Comparative Analysis of Position-Effect Variegation Mutations in Drosophila melanogaster Delineates the Targets of Modifiers

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ABSTRACT

In Drosophila melanogaster, heterochromatin-induced silencing or position–effect variegation (PEV) of a reporter gene has provided insights into the properties of heterochromatin. Class I modifiers suppress PEV, and class II modifiers enhance PEV when the modifier gene is present in fewer than two doses. We have examined the effects of both class I and class II modifiers on four PEV mutations. These mutations include the inversions In(1)w123 and In(2R)bw100, which are classical chromosomal rearrangements that typify PEV mutations. The other mutations are a derivative of brown (roman), in which brown reporters are inactivated by a large block of heterochromatin, and a P[white] transposon insertion associated with second chromosome heterochromatin. In general, we find that class I modifiers affect both classical and nonclassical PEV mutations, whereas class II modifiers affect only classical PEV mutations. We suggest that class II modifiers affect chromatin architecture in the vicinity of reporter genes, and only class I modifiers identify proteins that are potentially involved in heterochromatin formation or maintenance. In addition, our observations support a model in which there are different constraints on the process of heterochromatin-induced silencing in classical vs. nonclassical PEV mutations.

Gene expression depends on both intrinsic regulatory mechanisms, including enhancer–promoter interactions, and chromosomal context, including chromatin structure. Whereas intrinsic regulatory mechanisms are well defined molecularly, chromosomal context is difficult to assess and is sometimes revealed only by gene-silencing phenomena. Examples of gene silencing include X-chromosome inactivation and parental imprinting in mammals, telomere and mating-type silencing in yeast, as well as heterochromatin-induced gene silencing known as position–effect variegation (PEV) in Drosophila melanogaster (for review see Hendrich and Willard 1995). In PEV, chromosomal rearrangements that change the position of a gene so that it is placed near heterochromatin result in the variable expression of the gene. In contrast to gene-rich euchromatin, heterochromatin has comparatively few genes, remains condensed throughout the cell cycle, and is enriched in satellite and middle repetitive DNA sequences (reviewed in Csink et al. 1997). Although gene inactivation is a direct consequence of relocation to heterochromatin, it is not a normal function of heterochromatin, as evidenced by the presence of expressed genes in heterochromatin (Gatti and Pimpinelli 1992). Nevertheless, the ability to inactivate a gene is presumed to reflect an intrinsic difference between heterochromatin and euchromatin with respect to chromatin structure and gene expression.

Genic modifiers of PEV have been readily recovered in different screens (Dorn et al. 1993b; Locke et al. 1988; Sinclair et al. 1989, 1992; Wustmann et al. 1989) and have been considered a valuable tool in the study of heterochromatin. These modifiers have been categorized into two classes (Locke et al. 1988). Class I modifiers act to suppress PEV when only one dose of the wild-type gene is present and may enhance variegation in three doses. In genetic screens, class I modifiers were recovered as Suppressors of variegation [Su(var)s], which are gene mutations or deficiencies, and as chromosomal duplications that acted as Enhancers of variegation [E(var)s]. Because suppression of a PEV phenotype indicates a reduction in the ability of heterochromatin to silence a gene, Su(var)s were predicted to be mutations in genes that code for either structural components of heterochromatin or proteins that regulate heterochromatin components (Locke et al. 1988). Class II modifiers, isolated as mutations in E(var) genes, enhance variegation in one dose and may suppress in three doses. Therefore, E(var) genes might code for proteins that antagonize the silencing potential of heterochromatin or promote the formation of euchromatin (Locke et al. 1988).

More than 100 dosage-dependent modifiers of PEV have been identified (Locke et al. 1988; Reuter and Spierer 1992; Wustmann et al. 1989). Most of these
were identified by phenotypic suppression or enhancement of the chromosomal rearrangement In(1)wm4 (wm4). This inversion positions the white (w+) gene within 25 kb of pericentric heterochromatin, and the resulting mosaic inactivation of w+ produces patches of white tissue in a normally red eye (Tartof et al. 1984). The enhancement or suppression of this phenotype can be readily scored as more or less white tissue, respectively, in a genetic screen. Modifiers of PEV isolated using wm4 have been tested with other PEV mutations, such as the chromosomal rearrangements In(2R)bwDDe2, In(1)yβ, and T(2;3)Sb which affect the brown, yellow, and Stubble genes, respectively. Modifiers isolated using wm4 typically behave similarly with these other PEV mutations.

A different response to genic modifiers was reported for PEV associated with the brown dominant (bwD) mutation. bwD contains a large heterochromatic insertion into the coding region of the brown+ (bw+) gene, generating a null allele (Henikoff et al. 1993). The heterochromatin of the bwD insertion is able to silence a wild-type copy of the bw+ gene that is present on the homolog. This is referred to as trans-inactivation, and such dominant PEV is characteristic of all variegating alleles of brown. A genetic screen designed to discover dominant modifiers of bwD identified unlinked mutations that suppressed trans-inactivation but failed to detect comparable enhancer mutations (Talbert et al. 1994). A collection of these suppressors of bwD were tested for their effect on wm4, and 33 out of 37 were found to be typical class I modifiers [i.e., Su(var) mutations]. The notable absence of enhancers of bwD was not caused by an inability to detect enhancement of the phenotype because two dominant enhancer mutations, both involving a rearrangement of the bwD chromosome, were identified. In addition, the bwD mutation is enhanced in males that lack a Y chromosome (P. Talbert, personal communication), indicating that bwD responds to this modifier in a manner that is consistent with other PEV mutations (Spofford 1976). These observations suggest that classical PEV mutations (i.e., gross chromosomal rearrangements such as wm4) and nonclassical PEV mutations such as bwD exhibit differential responses to PEV modifiers, specifically to class II modifiers.

Failure to recover class II modifiers (i.e., E(var) mutations) in the collection of modifiers of bwD could be explained if the linkage enhancers of bwD were exceptionally strong, and therefore weaker effects would have been undetected. Alternatively, the lack of enhancers may be indicative of a bwD-specific property. For example, the bwD heterochromatin insertion is composed primarily of the simple sequence satellite (AAGAG)n (Csink and Henikoff 1996), which is present in heterochromatin (Lohe et al. 1993), and bound by GAGA protein in early embryos (Raff et al. 1994). This raises the possibility that the class II modifier Trithorax-like (Trl), which codes for the GAGA protein (Farkas et al. 1994), would modify the phenotype of bwD, but other modifiers would not. To differentiate between these and other possibilities, we examined a collection of PEV modifiers for effects on a panel of both classical and nonclassical PEV mutations. Our results indicate that insensitivity to class II modifiers is not restricted to the bwD mutation, but extends to two nonclassical PEV mutations, a derivative of bwD and a variegating w+ transgene insertion into heterochromatin. We rationalize these findings in terms of differences in targets of action between class I and class II modifiers. Additionally, our observations point to potentially different topological causes of heterochromatin-induced silencing in classical vs. nonclassical PEV mutations.

MATERIALS AND METHODS

Fly stocks: Flies were reared on standard cornmeal-molasses medium in vials or bottles at a constant temperature of 25°. Four PEV mutations were used in these experiments (Figure 1). The isolation and characterization of the Byron mutation has been described (Henikoff et al. 1995). P[wAR]B133-0923 (referred to in this paper as P[wA]B133) is a w+ transgene insertion in or near chromosome 2R heterochromatin from R. Levis, Syracuse University, NY. The collection of genic modifiers of PEV used in this study include both molecularly characterized mutations (ref-
TABLE 1  
Molecularly characterized modifiers of PEV

<table>
<thead>
<tr>
<th>Modifier</th>
<th>Protein product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Su(var)2-5</td>
<td>Heterochromatin protein 1, HP1, associates with heterochromatin and contains a chromodomain</td>
<td>Eisenberg et al. 1990</td>
</tr>
<tr>
<td>Su(var)3-6</td>
<td>Protein phosphatase 1, catalytic subunit</td>
<td>Baks et al. 1993</td>
</tr>
<tr>
<td>Su(var)3-7</td>
<td>Protein contains seven widely spaced zinc fingers</td>
<td>Cleard et al. 1995</td>
</tr>
<tr>
<td>Su(var)3-9</td>
<td>Protein contains chromodomain and trx homology</td>
<td>Tschierch et al. 1994</td>
</tr>
<tr>
<td>Class II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E(var)3-93D</td>
<td>Protein contains BTB domain and associates with polytene chromosomes</td>
<td>Dorn et al. 1993a; Dorn et al. 1994</td>
</tr>
<tr>
<td>E2F</td>
<td>Transcriptional activator, involved in cell cycle regulation</td>
<td>Seum et al. 1996</td>
</tr>
<tr>
<td>Tri</td>
<td>GAGA transcriptional activator</td>
<td>Farkas et al. 1994</td>
</tr>
</tbody>
</table>

RESULTS

Analysis of class I modifiers: We examined the effects of four molecularly characterized class I modifiers (listed in Table 1) on our collection of PEV mutations. As expected, all class I loss-of-function mutations suppressed and the class I duplication [E(var)39A] enhanced the variegation seen in w^m (Table 2, column 1; Figure 2, column 1). Similar results were obtained with bw^D (Table 2, column 2; Figure 2, column 2). Class I modifiers likewise affect variegation of the nonclassical PEV mutations Byron and P[wA^1]B133 (Table 2; Figure...
2, columns 3 and 4, respectively). However, one class I modifier, Su(var)3-9, did not suppress Byron and P[w^AR]B133 (Table 2, columns 3 and 4). A second allele, Su(var)3-905, similarly failed to suppress these nonclassical PEV mutations (data not shown).

### Analysis of class II modifiers:

Three class II modifiers that have been molecularly characterized (listed in Table 1) were also tested with our collection of PEV mutations. As previously described, w^md was significantly enhanced by all class II modifiers (Table 2, column 4). However, the presence of a putative null allele of Trl (Trl^R85) or the revertant allele (Trl^R4) had no consequence for the Byron phenotype. The two P element insertion alleles of Trl (Trl^13C and Trl^62), considered to be hypomorphic mutations, were also examined. The Trl^62 allele did not show enhancement of the Byron phenotype. Although the distribution of individuals into phenotypic rankings was significantly different in the presence of Trl^62, it was in a direction that indicates suppression, not enhancement. Trl^13C had an inconsistent effect in that males appeared to be enhanced using the rank distribution assay, whereas females were not affected. Enhancement in Trl^13C males was too weak to be detected using pigment assays (data not shown). Because we can detect background enhancement of w^md and bw^VDe2 by a revertant of Trl^13C, the weak enhancement attributable to this chromosome is suspect. Thus, we conclude that class II modifiers do not enhance the phenotype of Byron.

P[w^AR]B133 was also examined with the class II modifiers (Table 2, column 4). E(var)3-93D and E2F had no effect (Figure 2, column 4). The Trl^62 allele had no significant effect either; however, the number of P[w^AR]B133 individuals exhibiting an enhanced phenotype was increased in the presence of the null (Trl^R85), the hypomorphic (Trl^13C), and the revertant (Trl^R4) alleles. The observed enhancement of P[w^AR]B133 by the

### Table 2

**Effect of molecularly characterized modifiers**

<table>
<thead>
<tr>
<th>Modifier</th>
<th>w^md</th>
<th>bw^VDe2</th>
<th>Byron</th>
<th>P[w^AR]B133</th>
</tr>
</thead>
<tbody>
<tr>
<td>Su(var)205^02</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Dp(2;2)E39A</td>
<td>E</td>
<td>E</td>
<td>E</td>
<td>E</td>
</tr>
<tr>
<td>(&lt;0.0001)</td>
<td>(&lt;0.0001/ 0.0001)</td>
<td>(0.001/ 0.005)</td>
<td>(0.01)</td>
<td></td>
</tr>
<tr>
<td>Su(var)3-6^01</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Su(var)3-7</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Su(var)3-9^01</td>
<td>S</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.5/ 0.7)</td>
<td>(0.02E)</td>
<td></td>
</tr>
<tr>
<td>E(var)3-93D</td>
<td>E</td>
<td>E</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>(&lt;0.0001)</td>
<td>(0.006/ 0.04)</td>
<td>(0.1/ 0.2; 0.4/ 0.005S)</td>
<td>(0.5)</td>
<td></td>
</tr>
<tr>
<td>Trl^R85</td>
<td>E</td>
<td>E</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>(&lt;0.0001)</td>
<td>(0.001/ &lt;0.0001)</td>
<td>(0.5/ 0.6; 0.6/ 0.9)</td>
<td>(0.01)</td>
<td></td>
</tr>
<tr>
<td>Trl^13C</td>
<td>E</td>
<td>E</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>(&lt;0.0001)</td>
<td>(0.0004/ &lt;0.0001)</td>
<td>(-0.0001/ 0.6; 0.4/ 0.6)</td>
<td>(0.04)</td>
<td></td>
</tr>
<tr>
<td>Trl^62</td>
<td>E</td>
<td>E</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>(&lt;0.0001)</td>
<td>(0.001/ 0.02)</td>
<td>(0.065/ &lt;0.0001S; 0.075/ 0.3)</td>
<td>(0.05)</td>
<td></td>
</tr>
<tr>
<td>Trl^R4</td>
<td>E</td>
<td>E</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>(&lt;0.0001)</td>
<td>(0.05/ 0.01)</td>
<td>(1/ 0.7)</td>
<td>(0.003)</td>
<td></td>
</tr>
<tr>
<td>E2F^91</td>
<td>E</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.6/ 0.3)</td>
<td>(1/ 0.4)</td>
<td>(0.07S)</td>
</tr>
</tbody>
</table>

For Su(var)3-7, Df(3R)Ace/H1 was used (Reuter et al. 1987). S and E, suppression and enhancement, respectively. X, lack of expected effect. P values are shown in parentheses (male for w^md and P[w^AR]B133; male/ female for bw^VDe2 and Byron).
revertant allele TrlR4 is in keeping with the background enhancement that was detected for the classical PEV mutations. The fact that the independently derived Trl62 allele (i.e., with a different genetic background) does not cause enhancement indicates that the nonclassical PEV mutation P[wAR]B133 is not affected by Trl mutations. Thus, the class II modifiers used in this study have no effect on P[wAR]B133.

**Analysis of additional modifiers of unknown class:** A collection of 11 molecularly uncharacterized modifier mutations was used to further assess the effect of modifiers of PEV on the nonclassical PEV mutation Byron (Table 3). Seven of these modifiers were also tested with P[wAR]B133. Mutations in the putative class I modifier Su(var)2-1 result in hyperacetylation of histone H4 (Dorn et al. 1986). This mutation strongly suppresses both Byron and P[wAR]B133. Similarly, Su(var)208 strongly suppresses both nonclassical PEV mutations. Of nine E(var) mutations tested, six were without significant effect on the Byron phenotype (Table 3, column 1). The remaining three E(var) mutations had no effect on males, but they did enhance females. Five of the E(var) mutations were also tested on P[wAR]B133 (Table 3, column 2), and four had no effect. Our analysis of molecularly uncharacterized modifiers extends our observations made with the class I and class II modifiers: class I modifiers affect all PEV mutations, whereas class II modifiers affect classical PEV mutations but not nonclassical PEV mutations.

**DISCUSSION**

The comparative analysis of PEV modifiers presented here used a method that allowed detection of significant modification. Our results indicate a difference in susceptibility to modification between nonclassical and classical PEV mutations. As demonstrated in the studies of others, classical PEV mutations were sensitive to both class I and class II modifiers. In general, the nonclassical PEV mutations examined in this study
show the expected response to class I modifiers, but not to class II modifiers. This was true regardless of the dosage characteristics of the modifiers tested because both haplo-dependent and haplo-, triplo-dependent modifiers of a class behaved similarly. Our results confirm and extend inferences from previous studies that class II modifiers have no effect on nonclassical PEV mutations such as \( bw^0 \) (Talbert et al. 1994) or transgene insertions into heterochromatin (unpublished results cited in Wallrath and Elgin 1995).

Our results demonstrate that class I modifiers have a general role in heterochromatin-induced silencing in that they affect both classical and nonclassical PEV mutations. This is as expected if the target of class I modifier effects is heterochromatin. In contrast, class II modifiers only affect classical PEV mutations. One explanation for this observation is that the insensitivity of the nonclassical PEV mutations Byron and \( P[wA^R]B133 \) reflects an unusual composition of heterochromatin responsible for silencing. Compositional differences have been speculated to underlie differential responses to modifiers among classical PEV mutations (Bishop 1992; Lloyd et al. 1997). However, most classical PEV mutations respond to class II modifiers even though these mutations represent diverse heterochromatin breakpoints. Occasional failure of a classical PEV mutation to respond to a modifier does not extend to the class as a whole, as is the case for Byron and \( P[wA^R]B133 \). Furthermore, we do not think that the nonclassical PEV mutations used in our study are unusual because failure to respond to Trl mutations has been noted for multiple variegating transgene inserts (unpublished results cited in Wallrath and Elgin 1995).

Alternatively, the observed differences between classical and nonclassical PEV mutations in their response to class II modifiers may reflect differences in the mechanism of reporter gene inactivation. In the case of the \( w^+ \) reporter gene in \( w^{cn} \), the proximity of heterochromatin may create a competition between open and closed chromatin at \( w^+ \). Competition models for reporter gene inactivation have been previously described (Aparicio and Gottschling 1994; Elgin 1996). The outcome of competition can be seen as the variegated phenotype. Modifiers that alter the balance between maintaining an active transcriptional complex and forming heterochromatin will alter the phenotype. A common theme of class II modifiers appears to be the potential for involvement in chromatin architecture (De Robertis et al. 1996; Dorn et al. 1993a; Eberl et al. 1997; Farkas et al. 1994; Seum et al. 1996). Mutations that affect chromatin architecture would result in enhancement by decreasing the probability that an active transcriptional complex is formed at \( w^+ \), which in turn increases the probability that \( w^+ \) will be inactivated by heterochromatin. Thus, any modifier that affects the competition between maintaining an active transcriptional state and a heterochromatin-induced inactive state will affect the phenotype of \( w^{cn} \).

The same model would apply to the \( bw^+ \) reporter gene that is found in \( w^{cn} \) to the breakpoint of the inversion \( bw^{VDe} \). As is the case for all \( bw \) variegating mutations, \( bw^{VDe} \) is dominant and causes trans-inactivation of the \( bw \) gene on the wild-type homolog. Trans-inactivation is also sensitive to the effects of class II modifiers, as evidenced by the enhanced phenotype of \( bw^{VDe} \). Because homologous chromosomes are paired in Dipters (Metz 1916), the resulting formation of an inversion loop would bring the trans copy of the \( bw^+ \) reporter closer to a putative heterochromatic compartment (Figure 3). Proximity to a heterochromatic compartment is responsible for the susceptibility of the trans copy of the \( bw^+ \) reporter to class II modifiers. There would be an unstable balance between the formation of an active transcription complex on the trans copy of \( bw \) and the influence of trans-inactivating heterochromatin. As in the case of \( w^{cn} \), modifiers that alter the balance between maintaining an active state and a silenced state will affect the phenotype.

The mechanism of reporter gene inactivation in nonclassical PEV mutants may be qualitatively different than that for classical PEV mutants (Figure 3). In the case of \( bw^0 \) (and its derivatives), the \( bw^+ \) reporter gene present on the homolog may be inactivated because it is mislocalized to a heterochromatic compartment of the nucleus (Csink and Henikoff 1996; Dernburg et al. 1996; Talbert et al. 1994). A correlation between nuclear mislocalization and the degree of \( bw \) inactivation in the eye suggests that a heterochromatic compartment is not conducive to \( bw \) transcription (Csink and Henikoff 1996). The effect of decreasing the dose of a gene that encodes a protein involved in establishing an open chromatin state would be negligible if reporter gene expression is already compromised by virtue of its mislocalization to a heterochromatic compartment. The
The dose of a class I or class II modifier can affect the competition between establishing an active transcriptional complex at the $bw^+$ reporter gene or inactivation caused by heterochromatin association. A reduction in the dose of a class I modifier decreases heterochromatic association and results in phenotypic suppression. Conversely, a reduction in the dose of a class II modifier decreases the probability of an active state and therefore increases heterochromatic association leading to an enhancement of the phenotype. In the case of the nonclassical PEV mutation $bw^D$ (or its derivative, Byron), somatic pairing of the homologs, combined with association between the heterochromatic insertion of $bw^D$ and second chromosome heterochromatin, results in mislocalization of a $bw^+$ reporter gene to a heterochromatic compartment. The shaded oval represents a heterochromatic compartment with silencing properties. The force of heterochromatic association predominates, and therefore the dosage of class II modifiers is without phenotypic consequence. As for classical PEV mutations, heterochromatic associations in nonclassical PEV mutations are altered by changes in the dose of class I modifiers. Reducing the dose of a class I modifier decreases heterochromatic association and results in phenotypic suppression.

The same insensitivity would be observed for a transgene insertion in heterochromatin. In the case of $P[\text{wa}]B133$, the $w^+$ reporter is mostly inactivated (as indicated by the extreme variegation) because it is sequestered within a heterochromatic compartment, and so dosage changes in chromatin architectural proteins would not be limiting for transcription.

There were exceptions to the above-described generalizations regarding modifier mutations. Three of the 12 $E(\text{var})$ mutations enhanced the phenotype of Byron females (but not males), and one of these also enhanced $P[wA]\text{B133}$. In these cases, we predict that the corresponding mutation leads to a gain of function of a class I modifier gene. Alternatively, enhancement might be caused by a mutation in a gene that encodes a product directly involved in the negative regulation of heterochromatin. Another exception, the class I modifier $\text{Su}(\text{var})3-9$ did not suppress either Byron or $P[wA]\text{B133}$. This is surprising given that $\text{Su}(\text{var})3-9$ was shown to suppress associations between $bw^D$ and heterochromatin in larval brains (Csink and Henikoff 1996). Because $\text{Su}(\text{var})3-9$ mutations are in a transcribed region that is primarily expressed in embryos (Tschiersch et al. 1994), the product might not be present during development of the eye when the $bw$ gene is expressed. Consistent with this explanation, we found that $\text{Su}(\text{var})3-9^{101}$ was a weak suppressor of classical PEV mutations (data not shown). Although $\text{Su}(\text{var})3-9$ was suggested to play an important role in heterochromatinization (Tschiersch et al. 1994), our results suggest that its primary target of action is not heterochromatin.

How do our results fit with other well-studied silencing phenomena? The potential mechanistic similarities between class I modifiers of PEV and proteins involved in repression of homeotic gene expression [Polycomb group (Pc-G) gene products] have been discussed frequently (Moehrle and Paro 1994; Pirrotta and Rastelli 1994; Reuter and Spierer 1992). The class II modifiers $\text{Trl}$ and $E(\text{var})3-93D$ are mutations in genes of the Trithorax group, genes that encode proteins needed for the appropriate activation of homeotic genes. This observation led investigators to conclude that this overlap was noteworthy (Dorn et al. 1993a; Farkas et al. 1994). Our results provide an explanation. If the class II modifier effects reflect reporter gene sensitivity, then the loss of chromatin architectural proteins (such as encoded by $\text{Trl}$ and $E(\text{var})3-93D$) may affect susceptible homeotic genes by a similar mechanism. That is, loss of $\text{Trl}$ or $E(\text{var})3-93D$ alters the balance of an open vs. closed chromatin state, resulting in the increased probability of the inappropriate formation of a Pc-G or a heterochromatin-mediated silencing complex.
The large collection of mutations that can modify PEV phenotypes has been interpreted as an indication that heterochromatin-induced silencing is an inherently complex phenomenon (Reuter and Spierer 1992). In contrast, our comparative analysis of classical and nonclassical PEV mutations supports a simpler picture of PEV. We suggest that the complexity of modifiers may reflect the number of ways it is possible to affect gene expression. Class II modifiers act at the level of reporter gene expression; therefore, modification of gene expression is not informative with respect to the properties of heterochromatin. If PEV is to be used as a tool in the analysis of heterochromatin, the identification of modifiers that act on both classical and nonclassical PEV mutations would be more informative. Such an approach would distinguish proteins that affect reporter gene expression, either directly or indirectly, from those involved in packaging heterochromatin.

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