Interactions Among Dosage-Dependent Trans-Acting Modifiers of Gene Expression and Position-Effect Variegation in Drosophila

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Abstract

We have investigated the effect of dosage-dependent trans-acting regulators of the white eye color gene in combinations to understand their interaction properties. The consequences of the interactions will aid in an understanding of aneuploid syndromes, position-effect variegation (PEV), quantitative traits, and dosage compensation, all of which are affected by dosage-dependent modifiers. Various combinations modulate two functionally related transcripts, white and scarlet, differently. The overall trend is that multiple modifiers are noncumulative or epistatic to each other. In some combinations, developmental transitions from larvae to pupae to adults act as a switch for whether the effect is positive or negative. With position-effect variegation, similar responses were found as with gene expression. The highly multigenic nature of dosage-sensitive modulation of both gene expression and PEV suggests that dosage effects can be progressively transduced through a series of steps in a hierarchical manner.

Variation in dosage of chromosomes or chromosomal segments results in alterations of gene expression throughout the nucleus (Birchler 1979, 1981; Birchler and Newton 1981; Devlin et al. 1982, 1988; Sabl and Birchler 1993; Guo and Birchler 1994). Any one gene can be modulated by several regions of the genome. The range of these changes generally falls within the limits of an inverse or direct correlation between the chromosomal dose and the level of target gene expression. That is, a reduction from two to one copy often causes a twofold increase in expression. An increase in dosage of the same region from two to three copies causes reductions to two-thirds of the normal level. With direct correlations, chromosomal reductions result in 50% of normal expression and increases cause elevations to 150%. Although both types of dosage effects are found, the inverse correlations are more prevalent (Birchler et al. 1994; Begley et al. 1995; Bhadra and Birchler 1996; Bhadra et al. 1997a,b; Frolov et al. 1998).

The dosage effects of some regions have been traced to single loci. Using the white eye color locus as a monitor, numerous second-site modifiers have been recovered that exhibit a dosage effect on white expression and that fall within the range seen with chromosomal aneuploids. A fraction of these have been described in the literature (Rabinow et al. 1991; Birchler et al. 1994; Bhadra and Birchler 1996; Bhadra et al. 1997a,b; Frolov et al. 1998).

A similar situation occurs for position-effect variegation (PEV). PEV is the mosaic expression of genes brought near abnormal junctions of euchromatin and heterochromatin. There are scores of modifiers of PEV that either suppress or enhance the degree of mosaic expression (for reviews, see Spofford 1976; Weiler and Wakimoto 1995; Elgin 1996; Henikoff 1996). Several of the genes initially identified as dosage modifiers of white are also suppressors of PEV (Birchler et al. 1994; Bhadra and Birchler 1996; Bhadra et al. 1997b; Frolov et al. 1998). The modification of gene expression and PEV appears to be related, and indeed several genes affecting PEV have been identified as transcriptional regulators of some type (Dorn et al. 1993; Farkas et al. 1994; Tschiersch et al. 1994; Seum et al. 1996; Frolov et al. 1998).

Despite the fact that several genes or segmental aneuploids produce a dosage effect on a single monitored target gene, larger aneuploids as a general rule do not exceed the limits of inverse and direct correlations of gene expression with the varied dosage (Devlin et al. 1982, 1988; Birchler 1992; Guo and Birchler 1994). Hence, we wished to determine whether the combinations were cumulative, noncumulative, and/or epistatic with regard to both target gene expression and variegation. The results should aid in understanding interacting regulatory pathways affecting the two phenomena.

Two general trends emerge from our results. Increasing the number of mutant modifiers does not usually cause a multiplicative effect on gene expression. Although there are some combinations that are cumula-
tive, the overall tendency is for noncumulative action. Secondly, reducing the dosage of the functional alleles of an increasing number of modifiers produces a greater tendency for an inverse effect to occur.

MATERIALS AND METHODS

Flies were maintained on cornmeal-glucose-yeast-agar media at 25°C. The genetic mutations and chromosomal rearrangements are described in FLYBASE (http://morgan.harvard.edu/fb.html/).

Genetic recombination: The cytological locations of the five trans-acting modifiers [Inverse regulator-aEMS1 (Inr-a, cytological location 46D±47D), Lightener of white (Low, 39E7±F1), Ultramaleoverexpression (Um, 47A11), Weakener of white (Wow, 76D5-F), and Modifier of white EMS2 (Mow, 85F1±86C1)] used in this study were determined earlier (Rabinow et al. 1991; Birchler et al. 1994; Bhadra and Birchler 1996; Bhadra et al. 1997a,b). To analyze the combinations, it was necessary to generate other triple combination flies. described earlier (Bhadra et al. 1997a,b). Each modifier has a distinct effect on the dominant marker Tft used to generate other triple combination flies. For Northern analysis was performed as described earlier (Bhadra et al. 1994; Birchler and Bhadra 1994). Triplicate isolations of the four genotypes in each combination were electrophoresed on 1% agarose. Separate DNAase I and RNase A digestions confirmed that the upper and lower bands on ethidium-stained gels corresponded to genomic DNA and rRNA, respectively. In the same gel, a dilution series of identically prepared nucleic acid was electrophoresed. The photographic negatives of an ethidium-stained gel containing triplicate preparations from different modifier combinations were analyzed by laser scanning densitometry using the same parameters as described earlier (Hiebert and Birchler 1994; Bhadra et al. 1997a,b). The measurements of the dilution series were used to establish the standard curve (Pal Bhadra et al. 1998). The DNA/18S rRNA and the DNA/28S rRNA ratios were calculated from the densitometric scans. The relative ratios obtained from the males and females of different modifier combinations were not significantly different from the normal males and females (0±24 hr) (Pal Bhadra et al. 1998). Therefore, the lack of a significant alteration of rRNA level in any of the tested combinations shows rRNA to be a valid gel loading control.

RESULTS

Our laboratory is interested in second-site dosage-dependent modifiers of gene expression and their involvement with various phenomena. In particular, we are characterizing a collection of trans-acting regulators that have a positive or negative effect on the white eye color gene (Rabinow et al. 1991; Birchler et al. 1994; Bhadra and Birchler 1996; Bhadra et al. 1997a,b; Frollov et al. 1998). The white gene has been genetically and molecularly studied, and many aspects of its cis-acting regulatory features are known (Zachar and Bingham 1982; O'Hare et al. 1983). Numerous leaky and hypomorphic alleles at white facilitate characterization of its trans-acting modifiers at the phenotypic level. The five genes that have been selected for this study were described earlier (Rabinow et al. 1991; Birchler et al. 1994; Bhadra and Birchler 1996; Bhadra et al. 1997a,b). Each modifier has a distinct effect on white based on its interaction with different w alleles. Two white alleles, wcarrot (w^m) and wspotted (w^s), were used for an initial test of a combinatorial effect. w^m is a "point" mutation as determined by the absence of gross rearrangements detected by Southern analysis (Zachar and Bingham 1982). w^s has a 5′ regulatory lesion and shows a reduced level of pigment, lighter in females than males (Zachar and Bingham 1982; O'Hare et al. 1983; Davison et al. 1985). Each modifier has a positive or negative effect on the w^s phenotype. In contrast, three of the five modifiers alter the w^m eye color, with Inr-a and Low having no effect on it. Therefore, cis-acting regulatory sequences that are defective in w^s are required for the Inr-a and Low interaction.
Interaction of one to four copies of white modifiers with two white alleles and zeste

<table>
<thead>
<tr>
<th>Number of modifiers</th>
<th>Combination</th>
<th>w&lt;sup&gt;carrot&lt;/sup&gt;</th>
<th>w&lt;sup&gt;spotted&lt;/sup&gt;</th>
<th>zeste&lt;sup&gt;+&lt;/sup&gt;/w&lt;sup&gt;+&lt;/sup&gt;</th>
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</thead>
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<tr>
<td>1</td>
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<td>0</td>
</tr>
<tr>
<td>1</td>
<td>++ Ufo +; ++</td>
<td>++</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>Low + ++; ++</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>++ +; Wow +</td>
<td>−</td>
<td>−</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>++ +; + M ow</td>
<td>++</td>
<td>−</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>++ Inr-a; Wow +</td>
<td>0</td>
<td>0</td>
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<tr>
<td>2</td>
<td>++ Inr-a; + M ow</td>
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<tr>
<td>2</td>
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<td>++</td>
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</tr>
<tr>
<td>3</td>
<td>Low + Inr-a; Wow +</td>
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</tr>
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<td>3</td>
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<td>++</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>Low + Inr-a; Wow M ow</td>
<td>+</td>
<td>−</td>
<td>0</td>
</tr>
</tbody>
</table>

Males of each modifier combination were crossed separately with females carrying each w allele. The eye color of F<sub>1</sub> males was compared with that of balancer brothers. An elevation in pigment is designated by +, a reduction by −, and no effect by 0 in the w allele interaction and zeste column. (b s) represents brown sectors in the eye.

Eight double, four triple, and a quadruple combination of modifiers were tested against these two white alleles (Table 1). All the combinations either darken or lighten the eye colors of w<sup>or</sup> and w<sup>sp</sup> flies, with the exception of the Inr-a; Wow combination on w<sup>or</sup> and the Low; M ow, Low Inr-a; Wow, and Low Inr-a; M ow combinations on w<sup>sp</sup> (Figure 1 and Table 1). The two alleles respond differently to the combinations, and in neither case is the effect of modifier combinations predictable from their separate effects.

**Table 1**

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</tr>
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<td>−</td>
<td>−</td>
<td>0</td>
</tr>
<tr>
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<td>++ +; + M ow</td>
<td>++</td>
<td>−</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>++ Inr-a; Wow +</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>2</td>
<td>++ Inr-a; + M ow</td>
<td>++</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Low + ++; Wow +</td>
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<td>2</td>
<td>Low + ++; + M ow</td>
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<td>0</td>
</tr>
<tr>
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<td>+</td>
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<td>3</td>
<td>Low + Inr-a; Wow +</td>
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</tr>
<tr>
<td>4</td>
<td>Low + Inr-a; Wow M ow</td>
<td>+</td>
<td>−</td>
<td>0</td>
</tr>
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**zeste-dependent transvection and modifier combinations:** Transvection results in an altered gene expression due to allelic pairing. zeste is a pairing-dependent regulator of the white locus (Bingham and Zachar 1985; Bickel and Pirrotta 1990; Chen and Pirrotta 1993). Two doses of white, including its 5<sup>′</sup> regulatory sequences, are required for a phenotypic effect of zeste. The zeste protein binds at the 5<sup>′</sup> regulatory sequence to suppress white in the eye, resulting in a yellow eye color instead of wild-type red (Davis et al. 1985; Chen and Pirrotta 1993). None of the selected modifiers individually affects zeste. To examine whether zeste interacts with various combinations of modifiers, a series of genetic crosses were performed using males from each double, triple, and quadruple combination carrying the w<sup>118</sup> mutation in the X chromosome and females from a Dp(1;1)w<sup>118</sup>/z Dp(1;1)w<sup>118</sup> strain (Green 1963). The eye colors of nonbalancer and balancer males were compared. Data summarized in Table 1 reveal that in most cases the eye color of the balancer and nonbalancer males is similar, with the exception of three combinations: Low; M ow, Wow M ow, and Low; Wow M ow. In Low; M ow males, several patches of brown pigment are distributed throughout the eye (Figure 2 and Table 1). When the Wow mutation is added to this combination, a darker eye color is found along with brown patches (Figure 2). The eye color is the same in the triple combinations and in Wow M ow. Thus, these modifiers also interact nonadditively on the zeste phenotype.

**Position-effect variegation and modifier combinations:** Effect on the variegating white phenotype. In general, dosage-dependent PEV modifiers are separated into two classes, suppressors and enhancers. The effect of multiple modifiers on PEV in most cases is mainly additive (Locke et al. 1988; Elgin 1996; Henikoff 1996). That is, mutations in two suppressors more strongly suppress PEV than either one alone. We tested the effect of five modifiers in combinations on position-effect variegation, of which three alone are weak suppressors of PEV (Birchler et al. 1994; Bhadra and Birchler 1996; Bhadra et al. 1997b) and two others have no effect on the variegating phenotype (Rabinow et al. 1991; Bhadra et al. 1997a).

When a chromosomal inversion, such as In(1)w<sup>nth</sup>, relocates the white eye color gene next to heterochromatin, the expression of the white gene is silenced in some ommatidia but not in others. To examine the effects of different combinations of modifiers on this phenotype,
$w^{mh}$/ $w^{mh}$ females were crossed with males from multiple modifier stocks. The eye phenotype of each genotype of F$_1$ males was examined (Figure 1). Table 2 summarizes the results. The degree of suppression fluctuates in various combinations, but in general is noncumulative and epistatic. However, it is interesting to note that inclusion of Inr-a either with Low or Wow intensifies the suppression, although Inr-a alone does not suppress PEV. In contrast, Ufo, which also lacks any effect on variegation, reduces suppression by the Wow Mow combination. These effects of Ufo and Inr-a on position-effect variegation reveal that second-site modifiers that have no effect on their own, participate when they are combined with other genes that act as suppressors. In the absence of Inr-a and Ufo, the three modifiers Low, Wow, and Mow appear to act cumulatively (Figure 1).

The progenitor chromosomes of Low, Ufo, Wow, and Mow were found to have no effect on variegation (Birchler et al. 1994; Bhadra and Birchler 1996; Bhadra et al. 1997a,b). To determine the existence of any potential preexisting modifier on the Inr-a chromosome, we analyzed the second chromosome from the parental $w^+$ line for its effect on $w^{mh}$. The $w^{mh}$ females were mated to males carrying the progenitor second chromosome.

Figure 1.—Phenotypic effect of selected combinations of trans-acting modifiers on two different white alleles and $w^{mh}$. The genotypes and white alleles are noted.
chromosome, together with SM 6a, the same balancer chromosome present in the Inr-a stock. The amount of eye pigment in w\textsuperscript{m4h}; SM 6a/ + and w\textsuperscript{m4h}; +/+ male and females was compared (data not shown) and was not significantly different.

Effect on a variegating yellow rearrangement: In addition to the effect of modifiers on white variegation, we also examined the effect on yellow variegation among the \~80 bristles along the anterior margin of the wing blade in ln(1)\textsuperscript{y3P} flies. These results tested whether the effects of modifier combinations on position-effect variegation are general. An advantage of this study is that variegation events in individual cells can be easily detected and quantitated (Karpen and Spradling 1990; Bhadra and Birchler 1996; Bhadra et al. 1997b). After crossing +/Y; M/ Balancer (M represents the modifier combination) males to ln(1)\textsuperscript{y3P} females, the y and y\textsuperscript{1} bristles were counted in the experimental ln(1)\textsuperscript{y3P}/Y; M/ + males and their control ln(1)\textsuperscript{y3P}/Y; Balancer/+ brothers. For each genotype, the number of normal black (yellow\textsuperscript{+}) bristles on the anterior margin of one wing from each of 10 males was scored (Table 3).

### Table 2

<table>
<thead>
<tr>
<th>Number of modifiers</th>
<th>Combination</th>
<th>n</th>
<th>OD\textsubscript{abs} ± S.E.</th>
<th>Ratio</th>
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</thead>
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<tr>
<td>0</td>
<td>++++; ++</td>
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<td>0.267 ± 0.014</td>
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</tr>
<tr>
<td>1</td>
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<td>0.229 ± 0.039</td>
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</tr>
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<td>0.298 ± 0.021</td>
<td>1.10</td>
</tr>
<tr>
<td>1</td>
<td>Low + +; ++</td>
<td>4</td>
<td>0.591 ± 0.023*</td>
<td>2.25</td>
</tr>
<tr>
<td>1</td>
<td>+ + +; Wow</td>
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<td>0.656 ± 0.031*</td>
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<tr>
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<td>0.421 ± 0.007*</td>
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<td>1.295 ± 0.038*</td>
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<tr>
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<tr>
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<tr>
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<td>0.746 ± 0.021</td>
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<td>0.374 ± 0.005*</td>
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<td>1.549 ± 0.041*</td>
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<tr>
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<td>Low + In-r-a; Wow M ow</td>
<td>4</td>
<td>0.485 ± 0.018*</td>
<td>1.76</td>
</tr>
</tbody>
</table>

Alteration of w\textsuperscript{m4h} variegation is calculated from the OD\textsubscript{abs} values (mean ± SE) in a given (n) number of experiments, which were determined by eye pigment assays on adult male flies of each genotype. The ratio is the relative value compared to the segregating controls from each cross. The values indicated with asterisks are significantly different from those of control males at the 95% level of confidence.
The results reveal that various modifier combinations alter yellow variegation. In most combinations, the effect on the yellow variegation closely resembles the effect obtained with $w^{me}$. However, two combinations, Inr-a; Wow and Low Inr-a; Mow, have less effect on $y^{op}$ than on $w^{me}$. Overall, however, the results support the conclusion that most of the combinations act generally on PEV rather than specifically on $w^{me}$.

**Developmental alteration of white and scarlet mRNA:** An initial characterization of the five modifiers revealed that they altered the transcript levels of white and two other functionally related genes, scarlet and brown, either positively or negatively. These three eye color genes are involved in transport of pigment precursors from the hemolymph into the appropriate cell types. Each modifier is distinct from the others. Wow modulates the target gene expression either positively or negatively, depending on the developmental stage or tissue. In larvae, three of the five modifier mutations, Inr-a, Ufo, and Wow separately increased white transcripts to the twofold level or more, while in all double combinations the effects are more limited (Figure 3 and Table 4), especially for Ufo. Mow lowers white transcript levels even when combined with any of the three modifiers that individually increase it, but raises the white transcript level when combined with Low.

The combined effect of multiple modifiers on scarlet transcripts is distinct from their effect on white (Table 5). The influence of Mow in the double combinations is not as strong as found on white, with the exception of the Inr-a Mow combination. In some instances, such as Low; Wow, the combined effects were intermediate between the individual effects (Table 5).

We also measured the white and scarlet transcript abun-

### TABLE 3
**Influence of one to four modifiers on $y^{op}$ variegation**

<table>
<thead>
<tr>
<th>Number of modifiers</th>
<th>Combination</th>
<th>No. of yellow$^+$ bristles (mean no. ± S.D.)</th>
<th>Mean of the total no. of bristles</th>
<th>Percentage of yellow$^+$ bristles (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>+ + +; + +</td>
<td>13.3 ± 1.3</td>
<td>80.3</td>
<td>16.4</td>
</tr>
<tr>
<td>1</td>
<td>+ + Inr-a; + +</td>
<td>17.1 ± 1.4</td>
<td>80.3</td>
<td>21.1</td>
</tr>
<tr>
<td>1</td>
<td>+ Ufo +; + +</td>
<td>15.4 ± 1.3</td>
<td>80.5</td>
<td>18.9</td>
</tr>
<tr>
<td>1</td>
<td>Low + +; + +</td>
<td>38.1 ± 2.7*</td>
<td>79.9</td>
<td>48.4</td>
</tr>
<tr>
<td>1</td>
<td>+ + +; Wow +</td>
<td>51.3 ± 4.1*</td>
<td>80.0</td>
<td>64.1</td>
</tr>
<tr>
<td>1</td>
<td>+ + +; + Mow</td>
<td>42.1 ± 3.2*</td>
<td>80.4</td>
<td>51.7</td>
</tr>
<tr>
<td>2</td>
<td>+ + Inr-a; Wow +</td>
<td>31.5 ± 2.6*</td>
<td>80.7</td>
<td>38.3</td>
</tr>
<tr>
<td>2</td>
<td>+ + Inr-a; + Mow</td>
<td>28.8 ± 2.4*</td>
<td>80.1</td>
<td>36.2</td>
</tr>
<tr>
<td>2</td>
<td>Low + +; Wow +</td>
<td>47.4 ± 3.8*</td>
<td>79.7</td>
<td>59.5</td>
</tr>
<tr>
<td>2</td>
<td>Low + +; + Mow</td>
<td>53.9 ± 4.2*</td>
<td>80.1</td>
<td>66.8</td>
</tr>
<tr>
<td>2</td>
<td>Low + Inr-a; + +</td>
<td>21.7 ± 2.4*</td>
<td>80.0</td>
<td>26.9</td>
</tr>
<tr>
<td>2</td>
<td>+ + +; Wow Mow</td>
<td>36.5 ± 3.0*</td>
<td>80.8</td>
<td>45.1</td>
</tr>
<tr>
<td>2</td>
<td>+ Ufo +; Wow +</td>
<td>19.7 ± 3.1</td>
<td>80.4</td>
<td>24.4</td>
</tr>
<tr>
<td>2</td>
<td>+ Ufo +; + Mow</td>
<td>25.2 ± 1.9*</td>
<td>80.4</td>
<td>30.3</td>
</tr>
<tr>
<td>3</td>
<td>Low + Inr-a; Wow +</td>
<td>21.2 ± 1.7*</td>
<td>80.5</td>
<td>26.4</td>
</tr>
<tr>
<td>3</td>
<td>Low + Inr-a; + Mow</td>
<td>24.8 ± 2.1*</td>
<td>80.4</td>
<td>30.4</td>
</tr>
<tr>
<td>3</td>
<td>+ + Inr-a; Wow Mow</td>
<td>31.4 ± 2.7*</td>
<td>80.2</td>
<td>39.3</td>
</tr>
<tr>
<td>3</td>
<td>Low + +; Wow Mow</td>
<td>56.2 ± 4.1*</td>
<td>79.9</td>
<td>70.3</td>
</tr>
<tr>
<td>4</td>
<td>Low + Inr-a; Wow Mow</td>
<td>23.2 ± 1.8*</td>
<td>80.1</td>
<td>29.4</td>
</tr>
</tbody>
</table>

Suppression by different modifier combinations of $y^{op}$ variegation was determined by counting the number of yellow and black bristles between the first and second crossvein termini of the wings. Suppression increases the proportion of black to yellow bristles. The values indicated with asterisks are significantly different from those of segregating control males from each cross at the 95% level of confidence.

In double combinations: To discover whether the modifiers interact similarly on different target genes, we measured the steady-state levels of white and scarlet transcripts in the two segregating classes for males and females for each modifier combination. Total RNA was isolated from each genotype in four developmental stages: third instar larvae, early pupae (0–24 hr), middle pupae (24–48 hr), and eclosed adults (0–24 hr). The Northern profiles of each combination and their quantitative results are presented in Figure 3 and Tables 4 and 5.

In larvae, three of the five modifier mutations, Inr-a, Ufo, and Wow separately increased white transcripts to the twofold level or more, while in all double combinations the effects are more limited (Figure 3 and Table 4), especially for Ufo. Mow lowers white transcript levels even when combined with any of the three modifiers that individually increase it, but raises the white transcript level when combined with Low.

The combined effect of multiple modifiers on scarlet transcripts is distinct from their effect on white (Table 5). The influence of Mow in the double combinations is not as strong as found on white, with the exception of the Inr-a Mow combination. In some instances, such as Low; Wow, the combined effects were intermediate between the individual effects (Table 5).

We also measured the white and scarlet transcript abun-
Multiple Modifiers, Gene Expression and PEV

Figure 3.—Abundance of white transcripts in 12 selected combinations of modifiers at the larval stage. Northern blots of each genotype were hybridized with a ^32^P-labeled antisense RNA of white and then reprobed with antisense rRNA as a gel loading control (Bhadra et al. 1997a,b). M, heterozygous combination of the modifiers. The respective probes are noted.

dance in two different pupal stages. The larval-pupal transition significantly influenced the effect of the modifiers (Tables 4 and 5). In some cases, combinations that exhibited a direct correlative effect in larvae switch to an inverse effect, while inversely acting combinations in larvae act as direct correlative modifiers in pupae. Overall, the modulation in pupae is greater than in larvae. Similar to other stages of development, the extreme overexpression of scarlet transcripts in pupae caused by Ufo is eliminated when Ufo is associated with the Wow mutation. The mode of action of several double combinations is similar in the two pupal stages. In the Inr-a; Wow mid-pupal combination, white transcripts are reduced, while their respective individual effects each exhibit a significant elevation. In general, the double combinations exhibit noncumulative or epistatic relationships among the modifiers at the pupal stage (Tables 4 and 5).

The effect of double combinations on white transcript levels is minimal at the adult stage and the effects on scarlet are mostly noncumulative (Tables 4 and 5). Three of the five modifiers, Low, Wow, and Mow separately reduced white transcript levels, while such reduction is eliminated in their combinations. In several cases, variation of the white and scarlet transcripts at the adult stage is significantly different from that of the pupal stage (Tables 4 and 5). This result suggests that the developmental transition from pupa to adult also plays a role in modulating the combined effect of multiple modifiers on target genes.

In triple combinations: The quantitative effect on white transcripts was measured in each of four triple combinations (Tables 4 and 5). In two of these, Low Inr-a; Wow and Low; Wow Mow, white transcripts are overexpressed relative to their closest double combinations in larvae (Table 4). In the Low Inr-a; Wow combination, this effect also persists at the pupal stage, whereas, in the Low Inr-a Mow and Inr-a Wow Mow combinations, the level of white mRNA is considerably less elevated or reduced (Table 4). The pattern of scarlet transcripts in triple combinations closely resembles the effect on white (Table 5). However, there are instances where white transcript levels are elevated but scarlet is significantly reduced, and vice versa.

In the quadruple combination: The relative abundance of white and scarlet transcripts in the quadruple combination is increased to a lesser degree than in certain triple combinations (Figures 3 and 4 and Tables 4 and 5). The modulation for the quadruple modifiers is limited within a threefold range throughout development.

Sexual equivalence and modifier combinations: We also analyzed the difference of the combined effect of multiple modifiers between males and females. In a few combinations, the transcript levels of each gene (white and scarlet) exhibit sex-specific effects in larvae and adults (Figure 5). Six out of 13 combinations show a sex-specific effect on white in early pupae, while only the quadruple combination exhibits a differential level of scarlet expression between males and females.

DISCUSSION

In this study, we have generated double, triple, and quadruple combinations of dosage-dependent modifiers of the white eye color locus to gain information on their interaction properties. Taken altogether, there are examples of cumulative effects, cancellation by opposite effects, and epistatic interactions of one gene with another. In some cases, the combination of two directly correlative effects becomes an inversely correlative one,
TABLE 4

Effect of one to four modifiers on white gene expression in four different developmental stages

<table>
<thead>
<tr>
<th>Number of modifiers</th>
<th>Combination</th>
<th>Larvae</th>
<th>Early pupae (0-24 hr)</th>
<th>Mid-pupae (24-48 hr)</th>
<th>Adults</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>1</td>
<td>+ + Inra; ++</td>
<td>2.09 ± 0.14*</td>
<td>2.26 ± 0.09*</td>
<td>0.90 ± 0.02</td>
<td>1.02 ± 0.07</td>
</tr>
<tr>
<td>1</td>
<td>++ Ufo; ++</td>
<td>6.39 ± 0.31*</td>
<td>12.55 ± 0.39*</td>
<td>0.47 ± 0.01*</td>
<td>0.51 ± 0.02*</td>
</tr>
<tr>
<td>1</td>
<td>Low + +; ++</td>
<td>0.96 ± 0.01</td>
<td>1.74 ± 0.02*</td>
<td>2.48 ± 0.04*</td>
<td>1.29 ± 0.02</td>
</tr>
<tr>
<td>1</td>
<td>++ +; Wow +</td>
<td>2.04 ± 0.03*</td>
<td>2.49 ± 0.03*</td>
<td>0.55 ± 0.01*</td>
<td>0.45 ± 0.02*</td>
</tr>
<tr>
<td>1</td>
<td>++ +; + Mow</td>
<td>0.79 ± 0.03*</td>
<td>0.25 ± 0.01*</td>
<td>0.61 ± 0.02*</td>
<td>0.25 ± 0.01*</td>
</tr>
<tr>
<td>2</td>
<td>++ + Inra; Wow</td>
<td>1.75 ± 0.07*</td>
<td>1.47 ± 0.02*</td>
<td>0.17 ± 0.004*</td>
<td>0.35 ± 0.01*</td>
</tr>
<tr>
<td>2</td>
<td>++ + Inra + Mow</td>
<td>0.42 ± 0.02*</td>
<td>1.04 ± 0.04</td>
<td>0.43 ± 0.02*</td>
<td>0.38 ± 0.01*</td>
</tr>
<tr>
<td>2</td>
<td>Low + +; Wow +</td>
<td>0.91 ± 0.01</td>
<td>1.10 ± 0.02</td>
<td>0.88 ± 0.03</td>
<td>0.91 ± 0.03</td>
</tr>
<tr>
<td>2</td>
<td>Low + +; + Mow</td>
<td>2.01 ± 0.01*</td>
<td>1.61 ± 0.04*</td>
<td>1.12 ± 0.01</td>
<td>1.42 ± 0.02*</td>
</tr>
<tr>
<td>2</td>
<td>Low + Inra; ++</td>
<td>1.32 ± 0.03*</td>
<td>0.61 ± 0.01*</td>
<td>4.37 ± 0.06*</td>
<td>2.42 ± 0.05*</td>
</tr>
<tr>
<td>2</td>
<td>++ +; Wow Mow</td>
<td>0.24 ± 0.004*</td>
<td>0.51 ± 0.02*</td>
<td>3.73 ± 0.08*</td>
<td>3.81 ± 0.11*</td>
</tr>
<tr>
<td>2</td>
<td>++ Ufo; Wow +</td>
<td>0.36 ± 0.03*</td>
<td>1.96 ± 0.02*</td>
<td>1.86 ± 0.02*</td>
<td>0.32 ± 0.01*</td>
</tr>
<tr>
<td>2</td>
<td>++ Ufo; + Mow</td>
<td>1.08 ± 0.01</td>
<td>0.59 ± 0.01*</td>
<td>0.65 ± 0.02*</td>
<td>0.33 ± 0.01*</td>
</tr>
<tr>
<td>3</td>
<td>Low + Inra; Wow +</td>
<td>8.37 ± 0.07*</td>
<td>6.32 ± 0.12*</td>
<td>7.89 ± 0.05*</td>
<td>3.80 ± 0.09*</td>
</tr>
<tr>
<td>3</td>
<td>Low + Inra; + Mow</td>
<td>0.93 ± 0.03</td>
<td>0.58 ± 0.02*</td>
<td>0.19 ± 0.01*</td>
<td>0.15 ± 0.01*</td>
</tr>
<tr>
<td>3</td>
<td>++ + Inra; Wow Mow</td>
<td>1.12 ± 0.03</td>
<td>1.04 ± 0.02</td>
<td>0.61 ± 0.02*</td>
<td>0.73 ± 0.03*</td>
</tr>
<tr>
<td>3</td>
<td>Low + +; Wow Mow</td>
<td>3.99 ± 0.17*</td>
<td>4.12 ± 0.09*</td>
<td>1.07 ± 0.02</td>
<td>0.96 ± 0.02</td>
</tr>
<tr>
<td>4</td>
<td>Low + Inra; Wow Mow</td>
<td>2.29 ± 0.05*</td>
<td>1.69 ± 0.03*</td>
<td>0.42 ± 0.01*</td>
<td>0.84 ± 0.01*</td>
</tr>
</tbody>
</table>

Each value is the mean ratio ± SE (modifier combination/ + : +/+), calculated from three separate assays. The amount of radioactivity of each lane of Northern blots was measured using a Fuji Bas 2000 phosphorimager. Each mean value ± SE is based on three replicate Northern gels. The loading difference in each lane was corrected by reprobing each blot with the rRNA probe. To compare mutant/ + vs. the T(2;3) CyO, Tb-dv + genotype, the individual transcript/ rRNA ratios were calculated followed by the mutant/ + : +/+ ratios, which are presented in the table. *, significantly different from the value of 1.00 (P < 0.05).
### TABLE 5

Effect of one to four modifiers on scarlet gene expression in four different developmental stages

<table>
<thead>
<tr>
<th>Number of modifiers</th>
<th>Combination</th>
<th>Larvae Male</th>
<th>Larvae Female</th>
<th>Early pupae (0-24 hr) Male</th>
<th>Early pupae (0-24 hr) Female</th>
<th>Mid-pupae (24-48 hr) Male</th>
<th>Mid-pupae (24-48 hr) Female</th>
<th>Adults Male</th>
<th>Adults Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Inr-a; ++</td>
<td>0.94 ± 0.01</td>
<td>1.10 ± 0.03</td>
<td>1.15 ± 0.03</td>
<td>1.07 ± 0.01</td>
<td>3.15 ± 0.04</td>
<td>2.97 ± 0.03</td>
<td>1.47 ± 0.02</td>
<td>1.61 ± 0.04</td>
</tr>
<tr>
<td>1</td>
<td>Ufo; ++</td>
<td>1.13 ± 0.02</td>
<td>1.12 ± 0.03</td>
<td>3.80 ± 0.04*</td>
<td>6.83 ± 0.17*</td>
<td>2.24 ± 0.02</td>
<td>2.06 ± 0.01</td>
<td>1.24 ± 0.04</td>
<td>1.67 ± 0.02</td>
</tr>
<tr>
<td>1</td>
<td>Low; ++; ++</td>
<td>0.70 ± 0.01*</td>
<td>0.37 ± 0.01*</td>
<td>0.56 ± 0.01*</td>
<td>0.28 ± 0.004*</td>
<td>0.50 ± 0.02*</td>
<td>0.21 ± 0.01*</td>
<td>0.69 ± 0.02</td>
<td>0.33 ± 0.01*</td>
</tr>
<tr>
<td>1</td>
<td>+ + Wow</td>
<td>1.72 ± 0.01*</td>
<td>1.60 ± 0.02*</td>
<td>1.87 ± 0.02*</td>
<td>2.46 ± 0.03*</td>
<td>0.54 ± 0.01*</td>
<td>0.59 ± 0.01*</td>
<td>1.60 ± 0.01</td>
<td>1.76 ± 0.01*</td>
</tr>
<tr>
<td>1</td>
<td>+ + Wow</td>
<td>0.41 ± 0.01*</td>
<td>0.36 ± 0.004*</td>
<td>2.47 ± 0.16*</td>
<td>4.93 ± 0.21*</td>
<td>0.83 ± 0.05</td>
<td>0.74 ± 0.07</td>
<td>1.27 ± 0.04</td>
<td>0.78 ± 0.05</td>
</tr>
<tr>
<td>2</td>
<td>Inr-a; Wow</td>
<td>0.92 ± 0.03</td>
<td>0.87 ± 0.04</td>
<td>0.56 ± 0.02*</td>
<td>0.37 ± 0.02*</td>
<td>0.31 ± 0.01*</td>
<td>0.29 ± 0.01*</td>
<td>0.47 ± 0.01</td>
<td>0.53 ± 0.01*</td>
</tr>
<tr>
<td>2</td>
<td>Inr-a; Wow</td>
<td>0.52 ± 0.11*</td>
<td>0.45 ± 0.01*</td>
<td>2.41 ± 0.01*</td>
<td>2.19 ± 0.02*</td>
<td>1.97 ± 0.02*</td>
<td>1.88 ± 0.02*</td>
<td>0.87 ± 0.03</td>
<td>0.93 ± 0.03</td>
</tr>
<tr>
<td>2</td>
<td>Low; Wow</td>
<td>1.13 ± 0.04</td>
<td>1.10 ± 0.07</td>
<td>1.02 ± 0.02</td>
<td>0.97 ± 0.02</td>
<td>1.74 ± 0.04*</td>
<td>1.82 ± 0.03*</td>
<td>1.36 ± 0.04</td>
<td>1.38 ± 0.03*</td>
</tr>
<tr>
<td>2</td>
<td>Low; Wow</td>
<td>0.72 ± 0.14*</td>
<td>0.68 ± 0.01*</td>
<td>2.42 ± 0.09*</td>
<td>2.13 ± 0.04*</td>
<td>2.57 ± 0.03*</td>
<td>2.95 ± 0.03*</td>
<td>1.40 ± 0.03</td>
<td>1.34 ± 0.02*</td>
</tr>
<tr>
<td>2</td>
<td>Low; Inr-a</td>
<td>1.14 ± 0.04</td>
<td>0.72 ± 0.01*</td>
<td>0.45 ± 0.01*</td>
<td>0.57 ± 0.01*</td>
<td>0.29 ± 0.01*</td>
<td>0.34 ± 0.02*</td>
<td>2.26 ± 0.03</td>
<td>2.18 ± 0.03*</td>
</tr>
<tr>
<td>2</td>
<td>+ + Wow</td>
<td>2.82 ± 0.04*</td>
<td>1.96 ± 0.05*</td>
<td>0.52 ± 0.01*</td>
<td>0.46 ± 0.01*</td>
<td>0.63 ± 0.01*</td>
<td>0.31 ± 0.01*</td>
<td>2.53 ± 0.06</td>
<td>1.71 ± 0.04*</td>
</tr>
<tr>
<td>2</td>
<td>Ufo; Wow</td>
<td>1.07 ± 0.03</td>
<td>0.93 ± 0.02</td>
<td>0.62 ± 0.01*</td>
<td>0.68 ± 0.01*</td>
<td>1.52 ± 0.02*</td>
<td>1.71 ± 0.02*</td>
<td>0.85 ± 0.02</td>
<td>0.42 ± 0.01*</td>
</tr>
<tr>
<td>2</td>
<td>Ufo; Wow</td>
<td>1.15 ± 0.05</td>
<td>1.09 ± 0.01</td>
<td>2.40 ± 0.05*</td>
<td>2.11 ± 0.03*</td>
<td>2.57 ± 0.03*</td>
<td>2.85 ± 0.04*</td>
<td>0.72 ± 0.01</td>
<td>0.77 ± 0.02*</td>
</tr>
<tr>
<td>3</td>
<td>Low; Inr-a</td>
<td>7.65 ± 0.18*</td>
<td>7.12 ± 0.09*</td>
<td>8.62 ± 0.15*</td>
<td>8.46 ± 0.13*</td>
<td>1.58 ± 0.03*</td>
<td>1.10 ± 0.03</td>
<td>1.08 ± 0.03</td>
<td>0.97 ± 0.01</td>
</tr>
<tr>
<td>3</td>
<td>Low; Inr-a</td>
<td>8.91 ± 0.17*</td>
<td>7.84 ± 0.10*</td>
<td>2.66 ± 0.03*</td>
<td>2.45 ± 0.03*</td>
<td>6.57 ± 0.12*</td>
<td>2.14 ± 0.05*</td>
<td>1.90 ± 0.07</td>
<td>1.88 ± 0.06*</td>
</tr>
<tr>
<td>3</td>
<td>++ Wow</td>
<td>2.04 ± 0.05*</td>
<td>1.98 ± 0.05*</td>
<td>2.62 ± 0.09*</td>
<td>2.58 ± 0.10*</td>
<td>2.09 ± 0.04*</td>
<td>2.13 ± 0.12*</td>
<td>1.01 ± 0.03</td>
<td>1.12 ± 0.03</td>
</tr>
<tr>
<td>3</td>
<td>Low; Wow</td>
<td>0.96 ± 0.02</td>
<td>0.89 ± 0.02</td>
<td>0.41 ± 0.01*</td>
<td>0.58 ± 0.01*</td>
<td>0.88 ± 0.03</td>
<td>0.79 ± 0.02</td>
<td>2.46 ± 0.06</td>
<td>2.69 ± 0.06*</td>
</tr>
<tr>
<td>4</td>
<td>Low; Inr-a</td>
<td>2.52 ± 0.25*</td>
<td>2.41 ± 0.03*</td>
<td>1.44 ± 0.5*</td>
<td>0.70 ± 0.01*</td>
<td>2.11 ± 0.03*</td>
<td>4.28 ± 0.06*</td>
<td>1.17 ± 0.04</td>
<td>1.01 ± 0.04</td>
</tr>
</tbody>
</table>

Values are mean ratios ± SE of three individual assays per genotype. The radioactivity of the bands of the Northern blots were measured using a Fuji Bas 2000 phosphorimager. The loading difference in each lane was corrected by reprobing each blot with the rRNA probe. Each ratio was determined relative to +/+ controls as described in Table 4. *, values are significantly different from 1.0 with > 95% confidence.
and vice versa. However, despite the individual examples of specific types of interactions, considering all four developmental stages in both sexes for the two target genes studied, the overall trend is for noncumulative action. For example, although Low, Inr-a, Wow, and Mow all individually increase white expression in the mid-pupal stage between 1.5- and threefold, the quadruple combination still only increases white expression two- to threefold rather than the >16× level expected from independent action.

The second general trend, considering only Inr-a, Wow, Mow, and Low, for which a complete data set exists, is that with an increased number of modifiers in the combination, the likelihood increases that the net result is an inverse correlation between the dosage of the functional modifier alleles and target gene expression. With single modifiers, 26 of 64 data points (40% of the total, Ufo excluded) show an inverse effect. In the double combinations, 40% (38 out of 96) of the 96 data points (Ufo; Wow and Ufo; Mow not included) indicate an inverse response. However, with the triple and quadruple combinations, 55% (44 out of 80) of the 80 data points are now inverse effects (Figure 5). A row by column chi-square analysis of the null hypothesis that the single plus double vs. triple plus quadruple arrays are the same is rejected ($\chi^2 = 4.26$; d.f. = 1; $P < 0.05$). These two general trends are consistent with the results from larger aneuploids in which the predominant dosage effect is an inverse one (Devlin et al. 1988; Birchler et al. 1989), and that the magnitude of the effects more or less re-
main within the limits of an inverse correlation. Despite this potential connection from these data, it is probable that in many larger aneuploids more than four modifiers for one target gene are varied.

**Interactions:** Effect of zeste An interesting interaction involves the effect on zeste. The zeste gene affects pairing-dependent expression of white. When zeste is present with two copies of white, a lemon eye color results rather than the normal brick red. None of the white modifiers alone alters the eye color when zeste is modifying white. However, two double combinations—Low; M ow and Wow M ow—suppress the zeste-white interaction. The triple combination of Low; Wow M ow also suppresses zeste, but interestingly the Inr-a; Wow M ow returns to normal.

Effect on PEV: Of the five loci alone, Low, Wow, and M ow act as suppressors of PEV. We tested the combinations on two variegating rearrangements, w<sup>4</sup><sup>th</sup> and y<sup>p</sup>, to examine the generality of the effects, particularly since they were selected as modifiers of white. The results on the two rearrangements are quite similar.

The generalities formulated for the effects on gene expression also apply to PEV, primarily, in that the combinations do not exhibit strictly cumulative interactions. It is true in the case of PEV that the triple combinations are the strongest group of suppressors, but among these the two greatest both involve Low and M ow, which is the strongest dual combination. The quadruple combination drops back into the range of the single modifiers as did the effects on gene expression. Although Inr-a alone has no effect on variegation, in combination with Wow or Low a greater suppression occurs than with either alone. On the other hand Ufo, which has no effect by itself, will dilute the suppression caused by Wow or M ow. In contrast to the effects on gene expression, no combinations were found which switched the effect on PEV from suppression to enhancement.

**Why so many dosage-dependent modifiers of gene expression and PEV?** There are scores of dosage-dependent modifiers of PEV and gene expression. For PEV, Henikoff (1996) provided insight on this issue by noting that some modifiers are structural components of heterochromatin and others are regulatory genes that might control them. Indeed, many suppressors and enhancers of PEV have been cloned, and while they are a heterogeneous collection, all are reasonable candidates for having an involvement in the process as chromatin components or their regulators (Eissenberg et al. 1992; Garzino et al. 1992; Reuter and Spierer 1992; Tschi e r s c h et al. 1994; De R u b e r t i s et al. 1996; Tyler et al. 1996). The same could be noted for dosage modifiers of gene expression (e.g., Dorn et al. 1993; Farkas et al. 1994; Seum et al. 1996; Frolov et al. 1998). Indeed, there appears to be a significant overlap between the two.

The above-mentioned examples, as well as others (Botas et al. 1982; Weintraub 1993; Kennison 1995; Cubadda et al. 1997; Crews 1998), suggest that a disproportionately high fraction of regulatory genes are dosage sensitive. Over evolutionary time, genes involved with many aspects of gene expression from signal transduction to transcriptional regulation apparently have evolved to be rate limiting in the diploid state and as such would exhibit a dosage effect when assayed genetically. A hierarchy of dosage-sensitive regulation would contribute to the multigenic nature of these systems. If the regulatory genes most intimately involved with the expression of a structural gene are dosage dependent and in turn are controlled by dosage-dependent regulators and so on, the higher-order regulatory genes will still be dosage dependent on the expression of the monitored structural gene. Because a dosage effect can be transduced through a series of dosage-dependent steps, this situation could at least partially explain why so many dosage modifiers of gene expression and PEV exist. While individual genes in a hierarchical series might all affect the ultimate target gene, one would also expect examples of epistasis and an overall noncumulative effect in combinations, as found in this study.

**Relationship to the genetic control of quantitative traits:** As noted above, most mutations characterized in genetically studied organisms are recessive (see, for example, FLYBASE http://morgan.harvard.edu/fb.html/; Orr 1991; Thatcher et al. 1998). Yet the control of quantitative traits is governed disproportionately by genes that exhibit additivity in hybrids between the parental extremes (Tanksley 1993; Liu et al. 1996). Typically, quantitative traits are governed by multiple factors, which nevertheless often exhibit epistasis (Tanksley 1993; Damerval et al. 1994; Clark et al. 1995; Mackay 1996; Clark and Wang 1997; Laurie et al. 1997). The parallels to the multiple dosage-sensitive modifiers of gene expression, as exemplified here by the effect on the w<sup>4</sup><sup>th</sup> phenotype, are striking.

In a simplistic view, a phenotypic characteristic is controlled by a biochemical pathway. One enzymatic step in that pathway will be rate limiting by definition under a certain set of conditions and hence will exhibit a dosage effect on the phenotype when genetic variation is present at the locus encoding that enzyme. If this gene is governed by a dosage-dependent hierarchy, the phenotype will be governed by multiple dosage-sensitive factors (e.g., Byrne et al. 1996) that overall are not cumulative. To the extent that epistasis and interactions occur among the regulatory loci, such as was found in this study, these effects would be reflected in the phenotype. Of course, multiple biochemical pathways contributing to the phenotype would complicate the genetics further.

**Relationship to dosage compensation:** With dosage compensation, increasing the number of copies of a particular chromosomal segment leads to less product per copy of a gene included in that segment. Because the structural gene being monitored is increased in copy number at the same time, an overall equalization of total output per cell results. In addition, genes elsewhere...
in the genome may also be downregulated, leading to a reduction of their total product (inverse correlation) (Birchler 1979, 1981, 1996; Devlin et al. 1982; Birchler et al. 1989, 1990; Guo and Birchler 1994). These two responses appear to be related in that the dosage compensation results from the cancellation of a structural gene dosage effect by the inverse effect produced simultaneously (Birchler 1981; Birchler et al. 1990). If the same set of dosage-dependent modifiers are responsible for both dosage compensation, as well as the inverse effects on loci elsewhere in the genome, then they must not be generally cumulative and must favor an inverse dosage effect. While our study has analyzed in detail only four of the several modifiers of white, the results are consistent with these requirements on the whole.

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