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Journal

Genetics, 112(2)

ISSN

0016-6731

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Publication Date

1986-02-01

DOI

10.1093/genetics/112.2.249

EVIDENCE FOR REGULATORY VARIANTS OF THE DOPA DECARBOXYLASE AND ALPHA-METHYLDOPA HYPERSENSITIVE LOCI IN DROSOPHILA

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Manuscript received June 25, 1985
Revised copy accepted October 2, 1985

ABSTRACT

We have analyzed two variants of *Drosophila melanogaster* (R^S and R^E) which lead to the dual phenotype of elevated DDC activity and increased resistance to dietary alpha-methyldopa relative to Oregon-R controls. Both phenotypes show tight genetic linkage to the dopa decarboxylase, *Ddc*, and *l(2)amd* genes (*i.e.*, < 0.05 cM distant). We find that low (Oregon-R), medium (R^S) and high (R^E and Canton-S) levels of DDC activity seen at both pupariation and eclosion in these strains are completely accounted for by differences in accumulation of DDC protein as measured by immunoprecipitation. Genetic reconstruction experiments in which Ddc^+ and amd^+ gene doses are varied show that increasing DDC activity does not lead to a measurable increase in resistance to dietary alpha-methyldopa. This suggests that the increased resistance to dietary alpha-methyldopa is not the result of increased DDC activity but, rather, results from increased $l(2)amd^+$ activity. Both cytogenetic and molecular analyses indicate that these overproduction variants are not the result of small duplications of the *Ddc* and *amd* genes, nor are they associated with small (≥100 bp) insertions or deletions. Measurements of DDC activity in wild-type strains of Drosophila reveal a unimodal distribution of activity levels with the Canton-S and R^E strains at the high end of the scale, the Oregon-R control at the low end and R^S near the modal value. We conclude that accumulated changes in a genetic element (or elements) in close proximity to the Ddc^+ and amd^+ genes lead to the coordinated changes in the expression of the *Ddc* and *amd* genes in these strains.

LOCALIZATION and characterization of mutations which lead to altered gene regulation can identify elements involved in control of gene expression and reveal aspects of the mechanisms of control. In metazoans, lesions are frequently recovered which lead to moderate alterations in the level of gene expression. Some of these affect the temporal pattern of expression (*e.g.*, aldehyde oxidase, DICKINSON 1975; dopa decarboxylase, ESTELLE and HODGETTS 1984a, b) or the tissue specificity of expression (*e.g.*, amylase, DOANE *et al.* 1983; xanthine dehydrogenase, CLARK *et al.* 1984). However, most of the

Abbreviations: DDC = Dopa decarboxylase enzyme; cM = centimorgan; CRM = Cross-reacting material.
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regulatory mutants described in Drosophila, as well as in other metazoans, have been recovered as naturally occurring variants from various populations or strains rather than from direct mutagenesis (*e.g.*, in Drosophila, RABINOW and DICKINSON 1981; DOANE 1980; ESTELLE and HODGETTS 1984a; in mice, PAIGEN 1979; and in maize, CHANDLEE and SCANDALIOS 1984). LAURIE-AHLBERG *et al.* (1982) have suggested that naturally occurring polymorphisms of both *cis*- and *trans*-acting activity modifiers may provide an important source of variation for adaptive evolutionary change. However, few putative *cis*-acting activity regulators have actually been subjected to the detailed analysis necessary to distinguish between altered gene expression and structural alterations. We have examined low, medium and high activity variants of the *Ddc* and, possibly, *amd* genes to determine whether these changes might be due to altered gene regulation.

Dopa decarboxylase (DDC) (EC 4.1.1.26) in Drosophila is under stringent tissue and temporal control (LUNAN and MITCHELL 1969; MARSH and WRIGHT 1980) and is vital to the organism (WRIGHT, BEWLEY and SHERALD 1976). The *l(2)amd(amd)* gene is located immediately adjacent (0.002 cM) to the dopa decarboxylase gene (*Ddc*) and is functionally related to *Ddc* by virtue of its interaction with alpha-methyldopa (alpha MD) and by its affect on the cuticle. Mutants of the *amd* locus do not affect *Ddc* activity in any tissue, nor do they affect soluble phenol oxidase or dopamine acetyltransferase activity (MARSH and WRIGHT 1979). However, embryos homozygous for *amd* die at the embryonic/larval boundary exhibiting exceptionally friable cuticles and necrotic anal organs, suggesting a role in cuticle formation (WRIGHT 1977). The amd^+ product is required for resistance to dietary administration of structural analogs of dopa (*e.g.*, alpha-methyldopa). Although a large number of induced lethal alleles (50 for *Ddc* and 33 for *amd*) have been recovered (WRIGHT *et al.* 1982), none of these have yet been identified as alterations in noncoding regulatory elements. We have observed natural activity variants that lead to both elevated dopa decarboxylase activity and to increased resistance to dietary alpha-methyldopa relative to Oregon-R controls. In this report we examine the possible regulatory nature of these variants and document their affects on the level of activity of the Drosophila *Ddc* and *amd* genes. Specifically, we determined that the increases in DDC activity are completely accounted for by increased levels of DDC crossreacting material and not from a greater catalytic efficiency of the enzyme. Genetic reconstruction experiments suggest that the increased resistance to dietary alpha-methyldopa most likely results from increased expression of the amd^+ gene product rather than from the increase in DDC activity. Cytogenetic and molecular analysis indicates that the altered regulation in these variants results from accumulated changes in a genetic element (or elements) which affect the expression of both the *Ddc* and *amd* genes.

MATERIALS AND METHODS

Drosophila strains: The variant in the R^S strain was originally identified in a survey of 17 laboratory stocks with altered adult pigmentation. Young adults of a $speck^2$

blistered^2 stock (*sp*^{2}, 2-107 and *bs*^{2}, 2-107.3; LINDSLEY and GRELL 1968) had higher DDC activity and a higher LD50 to dietary alpha MD than Oregon-R-derived controls (SHERALD and WRIGHT 1974). The variant in the R^E strain that also increases both DDC activity and resistance to alpha MD was originally recovered as an alpha MD resistant line (RM1) from an EMS mutagenesis screen using a strain that was presumed to be isogenic for the Oregon-R6 second chromosome (SHERALD and WRIGHT 1974).

The chromosomes used in this study were placed in a common genetic background to minimize variation. The R^S, R^E and C1A (Oregon-R-derived control) strains are derived, respectively, from the S, R and C strains of SHERALD and WRIGHT (1974). The S, R and C strains had their first and third chromosomes replaced by chromosomes from an isogenic Oregon-R strain. The C strain was also made isogenic for a lethal free second chromosome from the Oregon-R strain. For this study, the R^S, R^E and C1A strains were constructed by recovering exchange events which replaced all of the second chromosome, except the 1.5 map units between *rdo* and *pr*, with the lethal-free second chromosome from the Oregon-R-derived C strain. Analysis of restriction site polymorphisms by blotting of genomic DNA from the different strains indicated that both the R^E nd R^S strains exhibit restriction site polymorphisms which are similar to the Canton-S strain and are unlike the Oregon-R-derived strains.

The Oregon-R and Canton-S lines derive from the Yale stock collection and have been maintained for 15 yr at the University of Virginia. The wild-type strains of *D. melanogaster* and one strain of *D. simulans* were obtained from the Bowling Green Drosophila Stock Center. These strains originate from widely separated geographical locations, but have been maintained as laboratory cultures for many years. Chromosomal aberrations used include the following: *Df(2L)TW130* = Df(2L)37B9-C1;37D1-2, *Df(2L)TW158* = Df(2L)37B2-8;37E2-F4. *Dp(2;1)C239* = Dp(2;1)7A-B;36C;39E. *Dp(2;1)AT:* New order = 1A-7A/36D1,2-37D1,-2/5A-20. *Dp(2;Y)H*1,36B4-37F/39C-40F. For a description of other genetic markers see LINDSLEY and GRELL (1968).

Resistance to dietary alpha-methyldopa: Food containing alpha-methyldopa was prepared by autoclaving dried yeast-agar-dextrose medium (CARPENTER 1950) for 30 min at 15 p.s.i., then adding 10 ml of Tegosept M (methyl *p*-hydroxybenzoic acid, 10% in 95% ethanol) and 10 ml propionic acid (0.5%) to 2 liters of food, followed by 40 ml ascorbic acid neutralized with sodium bicarbonate (5 mg ascorbate + 2.5 g bicarbonate/100 ml H_2O) to prevent breakdown of alpha MD in the food. When the food temperature had dropped to *ca.* 50°, the inhibitor was added as an aqueous solution, and the food was poured immediately (SPARROW and WRIGHT 1974).

To determine the LD50 of alpha MD, 50 or 100 eggs from flies of the appropriate genotype were placed on a piece of moist, dark blotting paper in a dairy creamer containing fresly made inhibitor-bearing food. The results are expressed as the percentage of hatched eggs surviving to eclosion relative to the number surviving in the absence of inhibitor. The data for *amd*^{H1}, R^E, and C1A (Figure 1) represent the means of four determinations, each involving over 300 eggs per inhibitor concentration. The data for R^S and d2 represent the means of two such determinations.

Animals with varying doses of *Ddc*$^+$ (Figure 3) were obtained by collecting eggs from a mating of *rdo hk Ddc*^{n8}*pr cn/CyO* females to *Dp(2;Y)H1; rdo hk Ddc*^{n7}*pr/Df(2L)TW130,rdo pr cn* males, and resistance to various concentrations of alpha-methyldopa was determined as described above. Survival of each genotype on the various concentrations of alpha-methyldopa is reported as percentage of survival relative to 100% survival of each genotype on standard food without inhibitor. *Dp(2;Y)H1* (HODGETTS 1980) is a duplication carrying a normal copy of *Ddc*$^+$ and all the genes in the deficiency region [*Df(2L)TW130*] and is present in all males from this cross. *Df/CyO* heterozygotes have curly wings and bright orange eyes resulting from the interaction of the two eye-color mutations, *pr* and *cn*. The duplication covers the entire deficiency region plus *rdo* and *hk*, but not *pr* or *cn*. The gene dose ratios for this cross are given with the genotypes and phenotypes in the legend to Figure 3.

To obtain animals with varying doses of *amd*$^+$ as shown in Figure 4, eggs were

collected from a mating of *l(2)amd^{H60}cn bw/CyO* females to *Dp(2;Y)H1; dp b amd^{H121} pr / Df(2L)TW130,rdo pr cn* males, and resistance to various concentrations of alpha-methyldopa was determined as described above. The gene dose ratios, phenotypes and genotypes for the cross in Figure 4 are given in the legend.

For the data shown in Figure 5, eggs were collected from matings of *rdo hk Ddc^{n8}pr cn/CyO* males to both *rdo hk Ddc^{n5}pr/CyO* and *rdo hk Ddc^{n1}pr/CyO* females. The progeny of these crosses include partially complementing heterozygotes with <5% DDC activity. Control genotypes tested in parallel on the same food include *Ddc^{n1}/CyO*, *Ddc^{n5}*/CyO and *Ddc^{n8}/CyO*, each with <50% activity (data not shown), as well as the double heterozygotes of two fully complementing lethals in nearby genes; namely *rdo hk l(2)37Ca^{1} pr/rdo hk l(2)37Cb^{1} pr* (shown as +/+ in the figure) and the parental genotypes *rdo hk l(2)37Ca^{1}pr/CyO* and *rdo hk l(2)37Cb^{1} pr/CyO* (data not shown). *l(2)37Ca* and *l(2)37Cb* are <0.01 cM proximal to *Ddc*. All five mutations were induced in the same parental chromosomes in the same screen. DDC activities are n5/n8 = 3.7 ± 1.5%; n8/n1 = 2.7 ± 2.3% (WRIGHT *et al.* 1982) and n8/CyO = 40%; n5/CyO = 33%; n1/CyO = 33%; l(2)37Ca^{1} or l(2)37Cb^{1}/CyO = 100% (WRIGHT, BEWLEY and SHERALD 1976).

DDC determinations: Crude extracts were prepared at about 3 mg/ml protein (*i.e.*, about ten 0–2-hr adults or white prepupae/ml) in 0.1 M phosphate buffer, pH 7.1, 0.3 M sucrose, and 0.2 mM phenylthiourea to prevent melanin formation. DDC enzyme activity was determined in triplicate by the micro-liquid-cation exchange assay of MCCAMAN, MCCAMAN and LEES (1972), with slight modifications. Protein was assayed by the method of LOWRY *et al.* (1951) using four replicates per homogenate. Typical activity in the Oregon-R control strain C1A was 12 pmol dopamine/30 min/μg protein for a signal of *ca.* 4600 cpm as assayed.

In control experiments for Figures 3 and 4, DDC-specific activity was determined in homogenates of flies bearing 1, 2 and 3 copies of the *Ddc^{+}* gene. One dose flies were *Df(2L)TW130, rdo pr cn/CyO* and *Df(2L)TW158/CyO*. Two doses were Canton-S; *Dp(2;1)C239*; *Df(2L)130,rdo pr cn/CyO*; *Dp(2;1)AT;Df(2L)TW130,rdo pr cn/CyO*; *Dp(2;Y)H1;Df(2L)TW130,rdo pr cn/CyO*, and three doses were *Dp(2;1)C239;+/CyO* and *Dp(2;Y)H1;+/CyO*. In all cases, DDC activity is a quantal function of gene dose.

Preparation of antibodies: DDC was purified from larvae approximately 200-fold by slight modifications of the method of CLARK *et al.* (1978). The most purified fractions were electrophoresed on preparative nondenaturing 7.5% acrylamide gels, stained briefly (5 min) in Coomassie blue, 50% methanol, 7% acetic acid at 37°, and destained 1 h at 37° in distilled H_2O. Portions of longitudinal slices were assayed for DDC activity. A single band giving >85% of all activity detected on the gel and containing an estimated 50 μg or less of DDC was lyophilized, crushed in a mortar and resuspended in PBS (0.15 M NaCl, 10 mM phosphate, pH 7.1).

Preimmune serum was collected on day 0 for controls. On day 3, rabbits were injected intramuscularly with a 1:1 mixture of acrylamide suspension and Freund's adjuvant, and a sample of serum (5 ml) was collected on day 25. On day 27, a booster of acrylamide suspension without adjuvant was administered intradermally, and 50 ml of serum was collected from fasted animals 9 days later, on day 35. This regimen gave a high titer of anti-DDC activity. Subsequent boosts were administered on day 66 and 388, followed by bleeding 7 and 8 days later, respectively. IgG was partially purified by 50% ammonium sulfate precipitation.

Precipitation assay: IgG was diluted in 0.5 M phosphate buffer, pH 7.1, and 5 μl of diluted IgG mixed with 20 μl DDC supernatant, incubated 2 h at 37° and centrifuged 5 min in an Eppendorf microfuge to precipitate antibody-antigen complexes. Supernatant (3 μl) was assayed in a 10 μl reaction mix, as above, in triplicate to determine the amount of DDC remaining in solution. Control experiments in which the pellet was resuspended and assayed revealed >99% of the DDC activity remaining in the pellet. Thus, the antibodies precipitate but do not inactivate the enzyme.

DNA analysis: DNA was prepared by a scaled-up version of the method of BENDER, SPIERER and HOGNESS (1983). Restriction digests employed a core buffer suggested by

P. O'FARRELL that gives final concentrations of 33 mM Tris-acetate, pH 7.9, 66 mM potassium acetate, 10 mM $MgCl_2$, 0.5 mM DTT (dithiothreitol) and 100 μg/ml BSA. Electrophoresis and blotting were performed as described by SOUTHERN (1975). Nick-translation was performed as described by RIGBY *et al.* (1977). Hybridization was in 50% formamide (MCB), 5× SSC, 1× Denhardt's solution, 10% dextran sulfate, 100 μg/ml salmon sperm DNA and 40 mM sodium phosphate, pH 6.8, at 42° for at least 16 h. Filters were washed at 65° in successive 500-ml 30-min washes of 0.1 SDS in 3× SSC, 1× SSC, 0.3× and, finally, 0.1× SSC, followed by drying and exposure to film.

RESULTS

SHERALD and WRIGHT (1974) identified two strains which exhibit the dual phenotype of elevated resistance to dietary alpha MD and elevated DDC activity relative to a carefully constructed Oregon-R control strain. Preliminary mapping localized these overproducer phenotypes to the general vicinity of the *Ddc* gene, which is known to be closely flanked by other genes affecting catecholamine metabolism, thus making them possible candidates for regulatory variants of the *Ddc* and/or other genes in this region. To determine whether the overproduction phenotypes result from *cis*-acting regulatory effects on *Ddc* or other genes, we have subjected the two overproducers (R^E and R^S), the Oregon-R-derived control chromosome (C1A) and a Canton-S strain to more detailed examination.

To test whether the differences in DDC levels between the R^E, R^S and Oregon-R derived C1A strains are due to alterations in contiguous control elements or to changes in the structural gene or at other loci, we determined (1) whether the overproduction element(s) is located close to the *Ddc* structural gene, (2) whether the product of the structural gene (*i.e.*, DDC) is altered and (3) whether the number of enzyme molecules produced is elevated in the overproducer strains.

Mapping overproducer phenotypes: Chromosome substitutions from the original isolates indicated that both the DDC overproduction and alpha MD resistance phenotypes were due to genetic variations located within the *rdo-pr* regions of both the R^E and R^S strains. To locate R^E more precisely, we mapped the DDC overproduction relative to *rdo* (2-53.1), *hk* (2-53.9) and *pr* 2-(54.5). Homozygous *rdo* $hk^+(R^E)pr/rdo^+hk(R^{E+})pr^+$ females were mated to *rdo* $hk(R^{E+})pr$ *cn* males. Eleven recombinants between *rdo* and *hk* and 12 recombinants between *hk* and *pr* were recovered, made homozygous and assayed for DDC activity. Six recombinants were *rdo* $hk(R^{E+})pr^+$, five were *rdo*$^+$$hk^+(R^E)pr$, seven were *rdo* $hk^+(R^E)pr^+$, and four were *rdo*$^+$$hk(R^{E+})pr$. One recombinant was recovered between *hk* and R^E (*i.e.*, *rdo* $hk^+R^{E+}pr^+$) indicating that R^E is proximal to *hk*. Since only one of the 12 exchanges in the *hk* to *pr* interval (0.6 cM) fell between *hk* and R^E, we conclude that R^E lies approximately 0.05 cM (1/12 × 0.6) proximal to *hk*. This location places the genetic change(s) responsible for R^E very close to the *Ddc*$^+$ locus, which has been estimated to be 0.025 cM proximal to *hk* (WRIGHT *et al.* 1981).

The DDC enzyme is not altered in R^E and R^S: To test whether the increased DDC activity is due to an alteration in the structural gene, the DDC from both the R^E and R^S strains was tested for *in vitro* thermostability and *in*

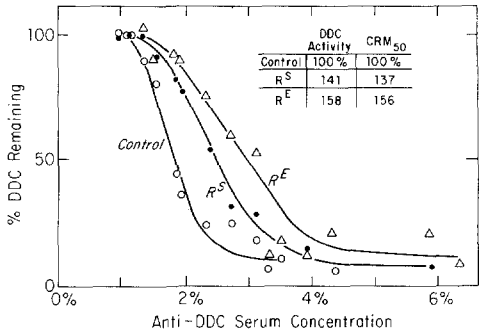

FIGURE 1.—Titration of DDC-CRM in the R^E and R^S overproducers. Crude extracts were prepared from the Oregon-R-derived control (C1A) and the overproducers R^E and R^S and were incubated with various dilutions of anti-DDC antiserum, as described in MATERIALS AND METHODS. After precipitating the antibody complexes, the amount of DDC activity remaining in the supernatant was determined. The overproducers show increased CRM relative to the control, which closely parallels the increase in enzyme activity.

vivo inhibition by alpha MD, and no differences were found. Mixtures of crude extracts showed additive activity, suggesting that the increased activity was not due to the presence of activators or absence of inhibitors (SHERALD and WRIGHT 1974).

Elevated activity is correlated with elevated cross-reacting material (CRM): To determine whether the elevated DDC activity was accompanied by an increase in the amount of DDC protein, we raised antiserum against Drosophila DDC, which precipitates but does not inactivate the enzyme. We found that the amount of anti-DDC CRM was increased to a similar extent as the DDC activity in homozygous R^S and R^E flies (Figure 1) and larvae (data not shown). Homozygous R^E elevates DDC activity 58% above Oregon-R controls and elevates DDC-CRM 56%, whereas homozygous R^S elevates DDC activity 41% and CRM 37%. Therefore, in these two strains, increased DDC activities are the result of increased levels of DDC protein.

Resistance to dietary alpha-methyldopa: Since the R^E, R^S and Canton-S strains all exhibit elevated DDC activity relative to Oregon-R, we compared the resistance of these strains to dietary alpha MD. In Figure 2, the survival of RE and RS is compared to that of the Oregon-R-derived control strain (C1A) and to the survival of a strain heterozygous for a dominant alpha-methyldopa hypersensitive mutant at the *amd* locus. The R^S strain shows moderately elevated resistance to alpha MD, whereas the R^E strain is resistant to concentrations of alpha MD at least twice as high as those which kill the Oregon-R-derived control (C1A). Similar studies (not shown) indicate that the LD50 of the Canton-S strain is approximately twice as high as the Oregon-R-derived control strain, suggesting that Canton-S is also an overproducer of the amd^+ gene product. Thus, Canton-S, R^E and R^S all exhibit elevated levels of DDC activity as well as increased resistance to dietary alpha MD relative to the Oregon-R-derived control.

Can the increased resistance to dietary alpha MD be accounted for by the

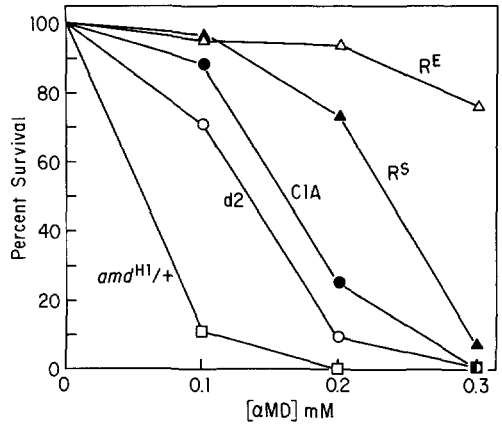

FIGURE 2.—Resistance of R^E, R^S and the Oregon-R-derived control (C1A) to alpha-methyldopa. Eggs were collected from the *rdo* R^E *pr*, *rdo* R^S *pr*, C1A, amd^{H1} *cn bw/SM5* and d2 strains and were placed on food containing increasing concentrations of alpha MD. The percentage of hatched eggs surviving to adulthood is plotted on the ordinate. The amd^{H1}*cn bw/SM5* strain carries a dominant alpha MD hypersensitive allele, amd^{H1}, and serves as a control for the alpha MD in the food. The d2 strain is a second control strain in addition to C1A, in which the *rdo-pr* region from a different Oregon-R strain (Oregon-R-CH) was inserted into the *C* chromosome.

increase in DDC activity, or does it indicate overproduction of a second gene product? All of the strains tested exhibit the dual phenotype of increased DDC activity and increased resistance to alpha MD. To determine whether increased DDC activity alone could account for the dual phenotype, we determined the resistance to dietary alpha MD of a series of strains in which the gene copy number of amd^+ and Ddc^+ was varied independently. Since this is accomplished by using chromosomal duplications, deficiencies and mutations, the dose of one gene can be varied uniquely, but the dose of the other gene is varied in concert with that of several other genes within the deficiency region (*i.e.*, 18 identified genes). In particular, all males carried *Dp(2;Y)H1* (duplicated for 36B4 to 37F and 39C to 40F; HODGETTS 1980) which contributes normal copies of Ddc^+ and amd^+ as well as a number of other genes in the duplicated region. The doses of Ddc^+ and amd^+ were varied by manipulating the number of noncomplementing null alleles of these genes in the genome. In some cases both *Ddc* and *amd* were reduced in concert by substituting the *Df(2L)TW130* (37B9-C1;37D1-2) chromosome for a normal second chromosome in the strain. Thus, three elements are being varied in these crosses, two well defined (*i.e.*, Ddc^+ and amd^+) and one less well-defined element (namely, the set of *ca.* 16 other genes in the region, designated as "flanking" genes in the figures and tables). It was not possible to control completely the background genome in these crosses. The details of each cross are described in the figure legends and the MATERIALS AND METHODS section.

To determine whether increased DDC activity can lead to increased resistance to dietary alpha MD, we constructed strains bearing one or two doses of Ddc^+ on a background of two or three doses of amd^+ and surrounding genes. These strains have 50% and 100%, respectively, of normal DDC activity (see

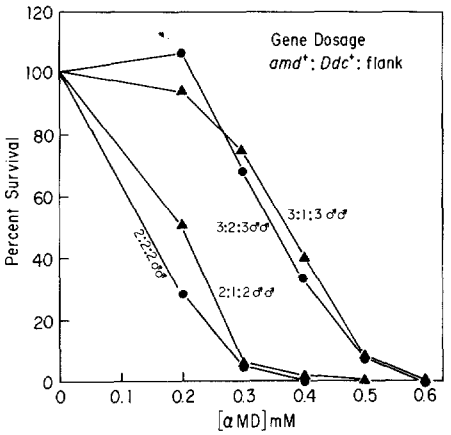

FIGURE 3.—Effect of various ratios of *amd*$^+$, *Ddc*$^+$ and 16 flanking genes in the 37B10-D1 region on resistance to dietary alpha-methyldopa. Progeny from the mating of *rdo hk Ddc*^{n8}*pr cn/CyO* females to *Dp(2;Y)H1; rdo hk Ddc*^{n7}*pr/rdo Df(2L)130 pr cn* males were tested for resistance to various concentrations of alpha-methyldopa, as described in the MATERIALS AND METHODS section. Progeny and gene dose ratios from the cross are given below.

Gene ratio	
amd$^+$:*Ddc*$^+$:flank	Male genotypes
2:1:2	*Dp;Df130/Ddc*^{n8}
2:2:2	*Dp;Df130/CyO*
3:1:3	*Dp;Ddc*^{n7}*/Ddc*^{n8}
3:2:3	*Dp;Ddc*^{n7}*/CyO*

The ratio column refers to the relative doses of *amd*$^+$ to doses of *Ddc*$^+$ to doses of all the flanking genes in the *Df(2L)TW130* region (*i.e.*, 16 flanking genes).

below). The survival of each of these strains as a function of concentration of alpha MD in the food is shown in Figure 3. Male larvae with two *Ddc*$^+$ genes are not more resistant than those with a single *Ddc*$^+$ gene on a background of either two or three doses of *amd*$^+$ and surrounding genes (compare Figure 3: 3*amd*$^+$:2 *Ddc*$^+$:3 flanking *vs.* 3:1:3 and 2:2:2 *vs.* 2:1:2). Therefore, doubling the dose of *Ddc*$^+$ from one to two does not result in a measurable increase in alpha MD resistance.

Next, we constructed strains with variable doses of *amd*$^+$ on a background of two or three doses of *Ddc*$^+$ and surrounding genes. These data (Figure 4) show that addition of a single dose of *amd*$^+$ on a two-dose background of *Ddc*$^+$ and surrounding genes leads to a substantial increase in resistance (*e.g.*, 1:2:2 males *vs.* 2:2:2 males). The same is true on a three-dose background, since 2:3:3 male larvae bearing two doses of *amd*$^+$ are more resistant than are 1:3:3 male larvae bearing only one dose of *amd*$^+$. These data indicate that the level of *amd*$^+$ activity strongly influences the degree of resistance to dietary alpha MD, irrespective of *Ddc*$^+$ dose.

If we compare the resistance of male larvae bearing two doses of everything (2:2:2 males) with that of larvae bearing two doses of *amd*$^+$, but three doses of *Ddc*$^+$ as well as three doses of each of the other genes within deficiency

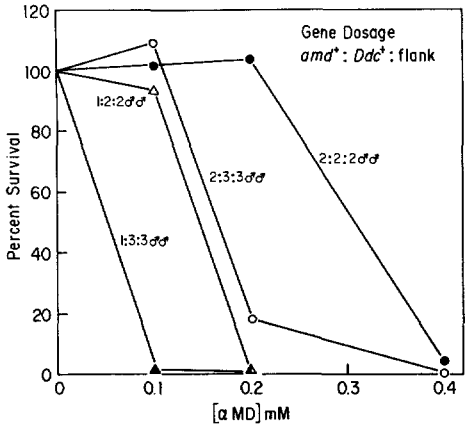

FIGURE 4.—Effect of various ratios of *amd*$^+$, *Ddc*$^+$ and 16 flanking genes in the 37B10-D1 region on resistance to dietary alpha-methyldopa. Progeny from the mating of *l(2)amd*^{H60}*cn bw/CyO* females to *Dp(2;Y)H1; dp b amd*^{H121} *pr/Df(2L)130,rdo pr cn* males were tested for resistance to various concentrations of alpha-methyldopa, as described in the MATERIALS AND METHODS section. The progeny and gene dose ratios produced from this cross are given below.

Gene ratio		Ratio of
amd$^+$:*Ddc*$^+$:*flank*	Male genotypes	normal:mutant *amd* alleles
1:2:2	*Dp;Df130/amd*^{H60}	1:1
1:3:3	*Dp;amd*^{H121}*/amd*^{H60}	1:2
2:2:2	*Dp;Df130/CyO*	2:0
2:3:3	*Dp;amd*^{H121}*/CyO*	2:1

The ratio column refers to the relative doses of *amd*$^+$ to doses of *Ddc*$^+$ to doses of all the flanking genes in the *Df(2L)TW130* region (*ca.* 16). The last column gives the ratio of normal and mutant *amd* alleles. If the *amd* protein functions as a dimer, the proportion of nonmutant dimers would be 1/4 in 1:1 animals, 1/9 in 1:2 animals, and 4/9 in 2:1 animals.

Df(2L)TW130 (*i.e.*, 2:3:3 males), we find that the larvae with increased DDC are not more resistant, but in fact are more sensitive to alpha MD than their two-dose counterparts. Comparison of the 1:2:2 males *vs.* the 1:3:3 males shows a similar phenomenon. Furthermore, the reciprocal case of decreasing DDC activity to approximately 5% of normal by the use of partially complementing heterozygotes (*e.g.*, *Ddc*^{n5}*/Ddc*^{n8}) (Figure 5) does not result in greater sensitivity to alpha MD. In fact, reduction of DDC activity to 5% leads to a slight increase in resistance to alpha MD. These observations support the conclusion that the level of *Ddc*$^+$ activity does not strongly influence the resistance to dietary alpha MD and that the increased resistance of the overproducer strains, R^E and R^S, cannot be due to the increase in DDC activity in those animals.

Do the overproducer phenotypes result from a gene duplication, insertion or deletion? Since *Ddc* and *amd* are so tightly linked, the dual phenotype of elevated resistance and elevated DDC activity might be the result of a small duplication for the two genes. Three lines of evidence argue against a duplication. First, no duplication is visible in cytological preparations, so that a duplication, if present, must be small (*e.g.*, less than *ca.* 30 kb).

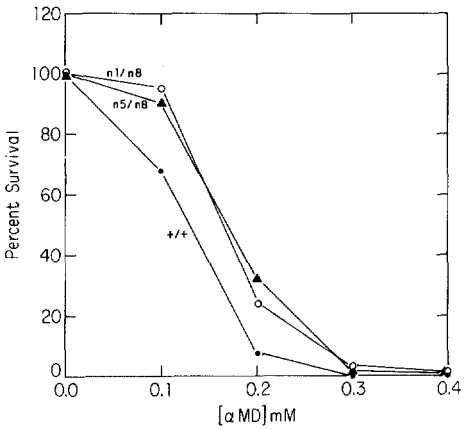

FIGURE 5.—The sensitivity of partially complementing heterozygotes of *Ddc* was determined relative to a series of controls. The heterozygotes (*i.e.*, Ddc^{n1}/Ddc^{n8} and Ddc^{n5}/Ddc^{n8}) have less than 5% of the normal DDC activity. Crosses and resulting genotypes are given in the MATERIALS AND METHODS section.

Second, Ddc^{+} activity is a quantal function of gene dose (HODGETTS 1975), so that flies with three doses of Ddc^{+} have 150% of the activity of normal two-dose flies. To confirm this observation, we measured DDC activity using several chromosomal aberrations (MATERIALS AND METHODS) in flies bearing one, two and three doses of Ddc^{+}. If the resistant strains were duplications for the Ddc^{+} locus, we would expect to see 200% of normal activity in R^S and R^E homozygotes. In fact, we see approximately 140% and 160%, respectively.

Third, a comparison of the restriction maps of R^E, R^S, the Oregon-R-derived control C1A and Canton-S over approximately 40 kb in the vicinity of *Ddc*, using whole genome DNA blots, revealed no novel restriction fragments which might suggest the presence of a duplication, or a small (*ca.* >100 bp) deletion or insertion in this region. These observations indicate that the altered levels of *Ddc* and *amd* expression are not associated with a duplication, nor are they correlated with an insertion of a mobile genetic element or a small deletion near these genes. One change in restriction pattern was observed with *Hin*dIII (Figure 6) that correlated with the known restriction site polymorphism between Canton-S and Oregon-R. A second more distal restriction site polymorphism between the strains was noted with *Eco*RI, where Oregon-R shows 4.2 and 3.2-kb fragments which appear as a single 7.4-kb fragment in R^E and R^S (Figure 7), as well as in Canton-S (not shown). The presence of the 1.2- and 1.8-kb *Hin*dIII restriction fragments in both R^E and R^S at coordinate +4 kb (Figure 6), and the fusion of the 4.2- and 3.2-kb *Eco*RI restriction fragments to a 7.4-kb fragment at coordinate −14.2 kb (Figure 7), indicate that both R^E and R^S exhibit the Canton-S-like restriction site haplotype.

We can find no evidence for a duplication, deletion or an insertion in either the R^S and R^E strains at the cytological or molecular level. Thus, we conclude that the increased DDC activity and increased resistance to alpha MD are the result of elevated expression of both *Ddc* and *amd* in these strains and that

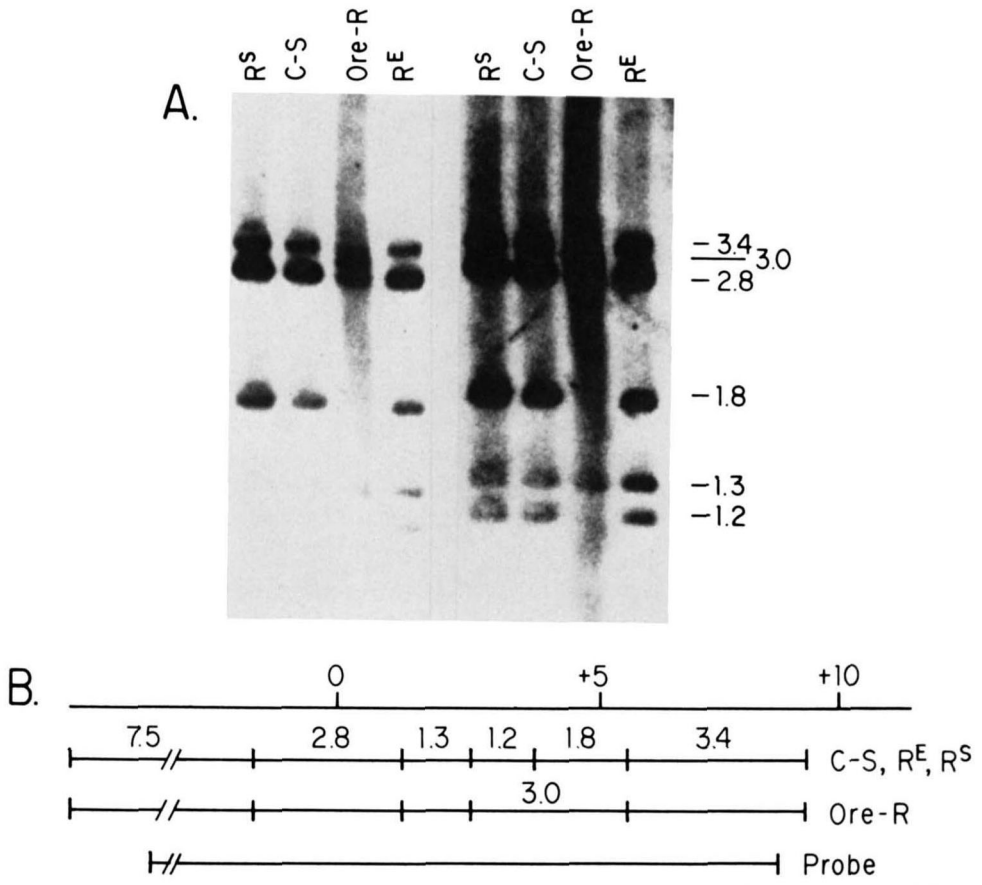

FIGURE 6.—A, DNA blot of R^E, R^S, Canton-S and Oregon-R. Genomic DNA prepared from adults, as described in the MATERIALS AND METHODS section, was cleaved with *Hin*dIII restriction enzyme, resolved on a 0.7% agarose gel and immobilized on nitrocellulose. The blot was probed with a nick-translated lambda clone that spans the entire *Ddc* gene. Two exposures of the same blot are shown for clarity. A restriction site polymorphism separates the contiguous 1.8- and 1.2-kb fragments of R^E, R^S and Canton-S. These fragments are missing in the Oregon-R lane and are replaced by a 3-kb fusion fragment. B, The location and sizes of the *Hin*dIII fragments and the probe are shown relative to a coordinate system in which the 0 point is central to the *Ddc* gene. Positive numbers proceed toward the centromere.

this altered expression is the result of accumulated changes in the control region(s) of these genes.

What is the magnitude of interstrain variation in DDC activity relative to Oregon-R, Canton-S, R^E and R^S? To determine the range of natural DDC activity variation in *D. melanogaster*, we examined 12 wild-type strains which had been collected from a variety of locations, as well as one line of *D. simulans*, for levels of DDC activity in emerging adults. Enzyme activity measurements (not shown) indicated a unimodal continuum of activity levels in different wild-type populations ranging between 80 and 180%, with the Canton-S strain at the peak and the Oregon-R as one of the lowest.

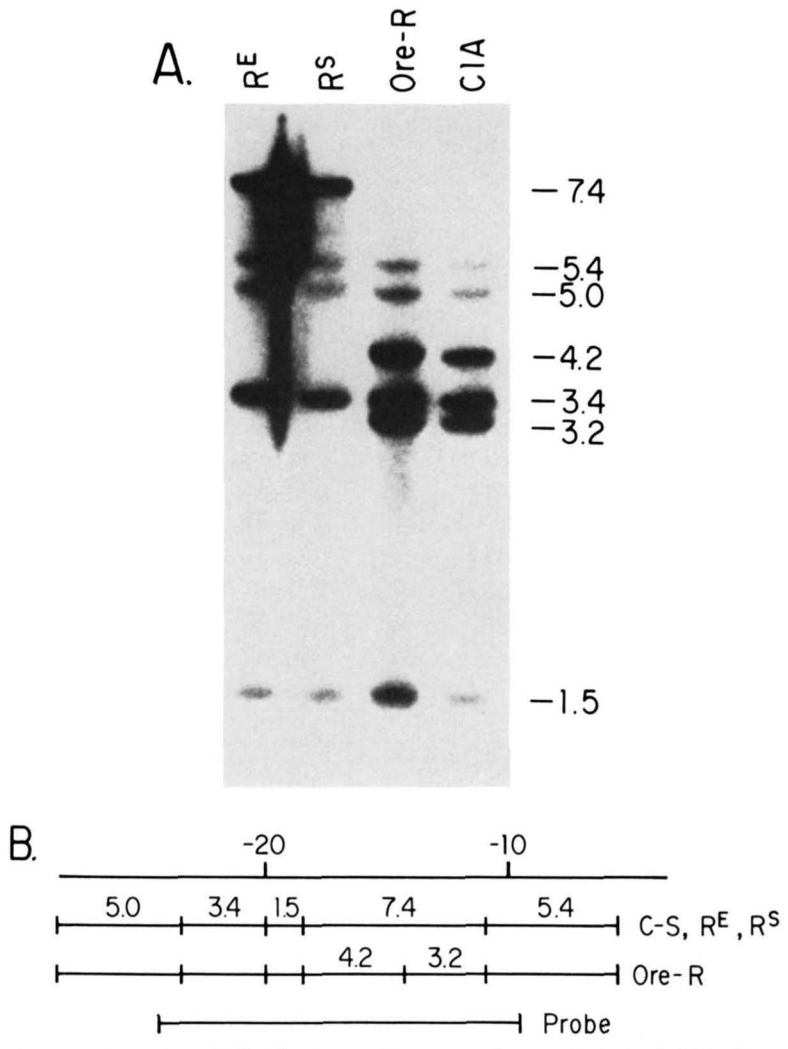

FIGURE 7.—A, DNA blot of R^E, R^S, Oregon-R and the Oregon-R-derived C1A. Genomic DNA prepared from adults, as described in the MATERIALS AND METHODS section, was cleaved with *Eco*RI restriction enzyme, resolved on a 0.7% agarose gel and immobilized on nitrocellulose. The blot was probed with a nick-translated lambda clone from the region distal to the *Ddc* gene. A restriction site polymorphism separates the contiguous 4.2- and 3.2-kb fragments of Oregon-R and C1A. These fragments are missing in the R^E and R^S lanes and are replaced by a 7.4-kb fusion fragment. B, The location and sizes of the *Eco*RI fragments and the probe are given relative to an arbitrary coordinate system where the *Ddc* gene is centrally located around 0 kb, and −10 kb is distal to the centromere and *Ddc*.

DISCUSSION

We have observed naturally occurring genetic variants which lead to both elevated DDC activity and increased resistance to dietary alpha MD. In light of the frequency of both linked and unlinked activity modifiers observed by LAURIE-AHLBERG *et al.* (1982), we examined the nature of the DDC overpro-

duction and elevated resistance in order to determine whether these altered activities result from altered regulation of the *Ddc* and/or *amd* genes, from changes in the structural gene(s) themselves or from alterations in transacting activity modifiers.

Chromosome substitution of all but the region immediately surrounding *Ddc* localized the DDC overproduction and alpha MD resistance phenotypes to the general vicinity of the *Ddc* and *amd* genes. Subsequent mapping by recombination relative to nearby markers (especially *hk*) places the overproduction in the immediate vicinity of *Ddc* (*i.e.*, both *Ddc* and R^E map <0.05 cM proximal to *hk*). Whole genome DNA blots revealed restriction site polymorphisms which are closely linked to *Ddc* and which suggest that R^E and R^S are related to the Canton-S strain as opposed to the Oregon-R strain. These analyses did not identify any restriction pattern changes which might be indicative of a duplication, or a small (≥100 bp) insertion or deletion in this region. We detected no structural alterations in DDC by any of several criteria. Furthermore, we find that the increases in DDC enzyme activity are paralleled by similar increases in the amount of CRM. Hence, we conclude that the elevated DDC activity is the result of altered regulation. We cannot exclude the possibility that a mutation in the protein sequence has occurred that makes the protein more resistant to degradation and yet does not alter the antigenicity, thermolability or inhibition by alpha MD. However, we favor the conclusion that increased levels of DDC activity result from increased levels of transcription, RNA processing or translation, and thus, these activity modifiers represent true regulatory variants of the *Ddc* gene.

Since elevated resistance to dietary alpha MD is correlated with elevated DDC activity in these strains, we asked whether the two phenotypes might have one cause; namely, that elevated DDC activity could confer an increase in resistance to alpha MD. To test this hypothesis, we examined the resistance to alpha MD of animals with varying doses of Ddc^+ or amd^+. The results from Figures 3, 4 and 5 show a slight negative correlation between doses of Ddc^+ and resistance to alpha MD and indicate that amd^+ is the only gene in this region with a strong positive effect on resistance to alpha MD. From these observations we conclude that the increase in Ddc^+ activity seen in R^E and R^S cannot account for the observed increase in resistance to dietary alpha MD relative to Oregon-R.

We observed (Figure 4) that animals with an extra dose of Ddc^+ and flanking genes are, in fact, more sensitive to alpha MD than their two- and one-dose counterparts (*e.g.*, 2:3:3 *vs.* 2:2:2 and 1:3:3 *vs.* 1:2:2). Several explanations for this are possible: (1) An extra copy of another gene(s) in the deficiency region may lead to increased sensitivity. None of the crosses reported here resulted in both *amd* and *Ddc* being held constant while the level of flanking genes was varied. However, the observation that stocks carrying extra flanking genes plus extra amd^+ are more resistant (*e.g.*, 3:2:3>2:2:2 and 3:1:3>2:1:2), whereas those carrying extra flanking genes plus extra Ddc^+ are more sensitive, tends to argue against this possibility with the caveat that the resistance conferred by an extra amd^+ dose in the former case may be masking an increased sen-

sitivity of lesser magnitude conferred by the extra flanking genes. (2) It is possible that excess DDC actually makes flies more sensitive to alpha MD, perhaps by metabolizing excess alpha MD to a toxic product. This is unlikely because 2:2:2 flies show the same sensitivity as 2:1:2 flies (and 3:1:3 = 3:2:3) and because flies with ≤5% DDC activity are not more resistant. This could be qualified by arguing that DDC levels above the normal ratio of *Ddc:amd* may lead to increased sensitivity. (3) A third possibility for the increased sensitivity of 2:3:3 and 1:2:2 flies is that the mutant *amd* alleles used may exhibit negative complementation. Some *amd* alleles show partial intracistronic complementation suggesting that amd^+ may function as a multimer, with at least two identical subunits. If only those enzymes comprised of all normal subunits were active, then amd^+ activity would be depressed in animals with one or more mutant alleles. Genetic precedence for this model is found in the *Ddc* locus, which exhibits both positive and negative complementation (WRIGHT *et al.* 1982). Examination of the legend to Figure 4 shows that the more sensitive genotypes have more mutant alleles and, thus, more potential for negative complementation than the comparison genotypes. If the number of mutant *amd* alleles in each genotype is indicated by asterisks, the decreased resistance of 2*:3:3 *vs.* 2:2:2 and 1**:3:3 *vs.* 1*:2:2 could reflect the greater potential for negative complementation, rather than the extra doses of Ddc^+ and flanking genes. This interpretation also accounts for the observation that variations in DDC activity below 100% of normal (such as seen in Figure 3 in 3:2:3 *vs.* 3:1:3 flies) have no affect on resistance because there are no mutant *amd* alleles segregating. These studies do not permit us to distinguish between the possible contributions of (1) increased DDC, (2) increases in flanking genes and (3) negative complementation in the comparison of 2:2:2 *vs.* 2:3:3 and 1:2:2 *vs.* 1:3:3.

With respect to the overproducers R^E and R^S, we conclude that the increased levels of DDC cannot account for the increased resistance to dietary alpha MD. Furthermore, among the *ca.* 16 flanking genes in *Df(2L)TW130* only the *amd* gene has an affect on resistance to dietary alpha MD of sufficient magnitude to account for observed resistance of R^E and R^S. Thus, since increased doses of amd^+ lead to increased resistance to alpha MD, we conclude (in the demonstrated absence of increased gene dosage) that the increased resistance of the R^E and R^S strains results from increased activity of the amd^+ locus.

In the absence of a direct physical assay for the amd^+ gene product, we cannot determine whether increased amd^+ activity results from increased expression of the amd^+ locus or from structural changes in the *amd* coding region. However, the close linkage (0.002 cM, or approximately 1–4 kb), the functional relation to the same metabolic pathway (catecholamine metabolism) and the observation that the production levels of *Ddc* and *amd* are linked in the naturally occurring variants that we have analyzed suggests the possibility of coordinate regulation, perhaps via a common potentiator of expression, such as an enhancer. The observation of naturally occurring coordinate overproduction supports the prediction of WILTON *et al.* (1982) that enzymes sharing a substrate will have correlated activity levels, although these observations will

have to be reconciled with the apparently normal regulation of Ddc^+ when placed in new chromosomal environments by transposon-mediated transformation (MARSH, GIBBS and TIMMONS 1985).

The distribution of DDC activities in 12 wild-type strains of *D. melanogaster* in our assay showed a modal value of approximately 140% relative to Oregon-R. The range and distribution of DDC activity variation was similar to that observed by AQUADRO *et al.* (1984) and by ESTELLE and HODGETTS (1984a) in second chromosomes recently extracted from several Eastern populations. Thus, the DDC activity of the Canton-S and R^E strains falls at the high end of the activity spectrum, whereas the activity of the Oregon-R-derived control strain (C1A) is one of the lowest observed, and the activity of the R^S strain is near the modal value. The observation that strains with up to 10 times the haploid level of DDC are viable under laboratory conditions (MARSH, GIBBS and TIMMONS 1985) suggests that there is no acute physiological barrier to greater levels of *Ddc* expression than those observed in R^E and in the Ddc^{+4} strain of ESTELLE and HODGETTS (1984a).

The R^S and R^E variants result in elevated CRM at the two major periods of Ddc^+ expression and, thus, are qualitatively different from the Ddc^{+4} overproducer strain described by ESTELLE and HODGETTS (1984a), which overproduced DDC at eclosion but underproduced it at pupariation. Thus, two classes of *Ddc* regulatory variants have been described suggesting a multifunctional control element for this gene. In addition, our evidence indicates the presence of regulatory variants which affect the adjacent *amd* locus.

Many putative regulatory mutations identified in metazoans derive from naturally occurring variants rather than from direct mutagenesis; *e.g.*, in *D. melanogaster* (CLARK *et al.* 1984; ESTELLE and HODGETTS 1984a; LAURIE-AHLBERG *et al.* 1982), as well as in *Mus musculus* (PAIGEN 1979) and *Zea mays* (SCANDALIOS and BAUM 1982). If regulatory structures in metazoans are redundant and small, such as the regulatory region of the *HIS4* locus in yeast (*e.g.*, three repetitions of approximately 8 bp; DONAHUE *et al.* 1983), one would expect that they would be refractile to classical mutagenesis and that the most likely variants to be identified would be those having moderate affects on the level of gene activity. Our observations are consistent with this model and suggest that the altered levels of Ddc^+ and amd^+ activity in the R^E, R^S and Oregon-R strains result from accumulated differences in the control region(s) for the *amd* and/or *Ddc* genes. Variants which lead to altered levels of gene product have been found to affect a variety of putative control elements, including contiguous control elements (*e.g.*, CLARK *et al.* 1984) and RNA processing signals such as seen in human globins (MANIATIS *et al.* 1980). A comparison of the nucleotide sequence relationships between the control regions of the R^E, R^S and Oregon-R strains will prove useful in defining the complexity of the regulatory apparatus for the *Ddc* and *l(2)amd* genes.

The authors would like to thank ANNE D. TOMSETT and P. M. TIMMONS for their expert technical assistance, as well as P. J. BRYANT, P. D. L. GIBBS and K. KONRAD for reading the manuscript. This work was supported by Public Health Service grant GM19242 to T.R.F.W. and,

in part, by Public Health Service grants GM28972 and PCM36485 to J.L.M. J.L.M. was supported, in part, by a Fellowship from National Institutes of Health training grant T-GM-01450.

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Communicating editor: V. G. FINNERTY