Baker, 1953), male fertility factors on the Y chromosome (Hackstein and Hochstenbach, 1995; Neuhaus, 1939), and the nucleolus organizer region (Baker, 1971). Not all euchromatic regions show the same efficiency in generating heterochromatic PEV. All regions of X chromosome euchromatin can cause heterochromatic gene variegation, but only the distal parts of the arms of chromosome 2 and 3. Breakpoints in the fourth do not induce PEV of heterochromatic genes, perhaps reflecting the fact that the fourth chromosome contains interspersed heterochromatic and euchromatic regions (Spofford, 1976). In two cases, heterochromatic genes showing variegation when removed from surrounding heterochromatin had this variegation eliminated when an additional rearrangement moved them back near a block of heterochromatin (Eberl *et al.*, 1993). This is one of the most intriguing observations of PEV, that genes normally located in hetrochromatin can be inactivated by being placed adjacent to euchromatin. Genes normally located in heterochromatic regions have some differences in molecular structure (Reugels *et al.*, 2000; Risinger *et al.*, 1997; Schulze and Wallrath, 2007; Schulze *et al.*, 2005). These observations suggest heterochromatic genes have evolved to not only tolerate being in a heterochromatic region but to actually require it for normal action.

## 3. Modifiers of the PEV phenotype

PEV induced by chromosomal rearrangements is strongly affected by *trans*-acting modifiers that increase the severity of the mutant phenotype (enhance variegation) or decrease the severity of the mutant phenotype (suppress variegation). Modifiers of PEV fall into two general classes, large-scale effects and single mutations. One large-scale effect is temperature. Rearing flies at higher temperature (29 °C) suppresses variegation (reduces the severity of the mutant phenotype) and rearing at lower temperature (16–18 °C) enhances variegation (increases the severity of the mutant phenotype). Gowen and Gay (1934) studied the phenotypic effects of temperature on PEV of *w*, *spl*, and *ec* caused by $w^{m1}$, $w^{m2}$, and $w^{m3}$. All of these genes showed more extreme mutant phenotypes at lower temperature. Kaufmann (1942) documented similar high- and low-temperature effects on PEV of $rst^3$. All other euchromatic genes tested showed similar results, indicating this is a general effect of PEV (Bahn, 1971; Benner, 1971; Schalet, 1969; Spofford, 1976; Wargent, 1972).

PEV phenotypes are modified by changes in the amount of heterochromatic sequences in the genome. This was first observed in studies in which adding an extra copy of the Y chromosome was found to suppress variegation

and eliminating the Y chromosome enhanced variegation (Gowen and Gay, 1934). A PEV phenotype is more severely mutant in XO versus XY males or in XX versus XXY females (Lewis, 1950; Spofford, 1976). This effect is seen in genes producing visible phenotypes and those examined by measuring enzyme phenotypes (Bahn, 1971; Gerasimova *et al.*, 1972). In extreme cases, a rearrangement may not show a variegation phenotype except when enhancing modifiers are present. One example studied by Gersh (1963) involved the inversion $In(1)$ $rst^3$, which gives a white variegated phenotype in XO males but not in XY males. Changes in the Y chromosome affect all PEV-inducing rearrangements (Lindsley *et al.*, 1960; Weiler and Wakimoto, 1995; Zhimulev, 1998). Changes in the amount of centric heterochromatin also modify PEV phenotypes in the same manner as the Y chromosome. The deletion of a significant portion of the centric heterochromatic region of the X enhanced PEV of the *y*, *w*, and *ac* loci (Panshin, 1938). The addition or subtraction of an entire X has about the same effect as the addition or subtraction of the Y chromosome (Grell, 1958; Hinton, 1949). Similar effects have been discovered for the centric heterochromatic regions of the autosomes. A second chromosome deficiency that removes heterochromatin from the right arm of the second chromosome, $Df(2R)M\text{-}S2^{10}$, enhances the PEV of several different genes in several different rearrangements (Morgan *et al.*, 1941). Duplications of the heterochromatic region of the right arm of the second chromosome suppress PEV phenotypes (Grell, 1970; Hannah, 1951). The fact that centric heterochromatin and the Y chromosome have similar effects on all euchromatic genes showing PEV indicates that this is a general effect of heterochromatin, and not due to specific sequences on the Y.

## 4. Single gene Su(var) and E(var) mutations

In several of the early studies of PEV, the severity of the phenotypes given by a PEV-inducing rearrangement varied from strain to strain, even when the strains did not differ for any obvious modifying factor (Hinton, 1949). A rearrangement, such as $In(1)w^{m4}$, that gives a PEV phenotype of 50% white eye in one strain could give phenotypes of 25% white eye or 75% white eye in other strains. These "genetic background effects" were believed to be due to unmapped dominant, single gene suppressor or enhancer mutations located throughout the genome (Schultz, 1941; Spofford, 1967, 1976). The first such modifier mutation to be isolated and characterized was a dominant suppressor mutation, *Su(var)*, on the third chromosome (Spofford, 1967). Individuals with an $In(1)w^{m4}$ PEV-inducing genotype that also had the *Su(var)* allele had almost completely red eyes, while siblings with the $Su(var)^+$ allele had light, variegated eyes, the standard PEV phenotype. The *Su(var)* allele had similar effects on the PEV phenotypes of *w*, *rst*, and *dm* given by the $In(1)w^{m4}$, $Dp(1;3)N^{264-58}$, and $In(1)rst^3$ chromosomes

(Spofford, 1967, 1973, 1976). The discovery of the *Su(var)* allele suggested that
there might be a number of genes whose mutation could modify PEV phenotypes.
This led to several experiments to recover suppressor and enhancer mutations
(Dorn *et al.*, 1993; Eissenberg *et al.*, 1990, 1992; Grigliatti, 1991; Locke *et al.*,
1988; Reuter and Wolff, 1981; Sinclair *et al.*, 1983, 2000; Wustmann *et al.*,
1989). A large number of dominant and recessive modifier alleles have been
isolated, and it is estimated that there are between 50 and 150 loci that can
mutate to give a PEV-modifying phenotype (Weiler and Wakimoto, 1995;
Schulze and Wallrath, 2007). The effects of different modifier mutations appear
to be similar; all suppressors reduce the severity of the PEV phenotype given by
all rearrangements, and all enhancers increase the severity of the PEV phenotype
given by all rearrangements (Fig. 1.3).

     The PEV phenotype of genes that normally reside in heterochromatic
regions shows a characteristic inverse response to modifiers of PEV. Temperature
effects on the PEV of the heterochromatic gene *lt* are the reverse of those on
euchromatic gene PEV, with higher temperatures producing a more mutant
phenotype (Gersh, 1949). While extra Y chromosomes suppress PEV of euchro-
matic genes, they act as enhancers of variegation of *lt* (Baker and Reim, 1962;
Schultz, 1936) and *ci* (Altorfer, 1967). Baker and Spofford (1959) studied the
effects of 14 free Y chromosome fragments and reported that the same regions of
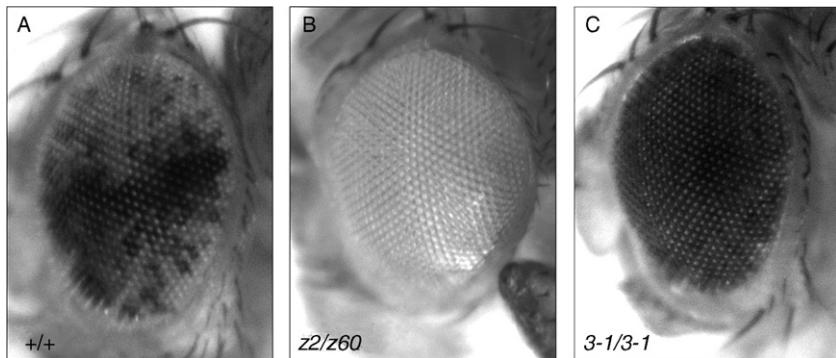


**Figure 1.3.** The effect of su(var) and e(var) mutations on PEV phenotypes. All individuals are
homozygous for the pericentric P insertion line P118E-10 that gives a *white* PEV
phenotype (Wallrath and Elgin, 1995). (A) The eye phenotype of an individual with
no modifier mutation is variegated, with the *white* marker gene actively expressed in
some cells but silenced in others. (B) An individual with a strong e(var) genotype, JIL-
$1^{z2}$/JIL-$1^{z60}$, shows complete silencing of *white* gene expression. (C) An individual
homozygous for a strong su(var) genotype, Su(var)3–1/Su(var)3–1, shows high levels
of *white* gene expression. Photos: X. Bao.

the Y that suppress the $w$ PEV phenotype given by $Dp(1;3)N^{264-58}$ enhanced the $lt$ PEV phenotype given by $T(2;3)ltm^{100}$ (Baker and Reim, 1962). Single-gene mutations that suppress euchromatic gene PEV enhance the PEV phenotype of heterochromatic genes, and single-gene mutations that enhance euchromatic gene PEV suppress the PEV phenotype of heterochromatic genes (Schulze and Wallrath, 2007; Spofford, 1976; Weiler and Wakimoto, 1995). The reason why heterochromatic gene PEV and euchromatic gene PEV should show such consistent and opposite reactions is one of the most intriguing characteristics of PEV.

A paradigm in genetics is that the normal function of a gene can be deduced from the phenotype given by mutant alleles. The observation that mutations of modifier genes alter the PEV phenotype, and that heterochromatin is involved in generating PEV phenotypes, suggested that the normal functions of modifier genes are part of a genetic system that is essential for the establishment, maintenance, or function of chromatin structure. This is supported by several observations. The products of most modifier genes that have been studied to date are either structural components of heterochromatin (Baksa *et al.*, 1993; Henikoff, 1979; James and Elgin, 1986; Locke *et al.*, 1988; Reuter and Spierer, 1992; Reuter *et al.*, 1982; Sinclair *et al.*, 1983), enzymes that modify histones or nonhistone chromatin proteins (Ebert *et al.*, 2006; Lerach *et al.*, 2006; Mottes *et al.*, 2000; Nishioka *et al.*, 2002; Schotta *et al.*, 2004), or nuclear architectural proteins (Bao *et al.*, 2007a; Yamaguchi *et al.*, 2001). For example, the product of the $Su(var)205$ gene is the HP-1 protein, a heterochromatic protein that is found in high concentrations in the chromocenter in salivary gland nuclei (Eissenberg *et al.*, 1990, 1992; James and Elgin, 1986). The product of the $Su(var)3-7$ gene is a large zinc-finger protein that is mainly associated with the pericentric heterochromatin (Reuter *et al.*, 1990). The JIL-1 protein is a tandem kinase that phosphorylates the serine 10 residue in the tail of histone 3 (Jin *et al.*, 1999; Lerach *et al.*, 2006; Zhang *et al.*, 2003) and a dominant gain of function allele of JIL-1 is one of the strongest supresors of PEV so far described (Ebert *et al.*, 2004). The majority are haplo-abnormal, showing the dominant modifying effect when the gene is present in only single copy, and some (10%) have both haplo- and triplo-abnormal effects, modifying PEV when present in either one or three copies. For example, a deletion of Su(var)205 suppresses the $In(1)w^{m4}$ PEV phenotype, giving a pure red eye, and a duplication giving a genome three copies of the locus enhances the PEV phenotype, giving a nearly pure white eye (Schulze and Wallrath, 2007; Weiler and Wakimoto, 1995). This sensitivity to dosage suggests that a balance in the amount of the products of these genes is important for chromatin structure formation and function.

# B. Transposon insertion PEV

Rearrangements that break and rejoin chromosomes are not the only method for placing a euchromatic gene adjacent to heterochromatin. In 1982, a genetically recombinant P element with an inserted cloned reporter gene ($ry^+$) was induced to transpose into a chromosomal site (Rubin and Spradling, 1982; Spradling and Rubin, 1982). The reporter gene in P element transformation is essentially a small piece of euchromatic sequence that is inserted into different regions of the genome. In an early transformation experiment (Hazelrigg *et al.*, 1984), two insertions of a transposon containing as a reporter a wild-type copy of the *white* gene were recovered that gave a white variegated eye phenotype. There was no indication that the inserted $w^+$ gene was altered in the transposition process and it was concluded that the variegation was the result of a position effect. One of these variegating insertions was located in the basal region of chromosome 2L, a heterochromatic region that was known to induce PEV in chromosomal rearrangements. The second was inserted near the telomere of the right arm of chromosome 3. This finding that genes inserted in a heterochromatic region by P element transformation can show a variegated phenotype was soon confirmed by other investigators (Steller and Pirrotta, 1985). In a genome-wide screen, Wallrath and Elgin (1995) recovered insertions with a white variegated phenotype in all of the heterochromatic regions known to induce PEV in rearrangements (the pericentric regions of the X, the second, the third, throughout the fourth, and in the Y chromosome). Variegating insertions were also recovered in or near the telomeres of all chromosomes (Wallrath and Elgin, 1995). Ahmad and Golic (1996) also recovered variegating P insertions in euchromatic regions at a site of intercalary heterochromatin. These results suggested that there was more to learn about position effects than had been suspected before, because P insertions can give a variegated phenotype in locations that do not give a PEV phenotype in chromosomal rearrangements (Fig. 1.4).

An important question about variegation produced by P element insertion is whether it is generated by the same mechanism as chromosomal rearrangement-induced PEV. One test of this is to determine the effect of known modifiers of PEV on the transposon insertion phenotype. Talbert *et al.* (1994) observed that the PEV phenotype of a transposon insertion in the telomeric region of 3R was not altered by modifiers of the PEV effect given by $w^{m4}$. Wallrath and Elgin (1995) showed the known PEV-modifying mutations, $Su(var)2$–$5^{02}$ and $Su$-$var(2)1^{01}$, suppressed the PEV phenotypes of all pericentric and fourth chromosome insertions, including insertions near the telomere of the fourth chromosome, but did not suppress the variegation produced by six different insertions located near the telomeres of 2R, 2L, or 3R. Comparing flies with XO and XXY genotypes showed that increasing the amount of heterochromatin suppressed the variegation phenotype of 13 inserts in the pericentric
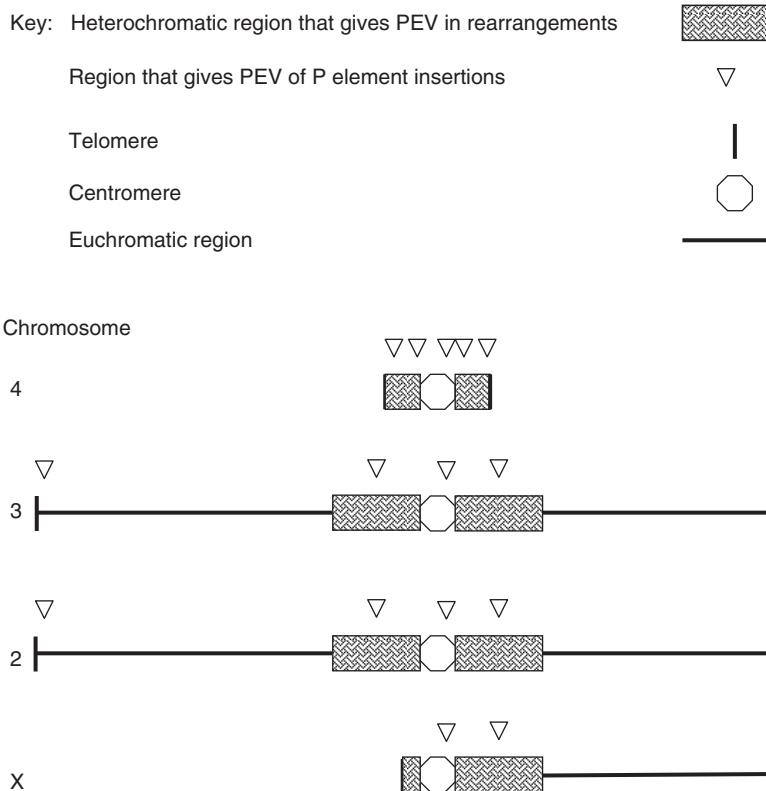
**Figure 1.4.** A diagram showing the chromosomal regions in which P element insertions showing a position-effect variegation (PEV) phenotype were recovered by Wallrath and Elgin (1995). Insertions were primarily recovered at centromeric and telomeric regions and distributed throughout the fourth chromosome.

heterochromatin or in the fourth chromosome, but had no effect on 7 insertions in or near the telomeres of 2R, 3L, or 3R (Wallrath and Elgin, 1995). A second test of PEV is to determine whether variegation is eliminated when the variegated allele is removed from its location. Ahmad and Golic (1996) demonstrated that if an inserted P element that is showing variegation in somatic cells is physically removed from the chromosome to an extrachromosomal circle using the flippase (FLP) recombinase system, the gene expression is restored. All of the insert lines they tested responded to modifiers of PEV (enhancement in XO males and suppression by the $Su(var)205^{502}$ mutation), but none of their insertions were located at a telomere. These results indicate that PEV generated by P

element insertions in nontelomeric locations behaves like chromosomal rearrangement PEV, but that inserts in the telomeric regions have different properties. These results suggest that there may be differences in the mechanisms that produce telomeric insertion PEV phenotypes.

## C. Pairing-dependent dominant PEV: *Trans*-inactivation

The first PEV-inducing rearrangements recovered gave mostly recessive phenotypes. For example, an individual heterozygous for the $In(1)w^{m4}$ chromosome and a normal chromosome ($In(1)w^{m4}/w^+$) has a wild-type phenotype because the breakpoint in the $In(1)w^{m4}$ chromosome has no *trans*-inactivation effect on the $w^+$ allele in the normal chromosome. Chromosomal rearrangements that give a dominant PEV phenotype are rare. One well-studied example consists of rearrangements that affect the *brown (bw)* gene. Several inversions with one breakpoint in heterochromatin and another in the 59C6–59F3 interval of chromosome 2R have been recovered that give a dominant variegating brown eye PEV phenotype (Schultz, 1936; Slatis, 1955). Flies that are heterozygous for such a rearrangement and a normal chromosome containing a wild-type allele of the *brown* gene have a variegating brown eye color phenotype. This indicates that the breakpoint has a *trans*-inactivation effect on the $bw^+$ allele in the normal chromosome. A dominant *brown* allele ($bw^D$) was discovered that is not associated with an inversion but which carries an insertion of a block of 1.6 mb of centromeric heterochromatin in the *bw* locus (Hinton, 1940, 1942; Hinton and Goodsmith, 1950; Slatis, 1955). Flies with a $bw^D/bw^+$ genotype have a *bw* PEV phenotype. Removal of the heterochromatic block by further rearrangement eliminates the PEV effect (Hinton and Goodsmith, 1950). The dominant $bw^D$ PEV phenotype is altered by suppressors of euchromatic gene PEV (Csink and Henikoff, 1996). These findings confirmed that the dominant *trans*-inactivation effect of $bw^D$ is due to the presence of adjacent heterochromatin, and that the effect is a PEV effect.

      Northern analysis indicates that $bw^D/bw^+$ individuals produce no detectable mRNA from either allele (Dreesen *et al.*, 1988; Henikoff and Dreesen, 1989). Thus, the dominant *bw* effect results from an inactivation of transcription of *bw* alleles in both chromosomes. The obvious question is how the insertion of a block of heterochromatin in one chromosome can affect the transcription of the allele on the other homologue. The key to this *trans*-inactivation effect is homologous chromosome pairing (Sass and Henikoff, 1999). When a fly has two *bw* variegating rearrangements with different breakpoints that disrupt somatic chromosome pairing, the PEV phenotype is also reduced (Henikoff and Dreesen, 1989). Confirmation that pairing is the key to *bw*-dominant *trans*-inactivation came when $bw^D$ was combined with duplications

containing extra copies of the $bw^+$ in other chromosomal locations, where they do not pair with the normal locus. In such cases, the inserted $bw^+$ is not inactivated by $bw^D$ (Dreesen et al., 1991).

The idea that somatic pairing of chromosomes is important for normal function and might be a factor in PEV is an old one (Ephrussi and Sutton, 1944; Henikoff, 1994). How might pairing with a chromosome containing $bw^D$ cause *trans*-inactivation of the $bw^+$ allele on the non-rearranged chromosome? The inserted heterochromatic block in $bw^D$ contains multiple copies of the AAGAG sequence, a sequence that is one of the most abundant in the genome and which is found on each chromosome, and which is especially common in the pericentric region of chromosome 2 (Lohe et al., 1993). Tracking the nuclear location of the *bw* insert using fluorescent *in situ* hybridization (FISH) shows that in the interphase nucleus, this block becomes associated with a region of the nucleus where the 2R-centric heterochromatin is localized (Csink et al., 2002; Dernburg et al. 1996). The silencing of $bw^D$ is thus correlated with the localization of the $bw^D$ allele and the paired $bw^+$ allele to a heterochromatic region of the nucleus. This suggested that the dominant $bw^D$ PEV effect is due to the $bw^D$ allele dragging the paired $bw^+$ allele on the other homologue to a nuclear compartment where heterochromatic pairing results in gene inactivation. In a further investigation, the expressions of a series of P element insertions at different locations along the normal 2R chromosome were assayed when paired with the $bw^D$ chromosome to determine the chromosomal extent of this *trans*-inactivation effect. An insertion 45 kb from the $bw^D$ insertion was inactivated but inserts 280 and 460 kb from *bw* were not inactivated (Csink et al., 2002). When the $bw^D$ insert was introgressed into *Drosophila simulans*, which has AAGAG sequences in the centric region of the X chromosome but not the second chromosome, the insert continued to associate with the centric region of the second chromosome, suggesting that the heterochromatic association effect is not sequence specific but is an association with the nearest large block of heterochromatin (Sage and Csink, 2003). Recent studies suggest that this heterochromatic-pairing *brown*-dominant PEV may not be unique, and that heterochromatic insertions in other euchromatic regions may show *trans*-association/inactivation (Sage and Csink, 2003; Thakar and Csink, 2005). This suggests that *trans*-inactivation by heterochromatic insertion may be an important mechanism of gene silencing.

## IV. GENOME ORGANIZATION AND PEV

The study of PEV has been instrumental in advancing our appreciation of the role that chromatin structure and genome organization play on the regulation of gene expression. In particular, the ability to isolate modifiers of the PEV-silencing effect in genetic screens resulted in identification of a large number of

chromosomal and nuclear architectural factors that are important for long-range control of gene expression (Table 1.1). Analysis of these factors has allowed us to develop a molecular understanding of the different processes contributing to the establishment or maintenance of active versus silent chromosomal domains and especially it has illuminated the critical role that heterochromatin plays in genome organization. In this section, we will review key aspects of chromatin structure and will examine some of the genes that affect PEV penetrance and the molecular mechanisms underlying the phenomenon. We will consider recent studies that underscore the link between heterochromatin formation and nuclear organization.

## A. Chromatin structure

The primary packaging unit of chromatin is the nucleosome, composed of 147 bp of DNA wrapped around a histone octamer composed of two subunits each of the core histones H2A, H2B, H3, and H4 (reviewed in Luger, 2006). The histone globular domains are arranged in the interior of the nucleosome, while their unstructured tail domains project outwards where they are targets for a range of posttranslational modifications, including acetylation, phosphorylation, methylation, ADP-ribosylation, ubiquitination, sumoylation, and biotinylation. These modifications have been proposed to regulate genomic function by influencing chromatin structure both by altering the biophysical contacts between DNA and histones and by providing specific binding sites for different classes of chromatin-binding proteins (reviewed in Kouzarides, 2007). Thus, the modified histone tails provide a signaling platform that integrates output from various signal transduction pathways ultimately specifying the level of higher order chromatin folding (Cheung et al., 2000; Fischle et al., 2003; Turner et al., 1992).

Hyperacetylation of histones H3 and H4 and methylation of H3K4 are correlated with the establishment of transcriptionally active chromatin across a wide range of species from yeast to Drosophila to human (Bernstein et al., 2005; Pokholok et al., 2005; Schubeler et al., 2004). Acetylation neutralizes the basic charge of the lysine residue, which is thought to reduce histone tail interaction with the acidic phosphate residues of DNA, making it more accessible for transcription (Wade et al., 1997), and acetylation of the H4K16 residue, in particular, has been shown to physically impede the formation of higher order folded structures as well as block nucleosome sliding by the Drosophila ATP-utilizing chromatin assembly and remodeling factor (ACF) chromatin-remodeling complex in vitro (Shogren-Knaak et al., 2006). But besides changing the biophysical properties of chromatin fiber behavior, lysine acetylation also generates specific docking sites for distinct bromodomain proteins that have been found in a variety of different transcriptional activators and chromatin-remodeling complexes; binding to these sites can be influenced

**Table 1.1.** Nuclear PEV Modifiers of $w^{m4}$

| Locus | Function | References |
|---|---|---|
| **Histone-modifying enzymes/histone variants** | | |
| JIL-1 | H3S10 kinase | Wang *et al.*, 2001; Ebert *et al.*, 2004; Bao *et al.*, 2007a |
| Su(var)3–9 | H3K9 methyltransferase | Reuter *et al.*, 1986; Schotta *et al.*, 2003b |
| G9a | H3K9 methyltransferase | Mis *et al.*, 2006 |
| HDAC1/RPD3 | Histone deacetylase | De Rubertis *et al.*, 1996; Mottus *et al.*, 2000 |
| Chameau | Histone acetyltransferase (HAT) | Grienenberger *et al.*, 2002 |
| Reptin | HAT complex | Qi *et al.*, 2006 |
| H2Av | H2A variant | Swaminathan *et al.*, 2005 |
| **Chromosomal proteins** | | |
| Su(var)2–5/HP1 | H3K9me-binding | James and Elgin, 1986; Eissenberg *et al.*, 1990 |
| HP2 | AT Hook protein | Shaffer *et al.* 2002 |
| D1 | AT Hook protein | Aulner *et al.*, 2002 |
| Su(var)3–7 | DNA binding (Zn finger) | Reuter *et al.*, 1990; Cléard and Spierer, 2001 |
| Modulo | DNA binding | Perrin *et al.*, 1998; Büchner *et al.*, 2000 |
| trl/GAGA factor | DNA binding | Dorn *et al.*, 1993; Farkas *et al.*, 1994 |
| Bonus | Nuclear receptor cofactor | Beckstead *et al.*, 2005 |
| mus209 | Proliferating cell nuclear antigen (PCNA); DNA replication and repair | Henderson *et al.*, 1994 |
| Cramped | Pc-G gene; DNA replication | Yamamoto *et al.*, 1997 |
| Zeste | Transvection | Hazelrigg and Petersen, 1992 |
| E(var)3–95E | E2F transcription factor; cell cycle | Seum *et al.*, 1996 |
| ORC2 | Replication; complexes with HP1 | Pak *et al.*, 1997 |
| HOAP | Complex with HP1/ORC; HMG-like | Shareef *et al.*, 2001 |
| DRE4/spt16 | Facilitates chromatin transcription (FACT) complex | Nakayama *et al.*, 2007 |
| **Nuclear and chromosome architecture** | | |
| Lamin Dm0 | Nuclear lamina/architecture | Bao *et al.*, 2007b |
| Su(var)2–10/PIAS | Nuclear organization | Reuter and Wolff, 1981; Hari *et al.*, 2001 |
| Piwi | RNAi | Pal-Bhadra *et al.*, 2004 |

*(Continues)*

**Table 1.1.** (*Continued*)

| Locus | Function | References |
|-------|----------|-----------|
| Homeless/Spn-E | RNAi | Pal-Bhadra *et al.*, 2004 |
| Aubergine | RNAi | Pal-Bhadra *et al.*, 2004 |
| mod/mdg4 [E(var) 3–93D)] | Boundary element | Dorn *et al.*, 1993; Gerasimova *et al.*, 1995 |
| BEAF-32 | Boundary element | Zhao *et al.*, 1995; Gilbert *et al.*, 2006 |
| Lip/Rm62 | Helicase | Csink *et al.*, 1994 |
| dCAP-G | Condensin subunit | Dej *et al.*, 2004 |
| gluon/SMC4 | Condensin subunit | Cobbe *et al.*, 2006 |
| wapl | Chromosome adhesion? | Verni *et al.*, 2000 |

by neighboring posttranslational modifications on the histone tail, thus providing a combinatorial complexity to the signaling process (Dyson *et al.*, 2001; Yang, 2004). Although histone acetylation is generally associated with active chromatin regions, acetylation of H4K12 is found in heterochromatic regions, suggesting that acetylation at particular sites may mediate specific effects on chromatin function (Turner *et al.*, 1992).

Whereas acetylation is, with few exceptions, associated with euchromatin, different methylated histone modifications have been found to play an important role in differentiating active (euchromatic) chromosomal domains from silenced (heterochromatic) domains (reviewed in Lachner *et al.*, 2003). In general, the "activating" modifications consist of methylated H3K4, H3K36, and H3K79, while the "silencing" modifications are composed of methylated H3K9, H3K27, and H4K20. Thus, identification of the enzymes affecting these modifications and elucidating the mechanisms underlying their targeting promised to provide significant insight in the specification of chromatin domains. Given that the early genetic and cytogenetic experiments indicated that PEV occurs when heterochromatin spreads into juxtaposed euchromatic sequences, it was anticipated that many of the modifiers of position effect would fall into the categories of histone-modifying and histone-binding proteins (Schotta *et al.*, 2003a).

## 1. Methylation and the spread of heterochromatin

Three *Su(var)* gene products that were of early interest due to their heterochromatic localization, their "haplo-suppressor/triplo-enhancer" behavior, and their physical and genetic interactions were SU(VAR)2–5 (also known as SU(VAR) 205, heterochromatin protein 1, or HP1) (Eissenberg *et al.*, 1990; James and Elgin, 1986; James *et al.*, 1989), SU(VAR)3–7 (Cléard and Spierer, 2001; Cléard

*et al.*, 1997; Reuter *et al.*, 1990), and SU(VAR)3–9, the latter of which is one of the strongest modifiers of PEV known, showing dominance over nearly all other enhancers of variegation tested (Reuter *et al.*, 1986; Tschiersch *et al.*, 1994; Wustmann *et al.*, 1989). Interestingly, one of the only two exceptions was $ptn^D$, which is a gain of function allele of *Su(var)3–9* that causes ectopic localization of the protein to euchromatic sites (Kuhfittig *et al.*, 2001). A critical advance in the field of heterochromatin biology came from the findings that (1) the mammalian orthologs of SU(VAR)3–9 encode a methyltransferase that specifically methylates H3K9 (Rea *et al.*, 2000), an activity that is conserved in *Drosophila* SU(VAR)3–9 (Czermin *et al.*, 2001; Schotta *et al.*, 2002) and (2) the chromodomain of HP1 binds to the methylated K9 of histone H3 (Bannister *et al.*, 2001; Lachner *et al.*, 2001). The silencing activity of HP1 was disrupted when its ability to bind methylated H3K9 was abolished by mutation (Jacobs *et al.*, 2001). Because the H3K9 methyltransferase SU(VAR)3–9 associates both with the H3K9 deacetylase HDAC1 (Czermin *et al.*, 2001) and with HP1 (Schotta *et al.*, 2002; Yamamoto and Sonoda, 2003), a spreading model for heterochromatin involving these components was proposed in which deacetylation of H3K9 clears the residue for methylation by SU(VAR)3–9 to enable HP1 binding, which, in turn, recruits additional SU(VAR)3–9 to methylate the adjacent nucleosome that provides another HP1-binding site in a self-propagating process (Grewal and Elgin, 2002; Schotta *et al.*, 2003b).

## 2. Initiation of heterochromatin formation

In recent years, a number of studies have indicated that RNAi-mediated silencing pathways can initiate the formation of heterochromatin (reviewed in Grewal and Rice, 2004; Matzke and Birchler, 2005; Zaratiegui *et al.*, 2007). This pathway was first identified in *S. pombe* where mutations in components of the RNAi pathway were found to disrupt centric heterochromatin silencing (Verdel *et al.*, 2004). Transcripts from repetitive elements in the centromeric region are processed into siRNAs that are incorporated into a RITS (RNAi-induced transcriptional silencing) complex that recognizes and binds homologous regions to initiate gene silencing mediated via H3K9 methylation (Verdel and Moazed, 2005; Verdel *et al.*, 2004). *Drosophila* centromeres are also composed of short satellite- and transposon-fragment repeats (Sun *et al.*, 1997) that are actively transcribed (Lakhotia and Jacob, 1974) and transgenes inserted into these regions show PEV (Cryderman *et al.*, 1998; Wallrath and Elgin, 1995; Wallrath *et al.*, 1996). Mutations in genes encoding the RNAi pathway Argonaute homologues *piwi*, *aubergine*, and the helicase *homeless* disrupt HP1 localization and suppress transgene silencing, indicating a similar mechanism for RNAi-mediated heterochromatin assembly operates in *Drosophila* as well (Pal-Bhadra *et al.*, 2004).

Mutants in *Argo2*, another RNAi argonaut family member, showed defects in centromeric heterochromatin assembly, including abnormal HP1 localization and defects in H3K9 methylation (Deshpande *et al.*, 2005).

## 3. Terminating heterochromatic spreading

That PEV is, by definition, a *variegated* phenotype indicates that chromosomal silencing can spread different lengths in different cells, and the question of what terminates the spreading is not well understood. Because of the "haplo-supressor/triplo-enhancer" behavior of certain key components of heterochromatin, some models have postulated that heterochromatic-promoting factors are present in limiting amounts and spreading continues until components are exhausted (Locke *et al.*, 1988; Zuckerkandl, 1974). Alternatively, a "boundary model" was proposed in which discrete sites promoted initiation and termination of heterochromatin (Tartof *et al.*, 1984), but discrete sites conferring these activities could not be identified. Recently, the examination of PEV in *JIL-1* mutants in *Drosophila* has revealed a role for H3S10 phosphorylation in antagonizing heterochromatic spreading that suggests both models may apply in different genomic contexts (Bao *et al.*, 2007b; Ebert *et al.*, 2004; Lerach *et al.*, 2006; Zhang *et al.*, 2006). The JIL-1 histone H3S10 tandem kinase localizes specifically to euchromatic interband regions of polytene chromosomes (Jin *et al.*, 1999; Wang *et al.*, 2001) and loss of JIL-1 results in an array of chromosomal defects, including disruption of banded regions that normally do not contain JIL-1 (Deng *et al.*, 2005). Alterations in band morphology in these mutants is accompanied by extensive spreading of the major heterochromatin markers H3K9me2 and HP1 to ectopic locations on the chromosome arms with the most pronounced increase on the X chromosomes (Zhang *et al.*, 2006). As would be expected, if *JIL-1* is required to "mark" euchromatic regions and terminate heterochromatic spreading, when a *white* reporter transgene is inserted into pericentric heterochromatin *JIL-1* loss-of-function alleles act as enhancers of variegation (i.e., the *white* gene is silenced; Bao *et al.*, 2007b). However, these very same *JIL-1* loss-of-function alleles act as *suppressors* of variegation for the $w^{m4}$ inversion chromosome (Lerach *et al.*, 2006). In this case, the redistribution of the major heterochromatin markers to ectopic locations on the chromosome arms in *JIL-1* loss-of-function mutants decreases the concentration of these components at the centromere, and the increased expression of the *white* gene reflects the reduced extent of pericentromeric silencing (Lerach *et al.*, 2006) (Fig. 1.5). A gain-of-function *JIL-1* allele *JIL-1*$^{Su(var)3-1}$, however, is able to mark the euchromatin and prevent silencing in both instances (Bao *et al.*, 2007b; Ebert *et al.*, 2004). JIL-1$^{Su(var)3-1[3]}$ is a C-terminal truncation that retains its ability to phosphorylate H3S10 but is mislocalized, showing a broad distribution on polytene chromosomes
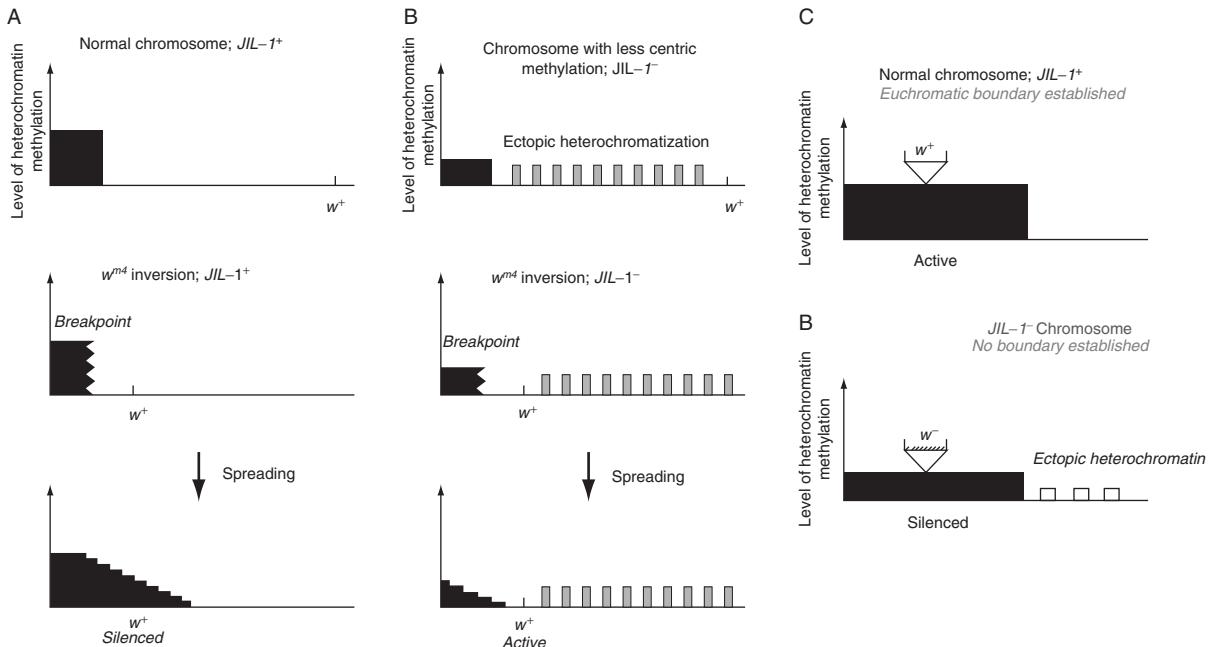
**Figure 1.5.** Three diagrams illustrating position-effect variegation (PEV) inactivation of genes by heterochromatic spreading. (A) In a genome with normal amounts of JIL-1 protein, heterochromatic spreading from the $w^{m4}$ inversion breakpoint often reaches the *white* gene; the gene is heterochromatized and is silenced. (B) In a genotype with significantly reduced JIL-1 levels, ectopic heterochromatization is widespread, resulting in lower levels of heterochromatic factors at the centromere. Therefore, heterochromatic spreading does not extend as far into the euchromatin and usually does not reach the *white* gene, leaving the gene euchromatic and active. (C) If a P element inserts a copy of the *white* gene into heterochromatin in a genotype where JIL-1 is present, JIL-1 often establishes a euchromatic chromatin structure at the *white* gene, the gene is protected from becoming heterochromatized, and the gene remains active. If the same insert is in a genome where JIL-1 is not present, the *white*

(Ebert *et al.*, 2004; Zhang *et al.*, 2006). Ectopic H3S10ph appears to restrict the formation of heterochromatin, both at the $w^{m4}$ inversion breakpoint and in the pericentric transgenes, resulting in a dominant suppression of variegation (Bao *et al.*, 2007b; Ebert *et al.*, 2004). Recently, H3S10 phosphorylation has been shown to displace HP1 binding to methylated H3K9 during mitosis (Fischle *et al.*, 2005; Hirota *et al.*, 2005). Likewise, JIL-1 kinase phosphorylation of H3S10 may displace or prevent HP1 binding, thereby terminating heterochromatic spreading at that site in order to establish a euchromatic domain (Johansen and Johansen, 2006). Consistent with this model, the $JIL\text{-}1^{Su(var)3-1}$ C-terminal truncation alleles are dominant over the triplo-enhancer effects of $Su(var)3$–$9$ and $HP1$ (Ebert *et al.*, 2004).

A second mechanism that operates to restrict heterochromatic spreading is the incorporation of the histone variant H3.3 into actively transcribed regions (Ahmad and Henikoff, 2002; Schwartz and Ahmad, 2005). Genome-wide profiling of histone H3.3 replacement patterns revealed that H3.3 is also enriched far upstream and downstream of active genes, but it was suggested this may reflect intergenic transcription that is now known to be widespread (Mito *et al.*, 2005). Recently, GAGA factor was found to interact with FACT (*fa*cilitates *c*hromatin *t*ranscription) complex to direct H3.3 replacement (Nakayama *et al.*, 2007). GAGA factor had originally been identified as the enhancer of variegation E(var)3–93D (Dorn *et al.*, 1993) and in this recent study, mutation in either GAGA factor or in DRE4/spt16, a subunit of FACT, enhanced $w^{m4}$ PEV (Nakayama *et al.*, 2007). Furthermore, the GAGA factor–FACT complex occupies a site where H3K4 methylation peaks and H3K9 methylation dips, suggesting that the remodeling activity of GAGA–FACT serves a barrier function against heterochromatic spreading and silencing (Nakayama *et al.*, 2007). The absence of such a barrier may result in expanded heterochromatic silencing, thus titrating heterochromatic factors away from the pericentromeric region resulting in an e(var) phenotype in the GAGA mutant.

## B. Nuclear organization

The phenomenon of $bw^D$ PEV in which chromosomal pairing brings a functional *bw* allele into a "silencing compartment" underscores the impact of nuclear organization on gene expression (Csink and Hennikoff, 1996; Csink *et al.*, 2002;

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gene is not protected. The establishment of ectopic heterochromatic sites throughout the chromosomes lowers the level of heterochromatic factors in the pericentric region. However, the basal level of pericentric heterochromatin, maintained by the RNAi system that generates heterochromatin, is sufficient to spread into the inserted *white* gene, the gene becomes heterochromatized, and is silenced.

Dernburg *et al.*, 1996). The functional consequences of long-range nuclear reorganization on gene expression were examined by FISH for three known variegating genes located on different chromosomes and in each case a strong correlation was observed between silencing and association with centromeric satellite sequences (Harmon and Sedat, 2005). Thus, changes in nuclear chromosomal organization have clear consequences on gene expression. What establishes the organization of the nucleus, however, is still not well understood (Jackson, 2003; Lanctôt *et al.*, 2007; Misteli, 2007).

Lamins are the best characterized nuclear architectural components to date and their role in chromosomal organization and gene expression is increasingly appreciated (reviewed in Hutchison, 2002; Goldman *et al.*, 2005). Association of genes with the nuclear lamina is generally linked to gene silencing (Andrulis *et al.*, 1998; Taddei and Gasser, 2004), although in some cases interaction with nuclear pore complex components is associated with transcriptional activation (Casolari *et al.*, 2004; Ishii *et al.*, 2002; Mendjan *et al.*, 2006). In a recent study, Pickersgill *et al.* (2006) identified ~500 genes that interact with lamin $Dm_0$ (the *Drosophila* B-type lamin) and found that they are transcriptionally repressed. Lamin-bound genes tend to be clustered in the genome, developmentally coregulated, have long intergenic domains, and lack active histone marks, suggesting that these regions likely comprise compacted chromatin (Pickersgill *et al.*, 2006). Consistent with lamins playing a role in epigenetic regulation of gene expression, a lamin $Dm_0$ C-terminal truncation mutant *Lam^{Ari3}* modified the PEV of *w^{m4}* (Bao *et al.*, 2007a). However, this allele shows an *enhancer* of variegation effect indicating *increased* silencing when the C-terminus is deleted, indicating that the role of lamin B in organizing chromatin domains is complex. Although interaction with lamin B is associated with gene silencing (Pickersgill *et al.*, 2006), the PEV studies suggest that lamin B's C-terminus may play a role in reversing silencing effects (Bao *et al.*, 2007a).

Interaction between nuclear lamins and chromatin is thought to regulate higher order chromatin organization and a direct physical interaction between lamin $Dm_0$ and histone H2A has been reported (Mattout *et al.*, 2006; Rzepecki *et al.*, 1998). In addition, different chromatin-organizing proteins have been found to interact with nuclear lamins and nuclear envelope proteins and the nature of these activities may facilitate the complex integration of various signaling pathways to influence genome organization and gene regulation (Gruenbaum *et al.*, 2005). Signaling to the chromatin may be mediated at several different levels besides physical association to the lamina. For example, the nuclear envelope protein LAP2$\beta$ recruits a histone deacetyltransferase to the periphery, thus triggering silencing histone modifications in this domain (Somech *et al.*, 2005).

The nuclear lamina has also been found to bind the ubiquitin ligase dTopors (Topoisomerase-I-interacting protein), which functions in concert with proteins that bind to a chromatin regulatory domain known as an insulator or boundary element (Capelson and Corces, 2005; Wei *et al.*, 2005; West *et al.*, 2002). Insulators block enhancer interactions (Geyer and Corces, 1992; Kellum and Schedl, 1992) and block the spreading of one chromatin state into another (Sun and Elgin, 1999). The *gypsy* insulator sequence was originally identified as a *gypsy* retrotransposon insertion that blocked enhancer function at the *yellow* locus by introducing a Su(Hw) (suppressor of Hairy wing)-binding site (Geyer and Corces, 1992; Parkhurst *et al.*, 1988; Roseman *et al.*, 1993). The Su(Hw) protein binds to BTB/POZ-domain proteins (Broad complex *T*ramtrack, *B*ric-abrac/*P*oxvirus and Zinc finger), CP190 (Pai *et al.*, 2004) and mod/mdg4 (Gerasimova *et al.*, 1995; Ghosh *et al.*, 2001) to form a complex with dTopors at the nuclear periphery that is thought to isolate regions of DNA in "insulator bodies" (Capelson and Corces, 2004; Gerasimova *et al.*, 2000). The presence of *gypsy* insulator sequences at endogenous sites in the genome suggests that this may provide a means to compartmentalize the genome and prevent heterochromatic spreading into active euchromatic regions (Parnell *et al.*, 2006; Ramos *et al.*, 2006). Consistent with a role in limiting heterochromatic spreading, *mod/mdg4* was originally identified as the enhancer of variegation *E(var)3–93D* (Dorn *et al.*, 1993).

Because there are significantly more chromosomal-binding sites for Su(Hw) and mod/mdg4 than genomic consensus Su(Hw) DNA-binding sites (Parnell *et al.*, 2006; Ramos *et al.*, 2006), it is not known whether the PEV modifier effects observed in the *mod/mdg4* mutant reflect activities related directly to *gypsy* insulator function, activities at other as-yet uncharacterized chromosomal sites, or is the indirect consequence of the nuclear reorganization that has been found to occur when *gypsy* insulator components are disrupted (Gerasimova *et al.*, 2000; Pai *et al.*, 2004). One plausible scenario is that if the *mod/mdg4* mutation compromises the insulator's barrier function to heterochromatic spreading, the resultant increased euchromatic distribution of heterochromatic factors reduces their levels at the pericentromeric regions resulting in decreased spreading at the $w^{m4}$ inversion and an E(var) phenotype, much as occurs for JIL-1 (Bao *et al.*, 2007b). Likewise, proper insulator function also depends on the presence of functional RNAi-mediated pathways, perhaps to ensure the generation of heterochromatin and thus the appropriate sequestration of heterochromatic components at centromeric regions. When *Argonaute* RNAi pathway genes are mutated, nuclear architecture is disrupted and insulator activity is decreased (Lei and Corces, 2006). Furthermore, CP190 interacts with Rm62/Lip (Lei and Corces, 2006), a DEAD-box helicase that has also been identified as a modifier of PEV and is required for dsRNA-mediated silencing, heterochromatin formation, and transposon silencing (Csink *et al.*, 1994; Ishizuka *et al.*, 2002). These

results underscore the relationship between genome organization/chromatin packaging of euchromatic sites and the levels of heterochromatic factors that are available to spread at pericentromeric sites (i.e., the inversion breakpoint).

Another nuclear domain besides the periphery that has been associated with silencing is the nucleolus (Lawrence and Pikaard, 2004; Lewis and Pikaard, 2001; Wang *et al.*, 2005; Yasufzi *et al.*, 2004) and chromosomal inversions that place the *white* gene adjacent to the nucleolar organizer region (NOR) exhibit PEV consistent with different levels of gene silencing (Spofford and DeSalle, 1991). Recently, it was found that heterochromatin-mediated silencing pathways including the RNAi pathway, HP1, and H3K9 methylation regulate nucleolar organization and structural integrity (Peng and Karpen, 2006). In most of the RNAi pathway mutants examined as well as in *Su(var)2–5/HP1* and *Su(var)3–9* mutants, heterochromatic repeated DNAs became dispersed and multiple, ectopic nucleoli were formed. In addition, significant amounts of extrachromosomal circles (ecc) of rDNA accumulated (Peng and Karpen, 2006). It was proposed that loss of heterochromatization in the NOR correlates with increased recombination or repair rates in this repetitive sequence region with subsequent accumulation of ecc rDNAs when errors occurred (Peng and Karpen, 2006). In support of this idea, mutation in *Ligase4*, a DNA repair enzyme, suppressed ecc generation (Peng and Karpen, 2006). A connection between DNA synthesis/repair and heterochromatin behavior has been inferred by a number of studies showing that mutations in different factors involved in these processes act as PEV modifiers (Table 1.1). Another mutation that disrupts nucleolar and chromosomal organization in *Drosophila*, *Su(var)2–10/dPIAS* (Hari *et al.*, 2001), also was originally identified as a modifier of PEV (Reuter and Wolff, 1981). Interestingly, a correlation between deregulation of chromatin-silencing and disruption of nuclear organization has also been observed in yeast (Teixeira *et al.*, 2002), suggesting that the link between heterochromatin behavior and nuclear organization is a general feature of eukaryotes.

# V. CONCLUDING REMARKS

Studies of the control of gene regulation that began with the discovery of PEV and the chromosomal conditions that cause it have brought us a long way toward understanding the role of chromatin structure in the regulation of gene expression. Studies of chromosome rearrangements and P element insertions demonstrated that a change in chromatin structure from euchromatic to heterochromatic leads to gene silencing and that this silencing effect has specific characteristics that are amenable to analysis. The identification of mutations that modify the PEV phenotype identified a number of factors that are part of or

regulate the formation or maintenance of heterochromatin and euchromatin. The findings of these studies indicate that control of gene expression is a complex process involving regulatory mechanisms that are integrated at multiple hierarchical levels ranging from the primary DNA sequence to the chromatin packaging of DNA and higher order chromosomal folding to the three-dimensional spatial organization of chromosomes within the nucleus (reviewed in van Driel et al., 2003; Misteli, 2007). A key component of this process may be the modification of histone N-terminal tails to "mark" a region of chromatin for the activation of a number of specific downstream responses (Fischle et al., 2003; Kouzarides, 2007; Strahl and Allis, 2000; Turner, 2002) such as the establishment of transcriptionally active chromatin (Brownell and Allis, 1996; Csordas, 1990; Turner, 2000) or higher level folding associated with silencing (Turner, 2002; Zhang and Reinberg, 2001). However, regulation of gene expression at the level of the nucleosome goes beyond the modification of histone tails. Genes that are actively transcribed are often subject to nucleosomal remodeling by chromatin-remodeling complexes (Becker and Horz, 2002; Workman and Kingston, 1998) and characterized by nucleosome-free or "DNase-hypersensitive" sites, whereas silenced or heterochromatic regions are characterized by tightly packed, ordered nucleosomal arrays (Elgin, 1988; Wallrath and Elgin, 1995). Besides the local remodeling of a small number of nucleosomes in the promoter region, larger scale nucleosomal remodeling is also implicated in the unfolding of large chromatin domains (Dietzel et al., 2004; Neely and Workman, 2002; Peterson, 2003; Tumbar et al., 1999). Current models suggest that within the nucleus, there exist regions of condensed silent chromatin interspersed with regions of decondensed, active chromatin. A clear example of this is found within the band-interband pattern observed in Drosophila larval polytene chromosomes (Labrador and Corces, 2002). The challenge is to identify the molecules and molecular mechanisms that determine how this organization is established and maintained and the signal transduction events that regulate this process.

A constructive way to think about heterochromatin is in quantitative terms. The Drosophila genome contains a finite number of repeated sequences that interact with the RNAi system to utilize a fixed quantity of heterochromatic factors that can bind to and heterochromatize a set length of chromatin. The concentration of these factors is normally high in the pericentric chromosomal regions, which serve as a reservoir for heterochromatic components. The amount of chromatin that is heterochromatic during interphase is a dynamic balance between the number of heterochromatic factors and the number of chromatin sites available for binding. Anything that increases the number of sites or decreases the number of factors will decrease the pericentric concentration and anything that decreases the number of sites or increases the number of factors will increase the pericentric concentration. Consider three types of

Su(var) phenotypes. (1) The addition of Y chromosomes or heterochromatic regions increases the sequences competing to bind heterochromatic factors. This increased competition lowers the concentration of heterochromatic factors in the pericentric heterochromatin. (2) Mutations that reduce the dose of genes that code for structural components of heterochromatin (e.g., HP1) also reduce the quantity of heterochromatic factors in pericentric regions. (3) JIL-1 proteins establish euchromatic regions that are resistant to heterochromatic factor binding. In loss-of-function JIL-1 mutants, ectopic-binding sites for heterochromatic factors are established throughout the genome that compete for heterochromatic factors. This reduces the concentration of heterochromatic factors in pericentric regions. In all three cases, the lower level of heterochromatic factors in pericentric regions means that heterochromatin cannot spread as far into euchromatin from a breakpoint and adjacent genes are not inactivated as often, resulting in a Su(var) phenotype. E(var) phenotypes result from mutations that increase the concentration of heterochromatic factors in the pericentric regions, leading to increased lengths of spreading from breakpoints.

The details of a number of the most interesting aspects of PEV remain to be explained. How does the regulation of genes normally residing in heterochromatic regions differ from that of genes residing in euchromatic regions? How does the PEV of genes inserted into telomeres by P element transposition differ from that of those inserted into pericentric regions? What controls nuclear compartmentalization and how does this normally act to control gene regulation? These and other questions are currently being actively investigated in a continuation of an examination that began more than 75 years ago with the observation of a novel color of the eyes of a fly.

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