Identification of *Trans*-dominant Modifiers of *Prat* Expression in *Drosophila melanogaster*

Nicolas Malmanche and Denise V. Clark[1]

Department of Biology, University of New Brunswick, Fredericton, New Brunswick E3B 6E1, Canada

Manuscript received December 11, 2002
Accepted for publication March 25, 2003

ABSTRACT

The first committed step in the purine *de novo* synthesis pathway is performed by amidophosphoribosyltransferase (EC 2.4.2.14) or Prat. *Drosophila melanogaster Prat* is an essential gene with a promoter that lacks a TATA-box and initiator element and has multiple transcription start sites with a predominant start site. To study the regulation of *Prat* expression in the adult eye, we used the *Prat:bw* reporter gene, in which the *Prat* coding region was replaced with the *brown* (*bw*) coding region. The pale-orange eye color of a single copy of *Prat:bw* prompted us to use a multicopy array of *Prat:bw* that was derived using P transposase mutagenesis and produces a darker-orange eye color in a bw^{D}*; st* genetic background. We used a 13-copy array of *Prat:bw* as a tool to recover dominant EMS-induced mutations that affect the expression of the transgene. After screening 21,000 F_1s for deviation from the orange eye color, we isolated 23 dominant modifiers: 21 suppressors (1 Y-linked, 5 X-linked, 4 2-linked, and 11 3-linked) and 2 enhancers (1 2-linked and 1 3-linked). Quantification of their effect on endogenous *Prat* gene expression, using RT-PCR in young adult fly heads, identifies a subset of modifiers that are candidates for genes involved in regulating *Prat* expression.

THE *Drosophila melanogaster Prat* gene encodes the first enzyme in the purine *de novo* synthesis pathway, amidophosphoribosyltransferase (EC 2.4.2.14; Clark 1994). Primer extension analysis showed that the *Prat* gene has multiple transcription initiation sites, although one predominant site is found in adult RNA. The promoter lacks a TATA-box and initiator element (D. Clark, unpublished observation). The 5′ untranslated region has a small intron with an alternative splice acceptor site while the rest of the gene is intronless (Clark *et al.* 1998). *Prat* mRNA is expressed in all stages of development, with low levels of expression during the three larval stages and high maternal expression in ovaries leading to mRNA accumulation in 0- to 2-hr-old embryos (Clark and MacAfee 2000; Malmanche *et al.* 2003).

In a screen for ethyl methanesulfonate (EMS)-induced mutations in region 84E1-2, five *Prat* alleles that had a phenotype described for other genes of the purine *de novo* synthesis pathway were recovered (Clark 1994). Analysis of the *Prat* alleles showed that a reduction of the enzyme activity to 40% of the wild-type level induces the "purine syndrome" phenotype. This is in contrast to genes for other enzymes of the pathway for which the purine syndrome phenotype is much less penetrant in mutants, even when there is no detectable enzyme activity (Henikoff *et al.* 1986c). This observation is consistent with the finding of studies of different organisms that *Prat* is the limiting step in the *de novo* purine pathway (Wyngaarden and Kelley 1983).

The purine synthesis pathway is regulated in part by Prat enzyme activity, which is stimulated by phosphoribosyl-pyrophosphate and inhibited by purine nucleotides (Holmes 1981). In addition, part of the regulation of the pathway occurs at the transcriptional level in *Escherichia coli*, *Bacillus subtilis*, and *Saccharomyces cerevisiae* (Daignan-Fornier and Fink 1992; Zalkin and Dixon 1992; Rolfes and Hinnebusch 1993). In the bacterial cases, negative regulation occurs in response to an increase in pathway end products whereas, in *S. cerevisiae*, positive regulation occurs in response to a decrease in pathway end products. In humans, the genes encoding Prat and the enzyme for steps 6 and 7 of the pathway are divergently transcribed and partly transcriptionally controlled by nuclear respiratory factor 1, which also controls expression of cytochrome genes (Chen *et al.* 1997). In addition, human inosine monophosphate mRNA levels are post-transcriptionally controlled by the availability of pathway end products (Glesne *et al.* 1991). Aside from these two cases, little is known about the genetic regulation of the *de novo* purine pathway in multicellular eukaryotes. In Drosophila, two genes encoding enzymes of the *de novo* purine pathway, *ade2* and *ade3*, have been characterized at the genetic and molecular levels (Henikoff *et al.* 1986a,b,c; Tiong *et al.* 1989; Tiong and Nash 1993), but the regulation of these genes at the transcriptional level or in relation to pathway end products has not been addressed.

In Drosophila, several dosage-sensitive second-site

[1]*Corresponding author:* Department of Biology, Bag Service 45111, University of New Brunswick, Fredericton, NB E3B 6E1, Canada. E-mail: clarkd@unb.ca

modifiers that act *in trans* to suppress or enhance gene expression have been described (Rabinow *et al.* 1991; Birchler *et al.* 1994; Bhadra and Birchler 1996; Bhadra *et al.* 1997b). The effect of such modifiers on expression of individual genes can be variable during the Drosophila life cycle and they can affect various loci differently. Since the genes corresponding to these modifiers would encode factors with a role in the expression of the genes they affect, we sought to isolate modifiers of *Prat* to identify *trans*-acting factors important for *Prat* expression. Furthermore, since *Prat* expression limits the purine synthesis *de novo* pathway in mammalian cells (Yamaoka *et al.* 1997), we predicted that some of the genes encoding regulatory factors could be identified by isolation of dosage-sensitive modifiers of *Prat* expression. In this study we describe the isolation of 23 modifiers of *Prat:bw* (2 enhancers and 21 suppressors). Results of pigment and mRNA assays show that a subset of the modifiers has an effect on *Prat* expression, demonstrating that the *Prat:bw* reporter gene is an effective tool for identifying candidate *Prat* regulatory genes.

MATERIALS AND METHODS

Drosophila stocks: Drosophila were cultured on standard media at 25°. The *Prat* alleles and the *P[ry$^+$; Prat:bw]* transgene are described by Clark (1994) and Clark *et al.* (1998), respectively. In this report, we used a 13-copy *Prat:bw* array inserted at 65A10 in a *bw^D; st* genetic background and we refer to it as *Prat:bw*. The *P[ry$^+$; bw$^+$]* transgene stock, described by Dreesen *et al.* (1991), and the *bw^D; st* stock were obtained from Steven Henikoff. The *T(2;3)Sb^v/CyO*, *In(1)w^{m4}* and *In(1)w^{m4}; Su(var)205^{E39A}/CyO* stocks were obtained from Amy Csink. All other fly stocks used in this study were obtained from the Bloomington (Indiana) Stock Center. Description and references for all the alleles described in this article can be found in Flybase (Flybase 1999).

Mutagenesis screen: *bw^D; st* males were starved for 2 hr and then fed on a 1% sucrose solution with 25 mm EMS (Sigma) for 12 hr. After mutagen treatment the males were crossed *en masse* to *bw^D; Prat:bw st/TM6b* females. The eye colors of the progeny were observed in 0- to 4-hr-old young adult flies under a dissecting microscope. Males and females showing a deviation from *bw^D; Prat:bw st/st* eye color were individually backcrossed to *bw^D; st*. When the F_2 showed a deviation from the *bw^D; Prat:bw st* eye color, the X or second chromosome was balanced using a *N^{55e11}/FM7c; bw^D; st* stock or a *bw^D Sp/CyO; st* stock, respectively. For F_2s showing no deviation, the third chromosome was balanced using a *bw^D; st/TM3, Sb st* stock and then the third chromosomes were retested for their effect on *bw^D; Prat:bw st/st* eye color. When applicable, the modifiers were then tested *inter se* for complementation with respect to their lethal recessive phenotype.

Genetic mapping: Chromosome 2- and 3-linked modifiers associated with an interesting effect on the endogenous *Prat* gene were meiotically mapped using linkage to recessive visible markers. A second chromosome marked with *al dp b Bl pr cn c bw^D* and a third chromosome marked with *ru h th st cu sr e* were used for recombination mapping of second- and third-chromosome-linked modifiers, respectively. To construct the second chromosome for mapping in a *bw^D; st* genetic background, *al dp b Bl pr cn c/CyO* females were crossed with *bw^D; st* males. In the F_1, heterozygous non-Cy females were crossed to *bw^D; st* males and, in the F_2, recombinant second chromosomes of white-eyed males were balanced using *bw^{D}Sp/CyO; st*. These stocks were then crossed to *al dp b pr cn c sp* flies to establish which recessive markers were present. For recombination mapping, modifier stocks were first crossed with the appropriate marker chromosome stock. In the F_1, heterozygous females were crossed to *bw^D Sp/CyO; st* for the second chromosome and to *bw^D; st/TM3, Sb st* for the third chromosome. In the F_2, individual heterozygous balanced males carrying potential recombinant chromosomes were crossed to *bw^{D}Sp/CyO; st* for the second chromosome and to *bw^D; st/TM3, Sb st* for the third chromosome for amplification. Between 200 and 250 recombinant chromosomes were tested for each individual modifier. For four members of the complex complementation group on the third chromosome, a total of 500 recombinant chromosomes were tested. Then, in the F_3, heterozygous balanced males carrying the potential recombinant chromosomes were tested in three crosses: (1) with the *Prat:bw* stock, to test for the dominant effect on eye color; (2) with the original modifier stock, to test for recessive lethal phenotype; and (3) with the marker chromosome stock, to test for the markers present on the recombinant chromosome. Once the chromosomal location of the modifiers was determined using recombination mapping, deficiency mapping for the associated recessive phenotype was completed using deficiency kits for the second and third chromosomes from the Bloomington Drosophila Stock Center.

Quantitative RT-PCR: Total RNA was extracted from 0- to 4-hr-old adult fly heads of the genotype *[Mod/+] ; bw^D; Prat:bw st* or *[Mod/+]; bw^D; P[ry$^+$; bw$^+$] st*. All samples for RNA extraction were collected in microcentrifuge tubes on ice, frozen in liquid nitrogen, and stored at −70°. RNA was extracted using Trizol reagent (Life Technologies/Invitrogen) and reverse transcriptase polymerase chain reactions (RT-PCR) were performed using RT-PCR beads (Ambion, Austin, TX). To establish quantitative RT-PCR conditions for each set of primers (*rp49*, *Prat*, and *bw*), a series of RT-PCRs with different numbers of PCR cycles for one concentration of RNA was performed to determine the range of cycles for which the output is in the linear range of amplification (Foley *et al.* 1993). These conditions were established using 0- to 4-hr-old adult fly head RNA from *bw^D; Prat:bw st* and *bw^D; P[ry$^+$; bw$^+$] st*. The established conditions are 16 PCR cycles for *rp49* primers, 23 PCR cycles for *Prat* primers, 23 PCR cycles for *bw* primers with *bw^D; P[ry$^+$; bw$^+$] st* RNA, and 21 PCR cycles for *bw* primers with *bw^D; Prat:bw st* RNA (Figure 1). The primer pairs (Life Technologies/Invitrogen) used were 5′ TCCGTTCCCGAGT CTGGGCACAGCGGC 3′ with 5′ fluo AGGCGGTGCAGT GGCCCGTTCCCTTG 3′ for *Prat*, 5′ ACTCACGCAACGGACG GTGCAGGACG 3′ with 5′ fluo AGGACCCCATAGCCAACAG CCGCCACC 3′ for *bw*, and 5′ CCAAGGACTTCATCCGC CACC 3′ with 5′ fluo GCGGGTGCGCTTGTTCGATCC 3′ for *rp49* (Foley *et al.* 1993). "Fluo" indicates fluorescein conjugated to the 5′ end of one of each of the primer pairs. The cDNA synthesis was performed at 42° for 30 min using 1 μg of total RNA and an oligo(dT) primer. PCR cycles were 1 min at 95°, 1 min at 66°, and 1 min at 72°. Following RT-PCR, the products were separated by agarose gel electrophoresis and blotted on Nylon membrane (Roche) by the capillary method (Sambrook *et al.* 1989). The products were detected using an antifluorescein-alkaline phosphatase antibody (NEN-Dupont) and CDP-star chemiluminescent substrate (New England Biolabs, Beverly, MA). The signal was detected using Kodak or Ultident X-ray film and films were scanned with an Epson Expression 626 scanner with a transparent film adaptor for measurement using Scion Image for Windows. Two and, for some modifiers, three replicate experiments were performed per genotype, beginning from independent RNA isolation.

The control RNAs were extracted once and an aliquot of the extract was used for each set of experiments (beginning from reverse transcription, PCR, and detection). Results from RT-PCR are shown in Table 2 as the ratio between *Prat* mRNA/*rp 49* mRNA in the control experiment *vs. Prat* mRNA/*rp 49* mRNA in the modifier background experiment. Each ratio indicates an independent measurement. As an example of the RT-PCR data analysis, the ratios with footnote *c* in Table 2 were obtained from analysis of the data shown in Figure 2.

Pigment assays: For pigment quantification, each modifier was crossed to the *bw^D; Prat:bw st* stock and to the *bw^D; P[ry^+; bw^+] st* stock. Adult flies were grouped into appropriate genotypes, aged for 5 days at 25°, frozen on liquid nitrogen, and vortexed, separating heads from the bodies. Prior to extraction, the heads were manually selected and collected in 30% ethanol (pH 2) and incubated for 3 days on a rotator at room temperature (Henikoff and Dreesen 1989). Ten heads per genotype were used per assay. Following centrifugation, the absorbance of the supernatant was measured at 480 nm using a Cary spectrophotometer. For each genotype, between 7 and 10 assays were performed. The mean, the standard deviation, and a *P* value for a *t*-test were then calculated.

Variegation: Modifiers were crossed with a *bw^+; st* stock to study their effect on *bw^D/ bw^+* eye-pigment variegation. Adult flies of the appropriate genotype were aged between 3 and 5 days prior to observation. An estimation of the modifier's effect on variegation was performed by visual observation and photography. For analysis of *Mod-Y-1* effects on *w^{m4}*, *w^{m4}; Su(var)205^{E39A}/CyO* females were crossed with *Mod-Y-1/+; bw^D; st* males, generating *Mod-Y-1/w^{m4}; bw^D/CyO; +/st* males, which were then crossed to *w^{m4}; +; +* females. The resulting *Mod-Y-1/w^{m4}; +/CyO; +/st (or +)* males could then be observed for modification of the *w^{m4}* phenotype in the absence of *bw^D* and *st*. Control males were generated in the same way using the parental *+; bw^D; st* stock males for the first cross. Flies were collected and aged at 25° for 10 days prior to photography. For analysis of *Mod-Y-1* effects on *Sb^v*, *Sb^v/CyO* females were crossed to *Mod-Y-1 ; bw^D; st* and *+; bw^D; st* males and, in the F_1, 14 thoracic bristles were scored for the Stubble phenotype (Csink *et al.* 1994). More Stubble bristles indicate a suppression of the *Sb^v* phenotype and a higher level of expression of the *Sb* allele. The data were represented as box plot data, generated using Minitab software.

RESULTS

Isolation of 23 dominant modifiers of *Prat:bw*: Our screen was designed with two stages. The first stage was to recover *trans*-acting dominant modifiers affecting the *Prat:bw* reporter gene phenotype. We used the *Prat* reporter gene, *P[ry^+; Prat:bw]*, which changes the eye color from white to pale orange in a *bw^D; st* genetic background (Clark *et al.* 1998). *Prat:bw* consists of an 8.9-kb genomic DNA fragment in which the *Prat* coding region has been replaced by an in-frame fusion between the amino-terminal *Prat* sequence and the *bw* coding region from a cDNA. Since the *Prat* coding region is uninterrupted by introns, we assume *Prat:bw* has retained the sequences necessary for *Prat* regulation and so the transcriptional activity of the *Prat* gene could be monitored in adult fly eyes using the *Prat:bw* reporter gene. We used a multicopy array of the transgene that was generated by several rounds of transposase mutagenesis. The level of pteridine pigment in eyes of flies carrying a multicopy array is directly related to the number of transgene copies within the array (Clark *et al.* 1998). In the present study, we used a 13-copy array of *P[ry^+; Prat:bw]*, localized on the third chromosome in 65A10, which will be referred to herein as *Prat:bw*. The 13-copy array produces a nonsaturated orange eye color, allowing us to screen for both enhancers and suppressors of eye color in a *bw^D; st* genetic background. The level of pteridine pigment in *Prat:bw* fly heads reflects *Prat* expression during some part of the prepupal and pupal stages and in young adult fly heads when pigment deposition occurs (Dreesen *et al.* 1988). The second stage of our screen was to test the isolated modifiers of *Prat:bw* eye color for an effect on the mRNA expression of the endogenous *Prat* gene. If the modifiers are loss-of-function mutations, then these would correspond to genes that are limiting for *Prat* expression.

We screened ~21,000 F_1 flies carrying EMS-mutagenized chromosomes for a deviation from the nonsaturated *Prat:bw* orange eye color in 0- to 4-hr-old young adult flies (see materials and methods). We isolated 23 modifiers of the *Prat:bw* eye color: 2 enhancers (1 2- and 1 3-linked) and 21 suppressors (1 Y-linked, 5 X-linked, 4 2-linked, and 11 3-linked). We call these modifiers *Mod(Prat:bw)m-n*, where *m* means linkage group and *n* means arbitrary number. Herein, we will refer to them simply as *Mod-m-n*. All X-linked modifiers were isolated from female F_1s, supporting the idea that spontaneously arising modifiers, at least for the X chromosome, were undetectable. We first sorted the 23 suppressors into three classes reflecting the strength of their effect on the *Prat:bw* pale-orange eye color in young adult flies (Table 1). The two enhancers increased pigment from pale orange to light red. Among the 21 suppressors, 15 reduced pigment from pale orange to yellow and 6 showed a weaker suppression of the pale orange. Complementation tests for recessive lethality revealed two complementation groups, one on the second chromosome with two alleles and one on the third chromosome with five alleles (see *Genetic mapping of dominant modifiers and their associated recessive phenotype*).

Pigment assays show that modifiers can be grouped into four classes: We quantified the effect of the 23 dominant modifiers on the pigment level of the *bw^D; Prat:bw st/st* and *bw^D; P[ry^+; bw^+] st/st* stocks. If a modifier affects some general aspect of transcription or affects expression post-transcriptionally, then we might expect a similar effect of the modifier on both transgenes. Otherwise, if a modifier has a different effect on *Prat:bw* than on *P[ry^+; bw^+]*, then it is acting through effects on sequences specific to the *Prat:bw* transgene.

Three main differences exist between the *Prat:bw* transgene and the *P[ry^+; bw^+]* transgene. First, while the *P[ry^+; bw^+]* transgene is a construct of an 8.4-kb genomic fragment from the *bw* locus that is sufficient to rescue the *bw* mutant phenotype (Dreesen *et al.*

TABLE 1

Effect of the modifiers on pteridine eye-pigment levels

Modifier	Class	Screen effect[a]	*n*	*bw^D; Prat:bw st/st*[b]	*t*-test *P* value[c]	Screen effect[a]	*n*	*bw^D; P[ry$^+$; bw$^+$] st/st*[b]	*t*-test *P* value[c]
Control			10	0.079 ± 0.003			10	0.137 ± 0.005	
Mod-1-1	1	ss	10	0.047 ± 0.006	10^{-9}	ss	10	0.077 ± 0.005	10^{-15}
Mod-1-2	1	ss	10	0.057 ± 0.007	10^{-6}	ne	10	0.114 ± 0.007	10^{-7}
Mod-1-3	1	ss	10	0.060 ± 0.004	10^{-8}	ws	10	0.098 ± 0.014	10^{-6}
Control			10	0.067 ± 0.003			10	0.133 ± 0.008	
Mod-1-4	1	ss	10	0.038 ± 0.005	10^{-9}	ss	10	0.070 ± 0.002	10^{-10}
Control			10	0.067 ± 0.004			10	0.135 ± 0.005	
Mod-1-5	1	ss	10	0.031 ± 0.004	10^{-13}	ss	10	0.043 ± 0.002	10^{-17}
Control			10	0.070 ± 0.005			10	0.136 ± 0.010	
Mod-2-1	1	ss	10	0.040 ± 0.0057	10^{-10}	ss	10	0.082 ± 0.0083	10^{-10}
Mod-2-2	1	ss	9	0.040 ± 0.0096	10^{-5}	ss	10	0.088 ± 0.0096	10^{-9}
Mod-2-5	2	se	9	0.100 ± 0.011	10^{-6}	we	10	0.190 ± 0.0087	10^{-10}
Control			10	0.068 ± 0.005			10	0.130 ± 0.010	
Mod-2-3	4	ws	10	0.050 ± 0.002	10^{-7}	ne	10	0.146 ± 0.009	0.001
Mod-2-4	1	ss	9	0.049 ± 0.003	10^{-8}	ne	10	0.114 ± 0.008	0.0006
Control			10	0.069 ± 0.006			10	0.122 ± 0.013	
Mod-3-6	1	ws	10	0.057 ± 0.005	0.00035	ne	9	0.112 ± 0.008	0.05
Mod-3-2	3	ws	10	0.066 ± 0.006	0.37	ne	8	0.132 ± 0.010	0.10
Mod-3-10	3	ws	8	0.070 ± 0.007	0.78	ne	9	0.126 ± 0.009	0.53
Mod-3-7	1	ss	10	0.058 ± 0.0003	0.0003	ss	10	0.070 ± 0.007	10^{-7}
Control			10	0.063 ± 0.004			10	0.136 ± 0.009	
Mod-3-1	2	se	7	0.075 ± 0.007	6×10^{-3}	ne	7	0.155 ± 0.010	0.002
Mod-3-11	3	ss	7	0.059 ± 0.004	0.07	ss	8	0.140 ± 0.008	0.32
Mod-3-9	3	ws	10	0.063 ± 0.005	0.99	ne	8	0.135 ± 0.010	0.80
Control			10	0.072 ± 0.004			10	0.139 ± 0.007	
Mod-3-3	1	ws	9	0.057 ± 0.006	1.4×10^{-4}	ws	8	0.121 ± 0.01	3×10^{-4}
Mod-3-4	4	ws	9	0.080 ± 0.006	0.0071	ss	8	0.107 ± 0.005	2×10^{-8}
Mod-3-5	1	ss	7	0.058 ± 0.004	10^{-5}	ne	7	0.128 ± 0.003	6×10^{-4}
Control			10	0.069 ± 0.004			10	0.123 ± 0.009	
Mod-3-8	3	ss	7	0.063 ± 0.009	0.175	ne	10	0.125 ± 0.005	0.55
Mod-3-12	3	ws	9	0.064 ± 0.004	0.043	ne	7	0.121 ± 0.019	0.80
Control			10	0.057 ± 0.004			10	0.148 ± 0.009	
Mod-Y-1	1	ss	10	0.033 ± 0.004	10^{-9}	ss	10	0.112 ± 0.007	10^{-9}

ss, strong suppressor; ne, no effect; ws, weak suppressor; se, strong enhancer; we, weak enhancer.

[a] The effect of the modifiers on eye color was observed for both transgenes on 0- to 4-hr-old young adult fly heads.

[b] Pigment values are expressed as mean absorbance at 480 nm ± standard deviation values obtained for *n* assays.

[c] The effect of the modifier on pigment levels was determined by performing a *t*-test. The *P* value represents the significance of the difference between the pigment value with and without the modifier at 95% confidence. Sets of modifier genotypes are grouped with their control genotype.

1991), the expression of *bw* in *Prat:bw* is under the control of the 5′ and 3′ regulatory sequences of the *Prat* gene. Second, for the *P[ry$^+$; bw$^+$]* transgene, the *bw* coding region has the original exon-intron organization of the *bw* locus, while those sequences are removed from the *Prat:bw* construct. The third difference is that the *P[ry$^+$; bw$^+$] st* stock has a single transposon insertion while *Prat:bw* is a transgene array of 13 *P* elements (Clark *et al.* 1998). Although some transgene arrays have been found to exhibit gene silencing (Peng and Mount 1995), the *Prat:bw* transgene arrays are not silenced and show copy-number-dependent expression (Clark *et al.* 1998). The absence of gene silencing is likely due to the presence of sufficient genomic se-

quence flanking *Prat:bw*, including the flanking gene sequences (Clark *et al.* 1998). Therefore, we predicted that modifiers of *Prat:bw* acting at the level of transcription would not be acting through modification of gene silencing due to the transgene array. Although we have some intriguing exceptions (see below), our finding that many modifiers affect the endogenous *Prat* gene's mRNA levels (see below) support this idea. Another difference between the transgenes is their insertion site; however, neither *P[ry$^+$; bw$^+$]* nor the *Prat:bw* transgene was found to be position sensitive (Dreesen *et al.* 1991; Clark *et al.* 1998).

The results of the pigment assays are summarized in Table 1. For this technique, we performed between 7 and 10 assays for each modifier in both genetic backgrounds (*Prat:bw* and *P[ry$^+$; bw$^+$]*). Assays were done on males and females for modifiers on the second and third chromosomes (although only the results from females are shown), on females for the X chromosome, and on males for the Y chromosome. For controls, we used flies derived from both transgene stocks crossed with the parental *bw^D; st* strain used in the EMS screen. We calculated the mean and standard deviation of the pigment value for each genotype. In addition, the *P* values for *t*-tests were calculated for comparisons between the presence and absence of the modifier in the same genetic background.

During the screen, the 23 modifiers fell into three classes when eye color was observed between 0 and 4 hr after eclosion, while the modifiers fell into four classes when tested for pigment values at 5 days: class 1 mutations suppress both *Prat:bw* and *P[ry$^+$; bw$^+$]* pigment levels (13 modifiers), class 2 mutations enhance both *Prat:bw* and *P[ry$^+$; bw$^+$]* pigment levels (2 modifiers), class 3 mutations affect *Prat:bw* eye color in 0- to 4-hr-old adult flies but do not show any effect on *Prat:bw* and *P[ry$^+$; bw$^+$]* pigment levels after aging the flies for 5 days (6 modifiers), and class 4 mutations enhance one transgene and suppress the other (2 modifiers).

In comparing the pigment levels of flies carrying the modifier to those that do not, the *P* values for all *t*-tests are highly significant ($P < 0.002$) for the 13 modifiers that suppress both transgenes (class 1), the two that enhance both transgenes (class 2), and the two class 4 modifiers (Table 1). One modifer, *Mod-3-6*, which showed a stronger effect on *Prat:bw* than on *P[ry$^+$; bw$^+$]* (Table 1), was also placed in class 1.

By definition, the six class 3 modifiers do not show any effect on pigment level for *bw^D; Prat:bw st* and they also do not show any effect on *bw^D; P[ry$^+$; bw$^+$] st* (Table 1). One explanation for class 3 is that, during the screen, mutations that suppress or enhance the *Prat:bw* eye color were selected when flies were young (between 0 and 4 hr) while, during the pigment test, flies were aged 5 days prior to analysis and this aging suppresses the modification of *Prat:bw* eye color.

Class 4 is composed of two exceptional modifiers, *Mod-2-3* and *Mod-3-4*. *Mod-3-4* enhances the eye color of *Prat:bw* and suppresses *P[ry$^+$; bw$^+$]* (Table 1), whereas *Mod-2-3* suppresses *Prat:bw* eye color and enhances *P[ry$^+$; bw$^+$]* (Table 1). While *Mod-3-4* was identified during the screen as a suppressor of *Prat:bw* eye color in young adult flies, the pigment measurements show that it has an inverse effect on *Prat:bw* eye color in aged flies.

In summary, visual scoring of the *Prat:bw* eye-color modification in young adult flies and the pigment measurements at 5 days correlate for 19 modifiers: the 13 class 1 modifiers were isolated as strong suppressors of *Prat:bw* eye color, the 2 class 2 modifiers as strong enhancers, and 4 of the 6 class 3 modifiers as weak suppressors. The existence of 4 modifiers that do not behave in this way illustrates that it is important initially to screen flies of the same age and that subsequent analysis of pigment levels at other ages can reveal modifiers with dynamic effects on the level of eye pigment.

RT-PCR analysis shows that a subset of modifiers also affects *Prat* expression: We developed a quantitative RT-PCR assay to determine the effects of the modifiers on gene expression at the mRNA level. For this technique we measured the level of three mRNAs in various genetic backgrounds: (1) the endogenous *Prat* mRNA in lines containing the transgenes, (2) the *bw* mRNA produced by the *Prat:bw* transgene, and (3) the *bw* mRNA produced by the *P[ry$^+$; bw$^+$]* transgene. In addition, *rp49* mRNA (O'Connell and Rosbash 1984) was coamplified with each of the other three mRNAs to provide an internal control (Foley *et al.* 1993). The levels of the three mRNAs were measured in the presence or absence of a modifier chromosome in adult female fly heads aged for 0–4 hr. The exception is the Y-linked modifier, *Mod-Y-1*, where males were assayed.

We chose to investigate the effect of each modifier on *Prat* expression in young adult fly heads for two reasons. First, we wanted to examine gene expression at the same stage used for isolation of the dominant modifiers of *Prat:bw*. Second, we wanted to determine if our selection of *Prat:bw* eye-color variants can lead to isolation of *trans*-modifiers of *Prat* expression rather than to mutations involved in the pigment pathway or in post-transcriptional regulation.

The *bw^D* allele is a null allele with a 2-Mbp heterochromatic insertion within the locus (Henikoff *et al.* 1995). Since the *bw* primers we used for RT-PCR correspond to sequences on the 5′ side of the heterochromatic insertion, it is possible that we were detecting some *bw* transcript from the *bw^D* locus in our RT-PCR experiments. The *bw* transcript was not detected by RT-PCR in homozygous *bw^D; st* adult fly heads, while the *rp49* mRNA was (data not shown), indicating that the mRNAs detected for both the *Prat:bw* and the *P[ry$^+$; bw$^+$]* transgenes are indeed derived only from the transgenes.

In determining the number of PCR cycles for quantification of the *bw* mRNA (Figure 1), we found that the *bw* mRNA is more abundant in *Prat:bw* than in *P[ry$^+$;*

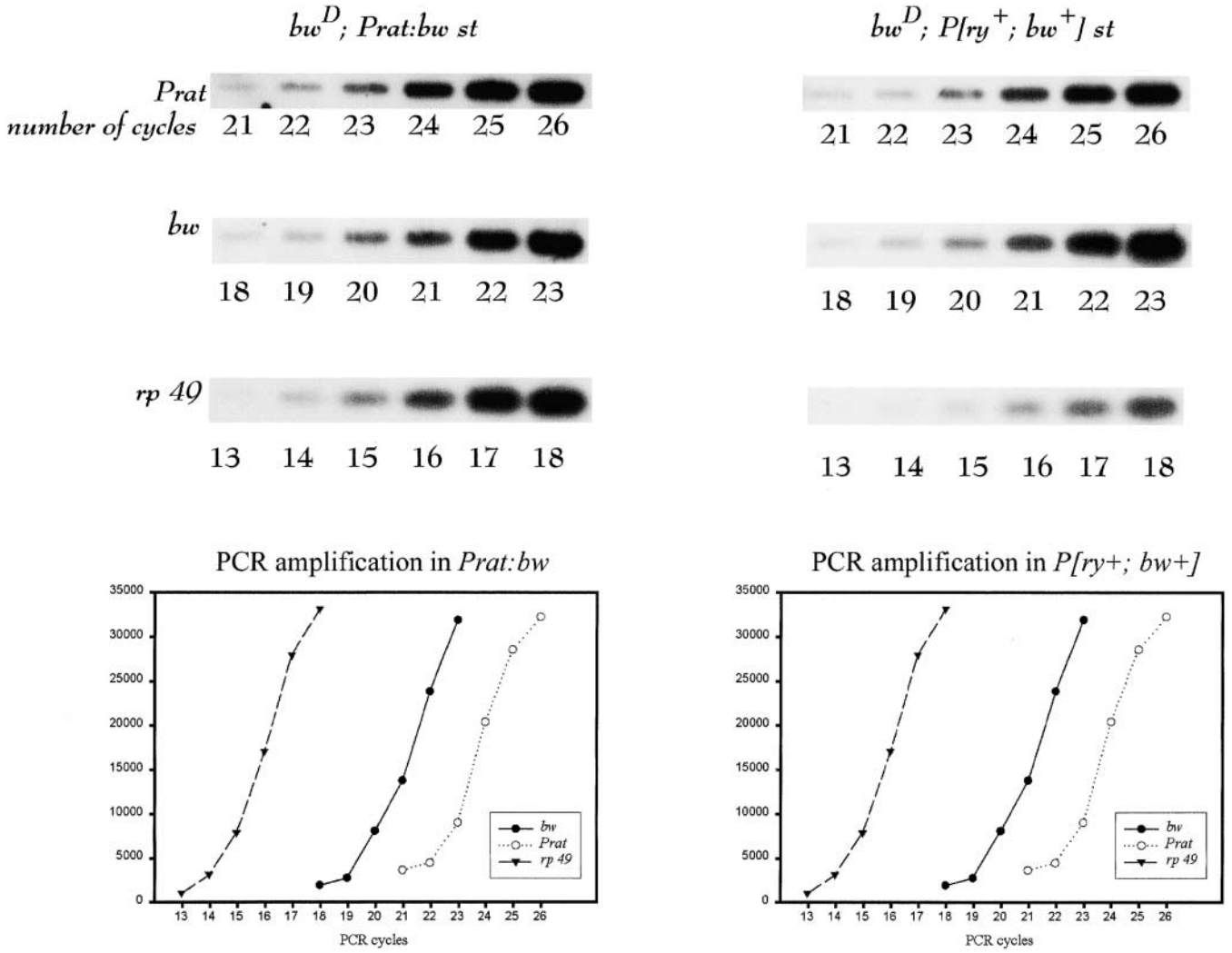

FIGURE 1.—Determination of the PCR conditions for the quantitative RT-PCR assays. The graphs show the yield of *Prat* and *rp49* mRNAs in two genotypes over several rounds of PCR. The yield of *bw* mRNA corresponds to the transgene in each genotype. For the RT-PCR assays, the number of PCR cycles for each primer pair was chosen in the linear range of amplification, so that the assays would be comparable. The values for the *y*-axis represent band density.

bw$^+$*]* flies, which is in contradiction to the level of pteridine pigments measured during our assay. Although this finding seems inconsistent, the pigment assay measures protein activity during the process of pigment deposition during 5 days in specific cells, whereas RT-PCR measures mRNA levels at one time in adult fly heads. One explanation is that *Prat* expression in adult fly heads occurs in cells where *bw* expression does not occur. An alternative explanation is that some of the transcripts derived from the *Prat:bw* transgene array are not functional.

Overall, the results from RT-PCR analysis show that, despite some inconsistencies between pigment assay and RT-PCR data, the original visual identification of modifiers of *Prat:bw* eye color is effective for recovery of *trans*-dominant modifiers of *Prat* gene expression. Specifically, the RT-PCR data indicate that 7/23 modifiers (5 suppressors and 2 enhancers) have a strong effect on *Prat* expression in both genetic backgrounds that correlates with the original eye-color observation and the pigment assay data. These are 5 class 1 modifiers (*Mod-3-5*, *Mod-3-6*, *Mod-2-1*, *Mod-2-2*, and *Mod-1-5*) and 2 class 2 modifiers (*Mod-3-1* and *Mod-2-5*). For *Mod-3-6*, the RT-PCR data show a strong decrease in the expression of the *Prat* endogenous gene in both genetic backgrounds (Table 2 and Figure 2A). There is also suppression of *bw* mRNA for both transgenes; however, there is more suppression for *Prat:bw* than for *P[ry*$^+$*; bw*$^+$*]*. For the chromosome 2 complementation group (see *Genetic mapping of dominant modifiers and their associated recessive phenotype*), *Mod-2-1* and *Mod-2-2*, and for the modifiers *Mod-3-5* and *Mod-1-5*, the RT-PCR results showed a reduction of *bw* mRNA for both transgenes that is consistent with the decrease in pigment level shown in the

TABLE 2

Effect of modifiers on *Prat*, *Prat:bw* and *P[ry$^+$; bw$^+$]* mRNA expression in 0- to 4-hr-old adult fly heads

	Transgene					
	Prat:bw			*P[ry$^+$; bw$^+$]*		
Modifier	Pigment effect[a]	RT-PCR *Prat*[b]	RT-PCR *bw*[b]	Pigment effect[a]	RT-PCR *Prat*[b]	RT-PCR *bw*[b]
Mod-1-1	ss	2.1; 2.3	2; 2.2	ss	1.4; 3	2.1; 2.3
Mod-1-2	ss	1; 1.4; 1.8	1.5; 1.5	ne	2.3; 2.5	1.6; 3
Mod-1-3	ss	0.6; 0.8; 0.9	0.7; 0.8	ws	0.6; 0.9	0.2; 0.4
Mod-1-4	ss	1.6; 3	1.4; 1.5	ss	2; 4	1.4; 3
Mod-1-5	ss	0.1; 0.2	0.1; 0.2	ss	0.1; 0.2	0.2; 0.2
Mod-2-1	ss	0.3; 0.5	0.3; 0.5	ss	0.4; 0.4	0.1; 0.1
Mod-2-2	ss	0.5; 0.7	0.5; 0.6	ss	0.1; 0.1	0.1; 0.2
Mod-2-3	ws	0.3; 0.7[c]	0.1; 0.4[c]	ne	0.1; 0.3[c]	0.2[c]; 0.4
Mod-2-4	ss	0.7; 1.3	0.1; 0.3	ne	0.9; 1	0.1; 0.1
Mod-2-5	se	1.3; 1.4; 1.5	5; 6.7	we	1.5; 1.7	1.5; 2
Mod-3-1	se	1.5; 1.5	1.8; 2	ne	1.5; 1.5	1; 1.1
Mod-3-2	ws	0.2; 0.3	0.9; 1.4	ne	0.2; 0.4; 0.6	0.3; 0.8
Mod-3-3	ws	0.6; 1.4	1.2; 1.4	ws	0.8; 1.1; 1.3	0.3; 0.3; 0.6
Mod-3-4	ws	0.2; 0.7	1; 1.3; 2.4	ss	2.5; 2.7; 2.8	0.7; 1.2
Mod-3-5	ss	0.1; 0.3	0.2; 0.3	ne	0.4; 0.5	0.1; 0.3
Mod-3-6	ws	0.1; 0.2[c]	0.1[c]; 0.3	ne	0.2; 0.5[c]	0.3; 0.5[c]
Mod-3-7	ss	1.3; 2.4; 2.7	0.7; 0.7	ss	3.1; 3.2	4.5; 5
Mod-3-8	ss	0.3; 0.4	0.7; 0.7	ne	0.1; 0.3	0.2; 0.4
Mod-3-9	ws	0.3; 0.5; 0.7	3; 5	ne	2; 2.9; 3	1.2; 1.4
Mod-3-10	ws	0.8; 1; 1.3	2; 2.7	ne	1.3; 1.3; 1.4	1; 1.2
Mod-3-11	ss	0.3; 0.4	0.8; 0.8	ss	1.6; 1.8	4.5; 5
Mod-3-12	ws	0.2; 0.3	0.2; 0.3	ne	0.8; 1	0.8; 1
Mod-Y-1	ss	1.6[c]; 1.9	1.2; 1.5[c]	ss	1.6; 1.8[c]	3.2; 3.5[c]

ss, strong suppressor; ne, no effect; ws, weak suppressor; we, weak enhancer; se, strong enhancer.

[a] The effect of the modifiers on eye color was observed for both transgenes in 0- to 4-hr-old young adult fly heads.

[b] RT-PCR values are expressed as the ratio between the level of gene x mRNA with and without the modifier relative to the *rp49* mRNA. Here, *rp49* was coamplified with each mRNA and its level was used to normalize the yield of mRNA detected for gene x. Gene x is the endogenous *Prat* mRNA in two different genotypes or gene x is one of the *bw* mRNAs derived from the *Prat:bw* or *bw*$^+$ transgene.

[c] Indicates the relative values obtained from the experiments shown in Figure 2.

pigment assays. As well, the suppression affects *Prat* mRNA expression in both genetic backgrounds. The class 2 modifiers *Mod-2-5* and *Mod-3-1* enhance *Prat* mRNA expression in both genetic backgrounds and this effect correlates with enhancement of pigment expression. However, while their effect on *Prat* mRNA is similar for both genetic backgrounds, their effect on *bw* mRNA varies, with a stronger enhancement of *Prat:bw* than of *P[ry$^+$; bw$^+$]* for *Mod-2-5* (Table 2) while, for *Mod-3-1*, there is no effect on *bw* mRNA in *P[ry$^+$; bw$^+$]* (Table 2). In spite of the latter inconsistencies, overall these 5 class 1 and 2 class 2 modifiers appear to be acting through a common mechanism for *Prat* and *bw* mRNA expression due to their similar effects on both types of construct and promoter.

Among the remaining modifiers, four (*Mod-2-3*, *Mod-3-2*, *Mod-3-8*, and *Mod-3-12*) show strong suppression of *Prat* expression in correlation with the young adult *Prat:bw* eye color, but in contradiction with the pigment assay data (Tables 1 and 2 and Figure 2A). Their strong effects on *Prat* expression and the correlation with the *Prat:bw* phenotype suggest that these four modifiers correspond to genes encoding *trans*-acting factors involved in *Prat* regulation of expression.

For the 12 remaining modifiers, 5 are enhancers of *Prat* expression in both genetic backgrounds (*Mod-1-1*, *Mod-1-2*, *Mod-1-4*, *Mod-3-7*, and *Mod-Y-1*). It is interesting to note that these 5 modifiers are strong suppressors of *Prat:bw* eye color in 0- to 4-hr-old adults and are suppressors of both *Prat:bw* and *P[ry$^+$; bw$^+$]* as measured by pigment assays. However, the enhancement of *bw* mRNA expression for both transgenes (except for *Mod-3-7* in *Prat:bw*) suggests a post-transcriptional effect associated with the modifier chromosomes.

Three modifiers display an opposite effect on *Prat* expression as a function of the genetic background

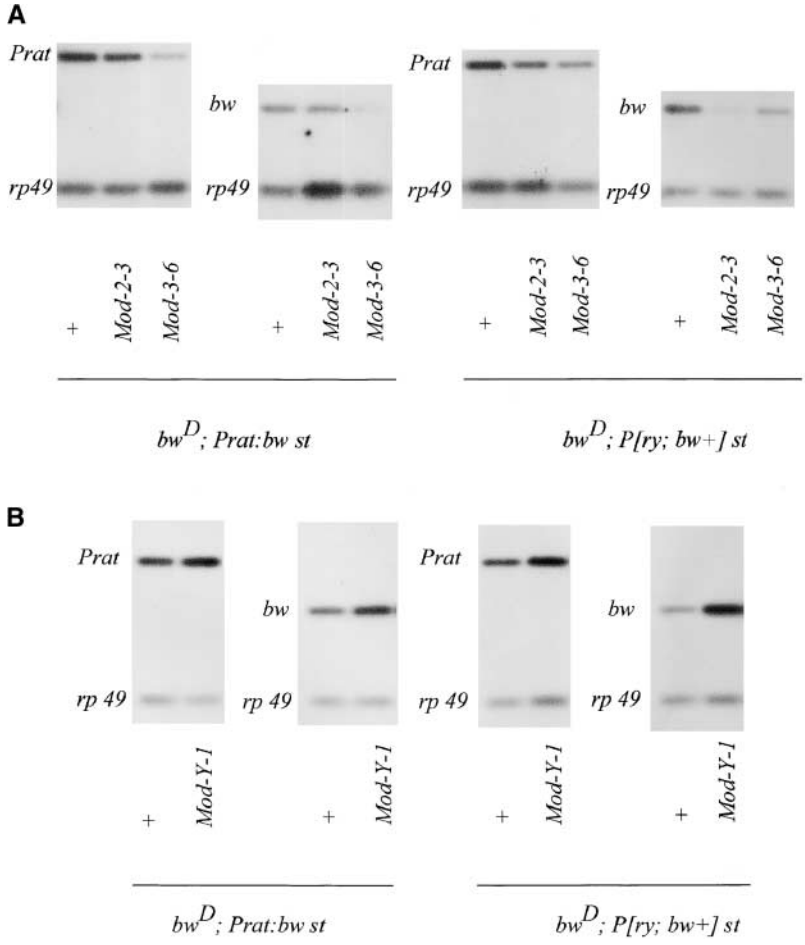

FIGURE 2.—Quantitative RT-PCR showing (A) the effect of *Mod-3-6* and *Mod-2-3* and (B) the effect of *Mod-Y-1* on endogenous *Prat* expression and on *bw* expression for both transgenes relative to the level of expression of *rp49*. The relative values obtained from these experiments are footnoted *c* in Table 2.

(*Mod-3-4*, *Mod-3-9*, and *Mod-3-11*). They are suppressors of *Prat* expression in the *Prat:bw* genetic background and enhancers in the *P[ry$^+$; bw$^+$]* genetic background. For these three modifiers, the RT-PCR results for both *bw* transgenes do not correlate with their initial isolation as suppressors of *Prat:bw* eye color.

Four modifiers do not modify *Prat* expression. For *Mod-1-3*, there is a weak suppression of *Prat* mRNA for both genetic backgrounds. The modifiers *Mod-2-4* and *Mod-3-3* reduce mRNA levels for both transgenes without affecting *Prat* mRNA levels (Table 2). *Mod-3-10* does not affect *Prat* significantly in either genetic background while it shows an enhancement of *bw* mRNA in *Prat:bw*. The specific effect of these three modifiers on *bw* transgene mRNA expression and pigment level suggests that they act through common sequences in the two constructs, the *bw* coding region, or somehow through the *ry*$^+$ marker present in both transgenes.

A subset of *Prat:bw* modifiers also modifies position-effect variegation: In Drosophila, some *trans*-acting dosage-sensitive modifiers of gene expression are also modifiers of position-effect variegation (PEV; BHADRA and BIRCHLER 1996; BHADRA *et al.* 1998). These findings reflect the relationship between the regulation of gene expression and chromatin structure. To test the possibility that some of our modifiers of gene expression are also modifiers of PEV, we examined their effects on the *bw^D/bw$^+$* variegation phenotype. Variegated expression of the wild-type copy of the *bw* gene can occur *in trans* to the *bw^D* allele. The inactivation of *bw* is thought to be due to the mislocalization of the *bw$^+$* allele to a heterochromatic compartment of the nucleus (CSINK and HENIKOFF 1996). These tests were facilitated by the fact that our modifiers were isolated in a homozygous *bw^D* background. A cross of the modifier stock to a *bw$^+$; st* stock allowed the visualization of the *bw^D/ bw$^+$* variegating pigment phenotype in the eye and thus allowed assessment of the dominant effect of the modifier on this model of variegation.

Among the 23 modifiers, 4 suppress *bw^D/bw$^+$* variegation (*Mod-Y-1*, *Mod-2-5*, *Mod-3-10*, and *Mod-3-12*) and 3 enhance *bw^D/bw$^+$* variegation (*Mod-1-5*, *Mod-3-4*, and *Mod-3-5*; Figure 3). For this experiment, note that the suppression of the *bw^D/bw$^+$* phenotype indicates an enhancement of *bw* expression while the enhancement of the phenotype indicates a suppression of *bw* expression.

Mod-Y-1 suppresses pigment levels for the two transgenes, yet it enhances mRNA levels for the transgenes and the endogenous *Prat* gene (Tables 1 and 2 and Figure 2B). The discordance between these two observa-

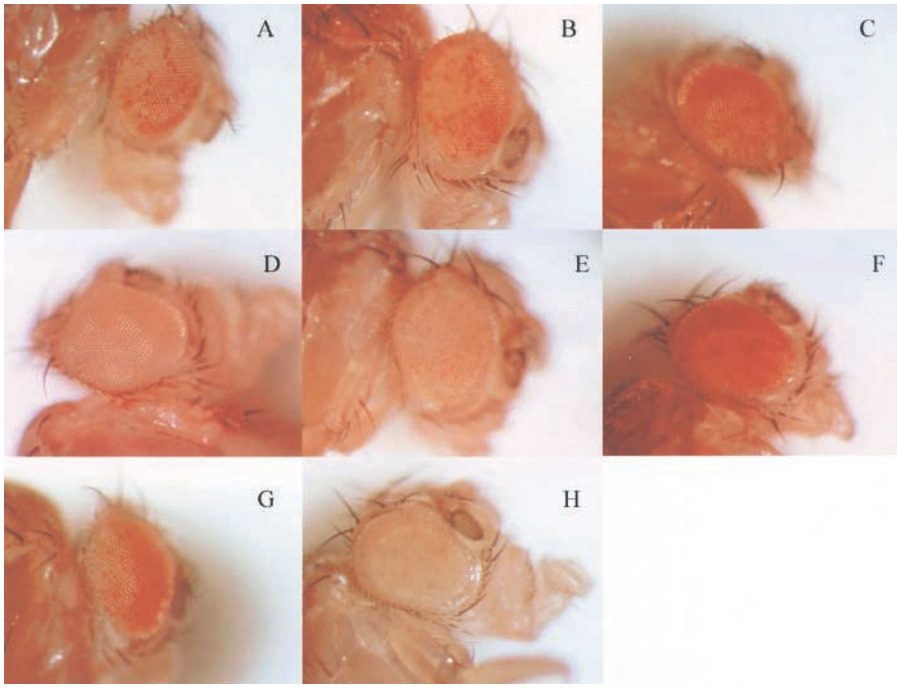

FIGURE 3.—Effect of several modifiers on $bw^D/+$ variegation. The phenotypes are shown for the genotypes (A) $bw^D/+$; *st*, (B) bw^D *Mod-2-5*/+; *st*, (C) bw^D *Mod-2-5*^{r110}/+; *st*, (D) $bw^D/+$; *Mod-3-4 st*/+ *st*, (E) $bw^D/+$; *Mod-3-5 st*/+ *st*, (F) $bw^D/+$; *Mod-3-10 st*/+ *st*, (G) $bw^D/+$; *Mod-3-12 st*/+ *st*, and (H) *Mod-1-5*/+; $bw^D/+$; *st*.

tions might be explained by the difference in sampling for pigment and mRNA. However, for a Y-linked modifier, an alternative explanation for the inconsistency between pigment suppression and mRNA enhancement, involving an effect on more than one gene, might be invoked. We were interested in determining whether *Mod-Y-1* has a general effect on PEV, so we investigated its effect on three variegating alleles: bw^D, w^{m4}, and Sb^v. Suppression of the variegating phenotype was observed for all three alleles (Figure 4). This finding correlates with the enhancement of mRNA levels that we observed for the two transgenes and for *Prat*. These results indicate that *Mod-Y-1* is an enhancer of gene expression. Changes in Y chromosome dosage have been shown to modify PEV, where an extra Y chromosome can suppress PEV (REUTER and SPIERER 1992). Thus, it is possible that *Mod-Y-1* is a chromosome rearrangement with multiple effects on gene expression. Cytological examination of larval neuroblast mitotic chromosomes showed no detectable rearrangement associated with the *Mod-Y-1* chromosome. In addition, the *Mod-Y-1* chromosome was not associated with elevated X-Y meiotic nondisjunction, nor did it display any recessive *bobbed* phenotypes in complementation tests with X-linked recessive *bobbed* alleles (D. CLARK, unpublished observations). Thus, although *Mod-Y-1* behaves as a general suppressor of PEV and as an enhancer of gene expression, it is not associated with any obvious chromosomal rearrangement involving the rDNA or the autosomes.

Mod-2-5 shows strong suppression of the bw^D/bw^+ variegated phenotype (Figure 3B). In addition, three recombinant chromosomes obtained during recombination mapping of the dominant modifier of *Prat:bw* eye color and of the recessive lethal phenotype (see below), show suppression of variegation (Figure 3C), indicating that this effect is linked with the modifier of *Prat:bw* expression.

Mod-3-4, *Mod-3-5*, and *Mod-1-5* enhanced the bw^D/bw^+ phenotype (Figure 3, D, E, and H, respectively). This finding is consistent with the suppression of *bw* expression observed for both genetic backgrounds for *Mod-3-5* and *Mod-1-5*, while *Mod-3-4* did not affect *bw* mRNA levels. Therefore, while the behavior of *Mod-3-4* is enigmatic, there is a correlation between the observed effects of *Mod-3-5* and *Mod-1-5* on the expression of transgenes, *Prat*, and bw^D/bw^+ variegation.

Mod-3-10 and *Mod-3-12* show a strong suppression of the bw^D/bw^+ variegated phenotype (Figure 3, F and G, respectively) while they were isolated as weak suppressors of *Prat:bw* eye color. While *Mod-3-10* and *Mod-3-12* do not modify *bw* expression in *P*[ry^+; bw^+], *Mod-3-10* is an enhancer of *Prat:bw* mRNA expression and *Mod-3-12* is a suppressor of *Prat:bw* mRNA expression.

Genetic mapping of dominant modifiers and their associated recessive phenotype: Sixteen modifiers are associated with a recessive lethal phenotype, 4 X-linked modifiers have no recessive lethal phenotype, 1 2-linked suppressor (*Mod-2-4*) has a recessive male sterile phenotype, 1 3-linked suppressor (*Mod-3-9*) has a recessive female sterile phenotype, and 1 is linked with the Y chromosome. *Inter se* crosses were performed on the 16 recessive lethal modifiers to determine the number of complementation groups. Two modifiers fell into one complementation group on the second chromosome and 5 fell into a complex complementation group on the third chromosome: a first subgroup consists of *Mod-3-1*, *Mod-3-2*,

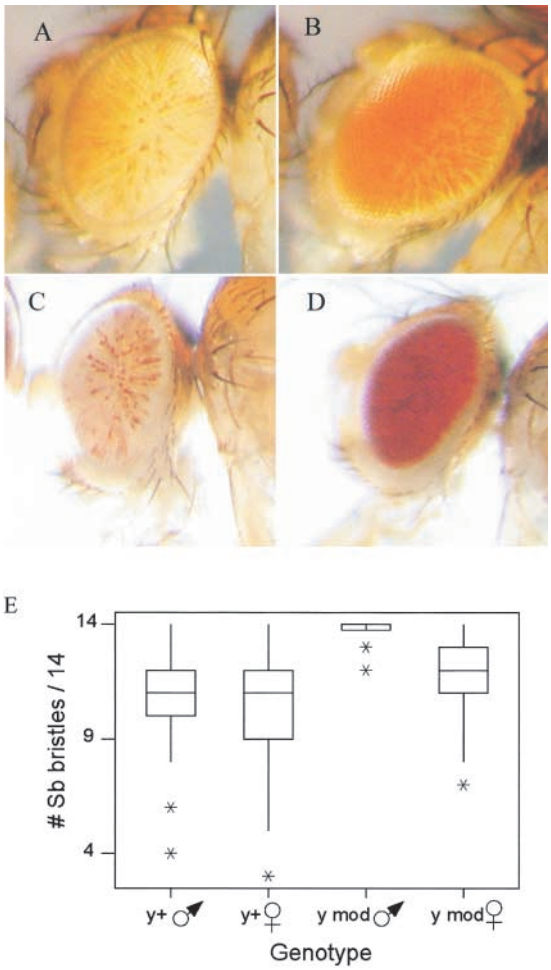

Figure 4.—Effect of *Mod-Y-1* on position-effect variegation. The phenotypes are shown for the following males: (A) bw^D/+; *st/st*, (B) *Mod-Y-1* ; bw^D/+; *st/st*, (C) w^{m4}/*Y*, and (D) w^{m4}/*Mod-Y-1*. (E) Box plot showing the effect of *Mod-Y-1* on Sb^1 variegation. The degree of suppression of Sb^1 was measured by scoring 14 thoracic macrochaetae for the presence of the stubble phenotype (Csink *et al.* 1994). The higher the proportion of stubble macrochaetae, the more suppression there is for the Sb^1 allele. "y+" males (n = 64) and females (n = 74) were the non-Cy flies derived from a cross between the +; bw^D; *st* parental strain males and Sb^1/*CyO* females. Likewise, "y mod" males (n = 58) and females (n = 87) were derived from a cross between *Mod-Y-1* ; bw^D; *st* males and Sb^1/*CyO* females.

and *Mod-3-3*, a second subgroup of *Mod-3-3* and *Mod-3-4*, and a third subgroup of *Mod-3-4* and *Mod-3-5*. These 5 modifiers could represent a single gene with some complementing alleles or there could be second-site mutations associated with some of the modifier chromosomes. The remaining 9 modifiers did not fall into a complementation group.

While for the second chromosome complementation group both alleles show a similar effect on *Prat* and *bw* expression, which correlates with the initial isolation and the pigment assay data, the third chromosome complementation group presents a more complex result. The three complementing alleles (*Mod-3-1* and *Mod-3-2* with *Mod-3-5*) show a similar effect in their visual isolation phenotypes, pigment assay, and RT-PCR results, whereas the effects of the two intercomplementing alleles (*Mod-3-3* and *Mod-3-4*) are not in relation to their visual isolation phenotype or the pigment assay data. This situation suggests that there are second-site mutations on the *Mod-3-3* and *Mod-3-4* chromosomes that affect the result obtained for these two alleles.

Modifiers were mapped that have an effect on *Prat* mRNA and an easily scored *Prat:bw* eye color. We focused on *Mod-2-3*, *Mod-2-5*, *Mod-3-6*, and the two complementation groups—*Mod-2-1* and *Mod-2-2* on the second chromosome and *Mod-3-1*, *Mod-3-2*, *Mod-3-3*, *Mod-3-4*, and *Mod-3-5* on the third chromosome—to determine whether alleles would colocalize on the genetic map. The dominant modifier phenotype was mapped by recombination and the recessive lethal phenotype was recombination and deficiency mapped. Mapping results from both approaches allowed us to determine whether there were second-site lethal mutations on the modifier chromosome. In some cases, recombinant viable dominant modifier chromosomes were retained to allow analysis of the recessive phenotype of the modifier in the absence of second-site mutations.

Mod-3-6 is linked with *h* on the left arm of the third chromosome (data not shown). Several recombinant chromosomes displayed the dominant modifier phenotype without the recessive lethal phenotype, indicating that one or more recessive lethal mutations are present on the modifier chromosome not associated with the modifier gene. The homozygous viable recombinant modifier chromosomes isolated during mapping do not show any obvious recessive phenotype. Using deficiencies for the third chromosome, the original *Mod-3-6* chromosome complements deficiencies uncovering the *h* region without any visible phenotype; however, it fails to complement three deficiencies on both arms of the chromosome [*Df (3L)emc-E12*, *Df(3R)Antp17*, and *Df(3R)p-XT103*], indicating second-site mutations on this chromosome.

For the second-chromosome complementation group consisting of *Mod-2-1* and *Mod-2-2*, both modifiers mapped between *cn* (42D) and *c* (52D) on the right arm of chromosome 2. Both modifiers fail to complement *Df (2R)stan1* (breakpoint: 46D7-9; 47F15-16), a deficiency localized between *cn* and *c*, and both modifiers complement two overlapping deficiencies, *Df(2R)X1* (breakpoint: 46C; 47A1) and *Df(2R)en-A* (breakpoint: 47D3; 48B02), suggesting that the modifier alleles are present in the cytological region 47A1-D3.

All five modifiers of the third chromosome complex complementation group, *Mod-3-1*, *Mod-3-2*, *Mod-3-3*, *Mod-3-4*, and *Mod-3-5*, mapped between the markers *th* and *sr*. No *cu sr Mod* recombinant chromosome was obtained, indicating the presence of the modifiers in the *cu* region on the right arm of the chromosome. All

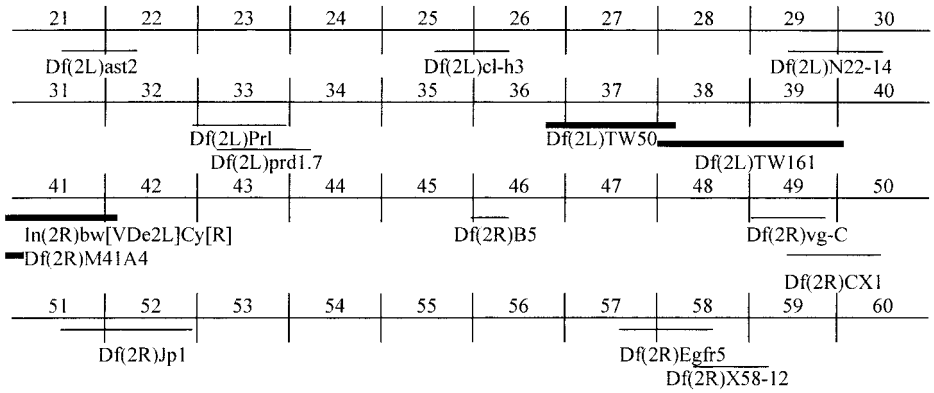

FIGURE 5.—Second-chromosome deficiency mapping of the recessive lethality associated with *Mod-2-3*. The cytological extents of the noncomplementing deficiencies are shown as horizontal lines under the cytological division(s). The thin lines represent deficiencies that fail to complement the modifier chromosome. The thick lines represent deficiencies that fail to complement the modifier chromosome and that colocalize with the recombination mapping of the dominant modifier of *Prat:bw*.

five members of the complementation group failed to complement a deficiency localized to the right of *cu*, *Tp(3,Y)ry 506-85C* (breakpoint: 87D1-2; 88E5-6). This result is consistent with the idea that the five members of the group are alleles of the same gene.

Mod-2-3 maps between the *Bl* (38B) and *cn* (42D) markers, which flank the centromere. *Mod-2-3* has a dominant effect on generation time, with a delay of 24 hr at 25°. During this mapping experiment, the extended generation time and a recessive lethal phenotype comapped with the modifier of *Prat:bw* (data not shown). Deficiency mapping showed that the *Mod-2-3* chromosome uncovers 15 lethal effects distributed over both arms of the chromosome (Figure 5). Among these deficiencies, four—*Df(2L)Tw50*, *Df(2L)Tw161*, *Df(2R)M41A4*, and *In(2R)bwVde2LCyR*—are located between the *Bl* and *cn* markers. Therefore, it remains possible that the dominant effect on generation time and the recessive lethal phenotype are independent of the *Prat:bw* dominant effect and are linked with one of the noncomplementing deficiencies present in this region. The high number of interactions of the modifier chromosome with the deficiencies used during the mapping is problematic in the mapping resolution of the modifier of *Prat:bw*. One explanation for this observation is that some of the deficiencies, rather than failing to complement recessive lethal second-site mutations on the *Mod-2-3* chromosome at all of these locations, have dominant synthetic effects with the delayed generation-time phenotype associated with the modifier chromosome.

Mod-2-5 shows a strong enhancement of *Prat:bw* eye color that maps near *bw* (59E) at the end of the right arm of the second chromosome. For three recombinant chromosomes carrying the modifier, we found a visible recessive phenotype that includes a modification of the wing shape (Figure 6). This phenotype has no relationship to the wing phenotype seen with the purine syndrome (CLARK 1994). Subsequently, deficiency mapping of the original modifier chromosome and a recombinant chromosome (*r110*) showed that both chromosomes displayed a wing phenotype (Figure 6) over *Df(2R)Px2* (breakpoint: 60C5-6; 60D9-10) and that both chromosomes complement *Df(2R)Px1* (breakpoint: 60B8-10; 60D1-02), indicating that the modifier of *Prat:bw* and the wing phenotype are localized in the region 60D to the right of *bw*.

DISCUSSION

The *Prat* gene encodes an enzyme that performs the first and limiting step in the purine *de novo* biosynthesis pathway. Characterization of the genes encoding enzymes of the pathway has been done in prokaryotic and eukaryotic species. Whereas several studies have addressed the regulation of the pathway at the enzymatic level, there is little information on the genetic regulation of the pathway available for multicellular organisms. The *D. melanogaster Prat* gene was previously characterized at the molecular and genetic levels (CLARK 1994) and a *Prat:bw* reporter gene was developed that changed the eye color from white to pale orange in a bw^{D};*st* genetic background (CLARK *et al.* 1998).

The alteration in eye color reflects *Prat* expression during the prepupal and pupal stages and during early adult development when pigment deposition occurs in Drosophila (STELLER and PIROTTA 1985; DREESEN *et al.* 1988). The *Prat* developmental profile of mRNA expression has been characterized (CLARK *et al.* 1998; CLARK and MACAFEE 2000; MALMANCHE *et al.* 2003). *Prat* expression occurs throughout development with variation in expression levels. The lowest level occurs during the second larval stage and expression is more abundant in females than in males, which corresponds to *Prat* expression in oocytes and its accumulation in the young embryo. Considering the complexity of *Prat* expression in development and the lack of molecular data on purine gene regulation in other multicellular organisms, we chose a genetic approach to identify factors with a role in *Prat* gene expression. Here, we reported the isolation and characterization of *trans*-acting dominant modifiers of the *Prat:bw* reporter gene.

The nonsaturated *Prat:bw* orange eye color allowed us to screen for both dominant enhancers and suppres-

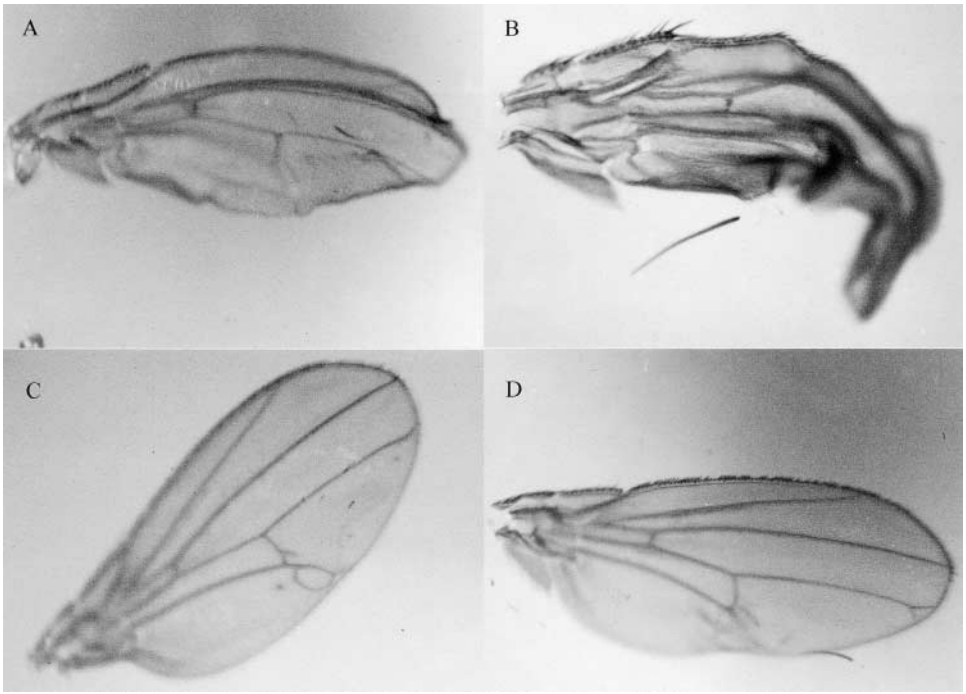

FIGURE 6.—*Mod-2-5* and *Mod-2-5^{r110}* wing phenotype. The phenotypes are shown for the following genotypes: (A and B) *Mod-2-5^{r110}* homozygotes showing the range of effect on wing shape, (C) *Mod-2-5^{r110}/Df(2R)Px2*, and (D) *Mod-2-5/Df(2R)Px2*.

sors of *Prat:bw* eye color. In an EMS mutagenesis screen for dominant modifiers of the *Prat:bw* orange eye color, we isolated two enhancers and 21 suppressors, 16 of which are single alleles. Following the screen, we quantified the specificity of the dominant modifiers for their effect on *Prat:bw* and *Prat* expression using pigment assays on 5-day-old adult fly heads and quantitative RT-PCR assays on 0- to 4-hr-old adult-fly-head mRNA. We also investigated their effect on chromatin structure by using the dominant phenotype associated with the *bw^D* allele.

The advantage to using an eye-color reporter gene in Drosophila is that it provides a simple and sensitive assay for gene expression, in which F_1 flies can be easily screened under a dissecting microscope and eye-color variants can be immediately mated without the need to establish stocks carrying each mutagenized chromosome. Eye-color reporter genes have been used in the past to identify genes involved in heterochromatin-mediated gene silencing (WEILER and WAKIMOTO 1995) and in intron splicing (PENG and MOUNT 1995), for example. Although it is difficult to assess whether *all* genes involved in the process will be identified, such as modifier genes not expressed in eye development, our screen provides us with a way to identify a subset of candidate regulatory genes, and thus with a starting point for further analysis of the genetic regulation of *Prat* and other purine genes.

The use of a *bw* reporter gene in our screen raises an issue about the interrelationship between the purine and eye-pigment pathways. The brown protein's function in the eye is to process pteridine pigment. Pteridines are synthesized from purine precursors that can be supplied from the purine *de novo* synthesis, salvage, and interconversion pathways. The issue is that we could have isolated modifiers that affect the purine or pteridine synthesis pathways, affecting *Prat:bw* and *bw* expression indirectly. Indeed, recessive mutations in *Prat* and other genes involved in GMP synthesis can have reduced pteridine eye pigment, along with a pleiotropic phenotype affecting other aspects of development called the purine syndrome. However, none of the *Prat:bw* modifiers has a dominant phenotype reminiscent of the purine syndrome. The screen was conducted in a wild-type genetic background for purine and pigment pathway genes, so a dominant gain-of-function mutation affecting one of these pathways would have to be isolated. Presumably, such a mutation would have other dominant effects on phenotype. This issue is addressed by the two-stage design of our screen, where we assess the affect of modifiers on endogenous *Prat* gene expression in the second stage, allowing us to distinguish between modifiers that have an effect on *Prat* expression and those that have an effect on eye-pigment synthesis or deposition.

In considering the effect of the modifiers on *Prat:bw* as measured by pigment assay and on *Prat* and/or *bw* as measured by RT-PCR, it is important to remember that the pigment synthesis and deposition process occurs over several developmental stages from prepupa to adult (STELLER and PIROTTA 1985; DREESEN *et al.* 1988). A modifier may affect the temporal dynamics of this process differently than the process of *Prat:bw* mRNA accumulation in young adults. Another factor

that could contribute to inconsistencies between pigment and mRNA measurements is that pigment from *P[ry$^+$; bw$^+$]* expression reflects expression in a specific cell type, whereas mRNA from *Prat:bw* expression likely reflects expression in a wider range of cell types in the adult head. For example, four modifiers of *Prat:bw* eye color did not affect *Prat* expression, indicating that some of the modifiers isolated during the screen are involved in post-translational processes such as pigment synthesis and/or transport rather than in the regulation of *Prat* expression.

Of the 23 dominant modifiers isolated during the screen, 7 modifiers show a correlation between the observed *Prat:bw* eye color, the pigment assay, and the RT-PCR results. Four additional modifiers show a correlation between the observed *Prat:bw* eye color and the *Prat* and *bw* RT-PCR results in a *Prat:bw* genetic background. Therefore, we consider these 11 modifiers of *Prat:bw* eye color to correspond to *trans*-acting factors involved in *Prat* expression. Among the remaining 12 modifiers, a group of 5 class 1 modifiers shows enhancement of *bw* and *Prat* gene expression in both genetic backgrounds even though they were isolated as suppressors of *Prat:bw* eye color in 0- to 4-hr-old adult flies and were found to be suppressors of pigment level at 5 days. On the whole, 19 of the modifiers have an effect on *Prat* expression in young adult fly heads, indicating that the use of the *Prat:bw* transgene array to recover *trans*-dominant modifiers of *Prat* expression was successful.

Most of the *Prat:bw* dominant modifiers also have an effect on *P[ry$^+$; bw$^+$]*, raising the possibility that these mutations have an effect on a more global aspect of gene expression. In yeast, three transcription factors play a role in expression of purine synthesis genes. For example, GCN4, which controls expression of many amino acid biosynthesis genes, upregulates purine gene expression in response to purine starvation (Mösch *et al.* 1991; Rolfes and Hinnebusch 1993). In humans, the transcription factor NRF1, which controls expression of cytochrome genes and other genes involved in respiration, plays a role in expression of the bidirectional promoter at the PRAT-AIRC locus (Chen *et al.* 1997). It therefore seems likely that in Drosophila the factors controlling expression of *Prat* will have wide-ranging effects on gene expression.

The *Prat:bw* modifiers may also have an effect on a more global aspect of gene expression by acting on chromatin structure. Multiple effects of *trans*-acting dominant modifiers have been described for several modifiers of the Drosophila *white* gene (Rabinow *et al.* 1991; Bhadra *et al.* 1997a,b). Some of these *white* modifiers also have an effect on chromatin organization, as demonstrated by their modification of PEV (Csink *et al.* 1994; Bhadra and Birchler 1996; Bhadra *et al.* 1998). A second possibility is that *Prat* and *bw* might be transcriptionally coregulated in the eye, perhaps due to the functional relationship between the genes as discussed above. Although *Prat* is likely to be expressed in the developing eye, there is no evidence for coordinated regulation of the purine *de novo* synthesis and pteridine transport pathways. In relation to our study, it will be of interest to follow the pattern of *bw* expression for both transgenes in comparison to wild-type *bw* and *Prat* expression from prepupa to adult to get an overview of the regulation of both transgenes at different developmental stages. This analysis would provide us with a basis from which to extend the analysis of the effect of our modifiers to different times during the process of eye-pigment deposition.

The regulation of gene expression involves the recruitment of *trans*-acting factors to chromatin and the formation of the transcriptional complex. The recruitment of *trans*-acting factors is related to chromatin structure and to the local nuclear environment in the region. Analysis of the behavior of the *white* modifiers described above has shown that the mechanisms of gene expression and chromatin organization are not exclusive processes. To examine the functional link between gene expression and chromatin organization with respect to our *trans*-acting dominant modifiers, we tested them for their effect on bw^D/bw^+ variegation.

Five of the modifiers affected bw^D/bw^+ variegation. *Mod-2-5* suppressed the bw^D/bw^+ phenotype, and a recombinant chromosome showed the same effect, indicating that the dominant modifier of *Prat:bw* and the modification of *trans*-dominant variegation are linked. In addition, deficiency mapping indicated that both the original modifier chromosome and the recombinant chromosome possess a recessive mutation in the *Df(2R) Px2* region with a wing-vein phenotype. These various properties of *Mod-2-5* will be useful for its characterization at the genetic and molecular levels.

Among the complex complementation group alleles on the third chromosome, two of the five members, *Mod-3-4* and *Mod-3-5*, enhance the bw^D/bw^+ phenotype whereas the other three, *Mod-3-1*, *Mod-3-2*, and *Mod-3-3*, show no obvious modification of the bw^D/bw^+ phenotype. Genetic mapping linked the five dominant modifiers to one deficiency, suggesting that they are all lesions in the same gene, even though there are complementing alleles and the enhancement of variegation does not always correlate with the dominant modification of *Prat:bw*. In addition, the alleles of this complementation group have different effects on *Prat:bw* and *Prat* expression. These findings will be taken into account in further genetic characterization of these alleles in an attempt to link the dominant effect on *Prat:bw* and the enhancement of the bw^D/bw^+ phenotype.

In addition to its use for the study of *Prat* regulation itself, the *Prat:bw* transgene array was used as a tool to study the general phenomenon associated with the variegation of transgene arrays (Sabl 1996). In classical PEV, the phenotype occurs as a result of chromosomal rearrangements involving heterochromatin and is often

followed using an eye-pigment gene. As well, expansion of a transgene into a multicopy array at a single site can produce a phenotype similar to classical heterochromatin-induced PEV (Dorer and Henikoff 1994). In contrast, the *Prat:bw* transgene array does not show a variegated phenotype and the polytene chromosomes of fly larvae carrying the array show a new and unknown structure resembling a dense puff (Clark *et al.* 1998). This cytological observation may be relevant to the organization of the *Prat:bw* transgene array and its copy-number-dependent expression.

We did not isolate *trans*-dominant modifiers inducing *Prat:bw* variegation. However, we did isolate two modifiers of *Prat:bw* expression for which no effect was observed for *Prat* or *P[ry$^+$; bw$^+$]*. As well, both modifiers are suppressors of *bw^D/bw$^+$* variegation. These observations lead us to think that both modifiers are involved in chromatin structure rather than in the control of gene expression. The effect of these modifiers may be linked with the unusual polytene structure observed for the *Prat:bw* multicopy array (Clark *et al.* 1998) or they could share a common mechanism of regulation associated with the *Prat:bw* insertion site.

In conclusion, we have demonstrated the effectiveness of using the *Prat:bw* transgene array as a reporter gene for isolation of dominant modifiers of *Prat* expression. We isolated 11 dominant modifiers of *Prat:bw* expression that display a similar effect on *Prat:bw* eye color and on *Prat* gene expression in young adult fly heads. An additional 5 modifiers strongly enhance *Prat* expression in contrast to their suppression of *Prat:bw* eye color. Our continued efforts to characterize these modifiers, including fine genetic mapping and studies of their effects on expression of other genes and times in development, will reveal their role in the regulation of *Prat* expression and other metabolic pathway genes.

The authors thank Amy Csink for help with analysis of *Mod-Y-1*, Nancy MacAfee for technical advice, André Breton for data analysis advice, and Roger Smith for help with imaging. We also thank anonymous reviewers for their helpful comments. This research was funded by an operating grant from the Canadian Institutes of Health Research to D.V.C.

LITERATURE CITED

Bhadra, U., and J. A. Birchler, 1996 Characterization of a sex-influenced modifier of gene expression and suppressor of position-effect variegation in *Drosophila*. Mol. Gen. Genet. **250:** 601–613.

Bhadra, U., M. Pal-Bhadra and J. A. Birchler, 1997a A sex-influenced modifier in Drosophila that affects a broad spectrum of target loci including the histone repeats. Genetics **146:** 903–917.

Bhadra, U., M. Pal-Bhadra and J. A. Birchler, 1997b A trans-acting modifier causing extensive overexpression of genes in Drosophila melanogaster. Mol. Gen. Genet. **254:** 621–634.

Bhadra, U., M. P. Bhadra and J. A. Birchler, 1998 Interactions among dosage-dependent *trans*-acting modifiers of gene expression and position-effect variegation in Drosophila. Genetics **150:** 251–263.

Birchler, J. A., U. Bhadra, L. Rabinow, R. Linsk and A. T. Nguyen-Huynh, 1994 *Weakener of white* (*Wow*), a gene that modifies the expression of the *white* eye color locus and that suppresses position effect variegation in *Drosophila melanogaster*. Genetics **137:** 1057–1070.

Chen, S., P. L. Nagy and H. Zalkin, 1997 Role of NRF-1 in bidirectional transcription of the human GPAT-AIRC purine biosynthesis locus. Nucleic Acids Res. **25:** 1809–1816.

Clark, D. V., 1994 Molecular and genetic analyses of Drosophila *Prat*, which encodes the first enzyme of *de novo* purine biosynthesis. Genetics **136:** 547–557.

Clark, D. V., and N. MacAfee, 2000 The purine biosynthesis enzyme PRAT detected in proenzyme and mature forms during development of *Drosophila melanogaster*. Insect Biochem. Mol. Biol. **30:** 315–323.

Clark, D. V., J. F. Sabl and S. Henikoff, 1998 Repetitive arrays containing a housekeeping gene have altered polytene chromosome morphology in Drosophila. Chromosoma **107:** 96–104.

Csink, A. K., and S. Henikoff, 1996 Genetic modification of heterochromatic association and nuclear organization in *Drosophila*. Nature **381:** 529–531.

Csink, A. K., R. Linsk and J. A. Birchler, 1994 The *Lighten up* (*Lip*) gene of *Drosophila melanogaster*, a modifier of retroelement expression, position effect variegation and white locus insertion alleles. Genetics **138:** 153–163.

Daignan-Fornier, B., and G. R. Fink, 1992 Coregulation of purine and histidine biosynthesis by the transcriptional activators BAS1 and BAS2. Proc. Natl. Acad. Sci. USA **89:** 6746–6750.

Dorer, D. R., and S. Henikoff, 1994 Expansions of transgene repeats cause heterochromatin formation and gene silencing in Drosophila. Cell **77:** 993–1002.

Dreesen, T. D., D. H. Johnson and S. Henikoff, 1988 The brown protein of *Drosophila melanogaster* is similar to the white protein and to components of active transport complexes. Mol. Cell. Biol. **8:** 5206–5215.

Dreesen, T. D., S. Henikoff and K. Loughney, 1991 A pairing-sensitive element that mediates *trans*-inactivation is associated with the *Drosophila brown* gene. Genes Dev. **5:** 331–340.

FlyBase, 1999 The FlyBase database of the Drosophila genome projects and community literature. Nucleic Acids Res. **27:** 85–88 (http://flybase.bio.indiana.edu).

Foley, K. P., M. W. Leonard and J. D. Engel, 1993 Quantitation of RNA using the polymerase chain reaction. Trends Genet. **9:** 380–385.

Glesne, D. A., F. R. Collart and E. Huberman, 1991 Regulation of IMP dehydrogenase gene expression by its end products, guanine nucleotides. Mol. Cell. Biol. **11:** 5417–5425.

Henikoff, S., and T. D. Dreesen, 1989 Trans-inactivation of the *Drosophila* brown gene: evidence for transcriptional repression and somatic pairing dependence. Proc. Natl. Acad. Sci. USA **86:** 6704–6708.

Henikoff, S., M. A. Keene, K. Fechtel and J. W. Fristom, 1986a Gene within a gene: nested Drosophila genes encode unrelated proteins on opposite DNA strands. Cell **44:** 33–42.

Henikoff, S., M. A. Keene, J. S. Sloan, J. Bleskan, R. Hards *et al.*, 1986b Multiple purine pathway enzyme activities are encoded at a single genetic locus in *Drosophila*. Proc. Natl. Acad. Sci. USA **83:** 720–724.

Henikoff, S., D. Nash, R. Hards, J. Bleskan, J. F. Woolford *et al.*, 1986c Two *Drosophila melanogaster* mutations block successive steps of *de novo* purine synthesis. Proc. Natl. Acad. Sci. USA **83:** 3919–3923.

Henikoff, S., J. M. Jackson and P. B. Talbert, 1995 Distance and pairing effects on the *brownDominant* heterochromatic element in Drosophila. Genetics **140:** 1007–1017.

Holmes, E. W., 1981 Kinetic, physical, and regulatory properties of amidophosphoribosyltransferase. Adv. Enzyme Regul. **44:** 215–231.

Malmanche, N., D. Drapeau, P. Cafferty, Y. Ji and D. V. Clark, 2003 The PRAT purine synthesis gene duplication in *Drosophila melanogaster* and *Drosophila virilis* is associated with a retrotransposition event and diversification of expression patterns. J. Mol. Evol. **56:** 630–642.

Mösch, H.-U., B. Scheier, R. Lahti, P. Mäntsälä and G. H. Braus, 1991 Transcriptional activation of yeast nucleotide biosynthetic gene *ADE4* by GCN4. J. Biol. Chem. **266:** 20453–20456.

O'Connell, P. O., and M. Rosbash, 1984 Sequence, structure, and

codon preference of the Drosophila ribosomal protein 49 gene. Nucleic Acids Res. **12:** 5494–5513.

Peng, X., and S. M. Mount, 1995 Genetic enhancement of RNA-processing defects by a dominant mutation in B52, the Drosophila gene for an SR splicing factor. Mol. Cell. Biol. **15:** 6273–6282.

Rabinow, L., A. T. Nguyen-Huynh and J. A. Birchler, 1991 A *trans*-acting regulatory gene that inversely affects the expression of the white, brown and scarlet loci in Drosophila. Genetics **129:** 463–480.

Reuter, G., and P. Spierer, 1992 Position effect variegation and chromatin proteins. Bioessays **14:** 605–612.

Rolfes, R. J., and A. G. Hinnebusch, 1993 Translation of the yeast transcriptional activator GCN4 is stimulated by purine limitation: implications for activation of the protein kinase GCN2. Mol. Cell. Biol. **13:** 5099–5111.

Sabl, J., 1996 Effects of repetitiveness, pairing and linkage on position-effect variegation in *Drosophila*. Ph.D. Thesis, University of Washington, Seattle.

Sambrook, J., E. F. Fritsch, and T. Maniatis, 1989 *Molecular Cloning Laboratory Manual*, Ed. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Steller, H., and V. Pirotta, 1985 Expression of the *Drosophila white* gene under the control of the *hsp 70* heat shock promoter. EMBO J. **4:** 3765–3772.

Tiong, S. Y. K., and D. Nash, 1993 The *adenosine2* gene of *Drosophila melanogaster* encodes a formylglycinamide ribotide amidotransferase. Genome **36:** 924–934.

Tiong, S. Y. K., C. Keizer, D. Nash and D. Patterson, 1989 Drosophila purine auxotrophy: new alleles of *adenosine2* exhibiting a complex visible phenotype. Biochem. Genet. **27:** 333–348.

Weiler, K. S., and B. T. Wakimoto, 1995 Heterochromatin and gene expression in Drosophila. Annu. Rev. Genet. **29:** 577–605.

Wyngaarden, J. B., and W. N. Kelley, 1983 Gout, pp. 1064–1114 in *Metabolic Basis of Inherited Disease*, Ed. 5, edited by J. B. Stanbury, J. B. Wyngaarden and D. S. Fredrickson. McGraw-Hill, New York.

Yamaoka, T., M. Kondo, S. Honda, I. Hiroyuki, M. Moritani *et al.*, 1997 Amidophosphoribosyltransferase limits the rate of cell growth-linked *de novo* purine biosynthesis in the presence of constant capacity of salvage purine biosynthesis. J. Biol. Chem. **272:** 17719–17725.

Zalkin, H. Z., and J. E. Dixon, 1992 *De novo* purine nucleotide biosynthesis. Prog. Nucleic Acid Res. Mol. Biol. **42:** 259–287.

Communicating editor: K. Golic