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# Studies of esterase 6 in *Drosophila melanogaster*

## XIV. Variation of esterase 6 levels controlled by unlinked genes in natural populations

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SUMMARY

The esterase 6 (*Est-6*) locus in *Drosophila melanogaster* is located on the third chromosome and is the structural gene for a carboxylesterase (E.C. 3.1.1.1) and is polymorphic for two major electromorphs (slow and fast). Isogenic lines containing *X* chromosomes extracted from natural populations and substituted into a common genetic background were used to detect unlinked factors that affect the activity of the *Est-6* locus. Twofold activity differences of esterase 6 (EST 6) were found among males from these derived lines, which differ only in their *X* chromosome. These unlinked activity modifiers identify possible regulatory elements. Immunoelectrophoresis was used to estimate quantitatively the levels of specific cross-reacting material in the derived lines. The results show that the variation in activity is due to differences in the amount of EST 6 present. The data are consistent with the hypothesis that there is at least one locus on the X chromosome that regulates the synthesis of EST 6 and that this regulatory locus may be polymorphic in natural populations.

## 1. INTRODUCTION

Classes of regulatory genes that function in activating or inactivating other genes may evolve at rates that differ from the genes they regulate (Wilson *et al.*, 1974, 1975; Maxson & Wilson, 1979). Wilson (1976) has suggested that 'regulatory evolution' parallels morphological divergence and tends to be of much greater magnitude than divergence at the protein sequence level. Carson (1975) believes that changes in regulatory sequences may be associated with rapid speciation observed in the Hawaiian *Drosophila*. Therefore, we believe a crucial step in understanding the processes of adaptation and speciation is to determine whether regulatory genes are polymorphic in natural populations.

Here we define regulatory genes as loci that control the timing or level of expression of other genes (Hedrick & McDonald, 1980). These regulatory genes may control structural genes in a number of ways, including differential rates of transcription, mRNA processing, translation, and post-translational modifications, intracellular compartmentalization, or differential rates of degradation.

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The *Est-6* locus in *D. melanogaster* is the structural gene for a carboxylesterase (E.C. 3.1.1.1) and is polymorphic for two major electromorphs (Wright, 1963). The combined frequencies of minor electrophoretic variants (including null alleles) seldom exceed 5% in all natural populations investigated (Wright, 1963; Girard, Palabost & Petit, 1977; Cavener & Clegg, 1981; Oakeshott *et al.* 1981). Structural variants of EST 6 map to position 36.8 on the third chromosome (Wright, 1963; Cochrane & Richmond, 1979). Mature virgin males have from 2 to 5 times the activity found in virgin females (Sheehan, Richmond & Cochrane, 1981). EST 6 is localized in the anterior ejaculatory duct of the adult male reproductive system and is transferred to the female prior to sperm transfer as a component of the seminal fluid (Aronshtam & Kuzin, 1974; Sheehan *et al.* 1979; Richmond *et al.* 1980). EST 6 from male seminal fluid influences female productivity and the timing of subsequent remating (Gilbert, Richmond & Sheehan, 1981; Gilbert & Richmond, 1982). EST 6 cleaves a male-specific lipid (cis-vaccenylacetate) that is transferred with EST 6 in the seminal fluid during mating. In the female's reproductive tract, this male-specific lipid appears to act as an anti-aphrodisiac and delays the female's time of remating (Mane, Tompkins & Richmond, 1983*a*).

Here we report the results of a study designed to quantify the amount of variability in the activity levels of the reproductive enzyme, EST 6, in natural populations of *D. melanogaster*. Our results are consistent with the hypothesis that there is at least one locus on the *X* chromosome which regulates the synthesis or degradation of EST 6, and that this regulatory locus may be polymorphic in natural populations.

## 2. MATERIALS AND METHODS

### (i) *General procedures*

*Culture conditions*: Wild-type stocks of *D. melanogaster* collected in Bloomington, Indiana, in September 1980 and September 1981, and laboratory stocks were used in these experiments. All stocks were maintained on standard cornmeal–agar–molasses medium at 25° and a 12:12 light–dark cycle.

*Sample homogenates*: Male flies to be analysed for enzyme activity were aged for three days and homogenized in groups of 30 in 1·0 ml of 0·1 M sodium phosphate buffer (pH 6·8). A Brinkman Polytron was used for homogenization. Crude homogenates were centrifuged at 15600 ***g*** for five minutes and the supernatant was stored at −20 °C. EST 6 shows no detectable loss of activity over a four-month period at this temperature (Mane *et al.* 1983*b*).

*Spectrophotometric assays*: EST 6 activity was measured as described previously (Cochrane & Richmond, 1979; Sheehan *et al.* 1979). Protein concentrations were determined by use of the Folin reagent using bovine serum albumin (Schwartz–Mann) as a standard (Lowry *et al.* 1951).

*Preparation of antisera*: The production of antisera specific to EST 6 has been described by Mane *et al.* (1983*b*). Fluorograms of SDS gels containing homogenates of anterior ejaculatory ducts from both *Est-6*$^{+}$ and *Est-6*$^{0}$ flies cultured in [$^{3}$H]leucine and precipitated with the rabbit antiserum reveal a single protein band at the molecular weight of EST 6, while no labelled protein band is present for similarly treated *Est-6*$^{0}$ flies (Tepper, Stein and Richmond, in preparation).

*Laurell rocket electrophoretic analysis*: Levels of specific cross-reacting material (CRM) were determined by one-dimensional electrophoresis (Laurell, 1966). Buffers, antibody concentrations, agarose concentrations, and voltage requirements have been previously described (Mane *et al.* 1983*b*). Subsequent staining for enzymic activity of the antigen–antibody complex is the same as that used by Tepper *et al.* (1982) for staining polyacrylamide disk gels. Laurell rocket electrophoresis revealed an approximately linear relationship between EST 6 concentration and rocket length (Mane *et al.* 1983*b*). No difference in immunological reactivity was observed among slow and fast isozymes and antisera.

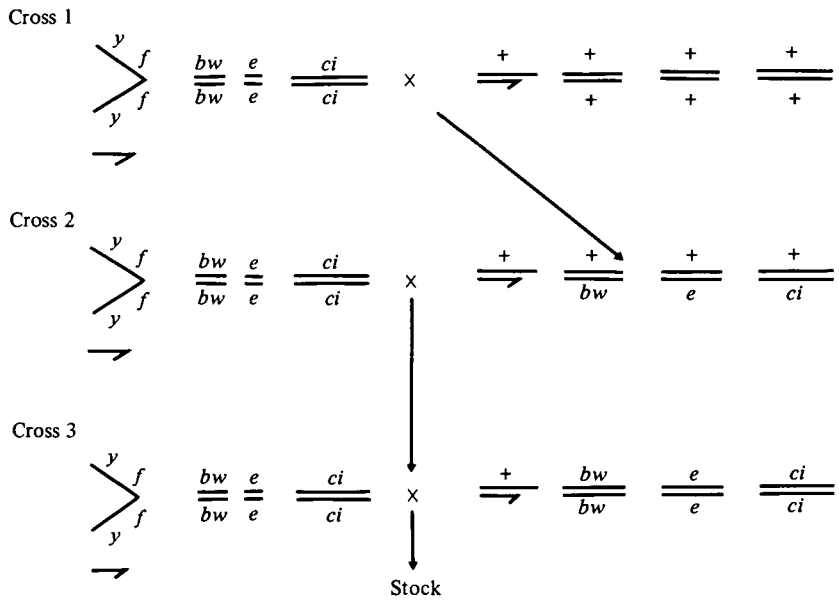


Fig. 1. Genetic scheme used to place *X* chromosomes derived from wild-collected males into a standard genetic background. Genotypes of the *X*, II, III, and IV chromosomes are shown. Genetic nomenclature follows Lindsley & Grell (1968). The attached-*X*, free *Y* stock is marked with: *y* = yellow body, *f* = forked bristles, both *X*-linked loci, *bw* = brown eye colour, 2nd chromosome; *e* = ebony body, 3rd chromosome; *ci* = cubitus interruptus (interrupted cubital view of wing), 4th chromosome.

(ii) *Extraction of wild* X *chromosomes*

This experiment was undertaken to extend our previous results for natural populations. Controls, spectrophotometric analysis, and statistical analysis have been described by Tepper *et al.* (1982). Briefly, wild-caught *D. melanogaster* males were crossed to attached *X*, free *Y* females (*yf*:) homozygous for recessive markers on all autosomes (*yf*:; *bw*; *e*; *ci*;, Lindsley & Grell, 1968; see Fig. 1). Each of 41 wild-caught males was crossed sequentially to three presumably isogenic females (inbred by full sib mating for 24 generations) to yield a total of 41 separate male lines with three replicate cultures for each original wild male. Nested analyses of covariance were used to adjust EST 6 activity to equivalent protein concentrations in each homogenate (Bliss, 1970; Steel & Torrie, 1970; Tepper *et al.* 1982).

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Table 1

((A) Protein concentration (mean mg/ml ± S.E.M.) and EST 6 activity (mean OD units/ml ± S.E.M.) for 41 lines differing only in their *X* chromosomes. The lines were isolated using the *yf*:; *bw*; *e*; *ci* stock. Mean EST 6 activities were adjusted to equivalent total protein concentration by analysis of covariance methods. (B) Nested analysis of covariance for data summarized in part A.)

(A) *Means*

| Line | No. replicates of each line | Protein | EST 6 activity | Adjusted EST 6 activity |
|---|---|---|---|---|
| 1 | 2 | 1·603 ± 0·094 | 25·60 ± 1·560 | 22·93 |
| 2 | 3 | 1·552 ± 0·067 | 32·04 ± 5·810 | 30·47 |
| 3 | 3 | 1·535 ± 0·026 | 34·67 ± 1·830 | 33·47 |
| 4 | 1 | 1·446 ± 0·106 | 22·71 ± 6·750 | 23·43 |
| 5 | 2 | 1·911 ± 0·240 | 52·44 ± 5·050 | 43·12 |
| 6 | 3 | 1·452 ± 0·062 | 40·45 ± 2·000 | 41·04 |
| 7 | 3 | 1·334 ± 0·167 | 35·14 ± 6·350 | 38·28 |
| 8 | 2 | 1·543 ± 0·052 | 39·24 ± 1·020 | 37·87 |
| 9 | 3 | 1·476 ± 0·086 | 27·30 ± 4·720 | 27·37 |
| 10 | 3 | 1·274 ± 0·061 | 31·00 ± 2·450 | 35·44 |
| 11 | 3 | 1·429 ± 0·138 | 34·70 ± 4·360 | 37·79 |
| 12 | 3 | 1·609 ± 0·136 | 37·59 ± 2·220 | 34·79 |
| 13 | 3 | 1·641 ± 0·060 | 26·63 ± 1·080 | 23·13 |
| 14 | 3 | 1·528 ± 0·082 | 27·51 ± 2·530 | 26·46 |
| 15 | 2 | 1·778 ± 0·235 | 29·16 ± 3·230 | 22·71 |
| 16 | 3 | 1·329 ± 0·044 | 20·34 ± 4·280 | 23·59 |
| 17 | 3 | 1·335 ± 0·041 | 18·81 ± 2·250 | 21·93 |
| 18 | 3 | 1·509 ± 0·040 | 27·08 ± 2·590 | 26·44 |
| 19 | 3 | 1·398 ± 0·024 | 18·10 ± 2·440 | 19·85 |
| 20 | 3 | 1·397 ± 0·046 | 17·83 ± 2·950 | 19·61 |
| 21 | 3 | 1·356 ± 0·035 | 10·69 ± 0·600 | 13·36 |
| 22 | 2 | 1·705 ± 0·077 | 23·96 ± 2·500 | 19·08 |
| 23 | 3 | 1·302 ± 0·084 | 17·20 ± 2·430 | 21·03 |
| 24 | 1 | 1·569 ± 0·102 | 41·40 ± 1·330 | 39·47 |
| 25 | 3 | 1·509 ± 0·063 | 23·57 ± 1·310 | 22·93 |
| 26 | 2 | 1·187 ± 0·041 | 9·14 ± 0·540 | 15·45 |
| 27 | 3 | 1·376 ± 0·074 | 17·85 ± 0·500 | 20·08 |
| 28 | 1 | 1·641 ± 0·116 | 24·45 ± 7·060 | 20·95 |
| 29 | 3 | 1·468 ± 0·078 | 25·96 ± 4·130 | 26·20 |
| 30 | 2 | 1·606 ± 0·112 | 22·27 ± 5·470 | 19·53 |
| 31 | 2 | 1·580 ± 0·111 | 21·66 ± 3·500 | 19·49 |
| 32 | 3 | 1·451 ± 0·061 | 19·79 ± 2·300 | 20·41 |
| 33 | 2 | 1·426 ± 0·095 | 22·39 ± 2·320 | 23·55 |
| 34 | 3 | 1·341 ± 0·063 | 21·90 ± 2·060 | 24·89 |
| 35 | 2 | 1·394 ± 0·102 | 20·51 ± 0·760 | 22·35 |
| 36 | 3 | 1·522 ± 0·037 | 23·84 ± 3·590 | 22·92 |
| 37 | 1 | 1·271 ± 0·062 | 18·75 ± 5·150 | 23·25 |
| 38 | 1 | 1·458 ± 0·022 | 16·20 ± 0·425 | 16·66 |
| 39 | 2 | 1·725 ± 0·151 | 28·40 ± 4·000 | 23·10 |
| 40 | 3 | 1·307 ± 0·081 | 16·41 ± 1·820 | 20·14 |
| 41 | 3 | 1·382 ± 0·050 | 16·77 ± 1·850 | 18·87 |

Table 1 (*cont.*)

(B) *Nested analysis of covariance*

| Source | Sums of squares and products D.F. | $P^{2}$* | $P \times E$† | $E^{2}$‡ | Adjusted mean squares for EST 6 activity D.F. | *MS* | *F* | *P* |
|---|---|---|---|---|---|---|---|---|
| Among lines | 40 | 4·021 | 136·439 | 14591·95 | 40 | 261·80 | 3·97 | < 0·001 |
| Among replicates within lines | 61 | 5·078 | 97·025 | 5860·39 | 61 | 65·85 | 3·17 | < 0·001 |
| Error | 102 | 1·766 | 38·151 | 2922·51 | 101 | 20·76 | | |
| Total | 203 | 10·865 | 271·615 | 23374·86 | 202 | | | |

* Sum of squares for protein concentration.
† Sum of products of protein concentration × EST 6 activity.
‡ Sum of squares for EST 6 activity.

## 3. RESULTS

### (i) *EST 6 activity in co-isogenic lines differing only in their X chromosomes*

An analysis of covariance was used to adjust the mean EST 6 activities (Table 1 A) of each line to an equivalent concentration of total protein (Table 1 B). This method uses the actual relationship between protein concentration and enzyme activity to adjust activity levels, rather than assuming a perfect correlation between the variables (see Tepper *et al.* 1982).

The nested analysis of covariance (Table 1 B) tests the hypothesis that there is no significant difference between the 41 male lines, which differ only in the *X* chromosome they possess. Table 1 B shows that this hypothesis is clearly rejected ($P < 0{\cdot}001$). These data suggest that natural populations of *D. melanogaster* may be genetically variable for factors carried on the *X* chromosome which influence EST 6 activity. These results agree with our previous findings suggesting that EST 6 activity is influenced by factors on the *X* chromosome (Tepper *et al.* 1982).

Table 1 also shows that there is a significant variance ($P < 0{\cdot}001$) among replicates within lines. This result suggests that either large environmental differences exist between cultures, isogenic females were not genetically identical, or some combination of these two possibilities contributed to the significant between-culture variance. The error mean square used to test the significance of the between-culture variance in these experiments arises from variance between replicate homogenates and replicate assays of any given homogenate. In our hands the amount of variation associated with these factors seldom exceeds 10 % of the mean.

### (ii) *Stability of EST 6 activity in X chromosome substitution lines*

The mean adjusted EST 6 activities listed in Table 1 A differ by a maximum of 223 %. This result suggests that the genetic composition of the *X* chromosomes varies significantly (Table 1 B). Given the large number of significant genetic differences between male lines, it is important to determine whether these differences are stable through time. The temporal stability of differences in enzyme activity attributable to non-structural loci is largely unexplored. Reanalysis of a

subset (chosen as representative of the range of activity from Table 1A) of our original 41 male lines (Fig. 2) has confirmed that the EST 6 activity differences are relatively stable over a nine-month period. In this experiment involving male sublines 3, 5, 6, 9 and 30, EST 6 activity was examined over a nine-month period at four different times, including the original data set (Table 1A). At each time, four sets of 30 male flies each were homogenized in buffer and assayed spectrophotometrically as previously described (see Materials and Methods).

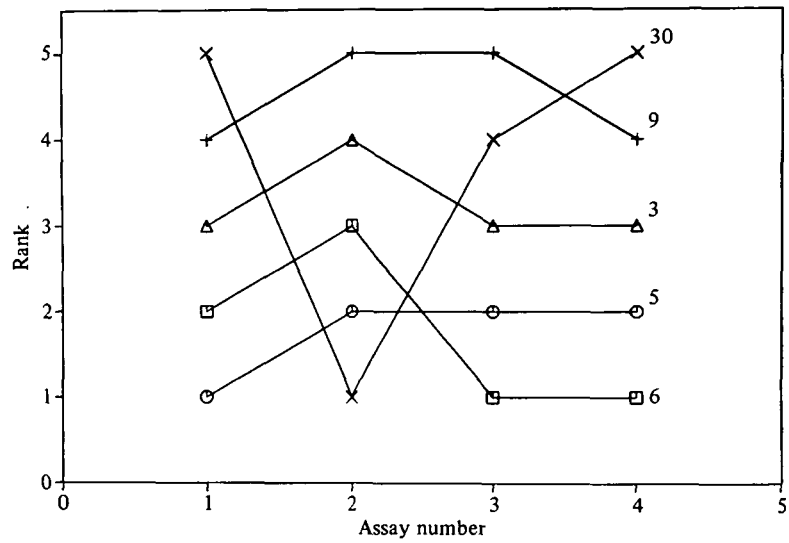


Fig. 2. Reanalysis of a subset of our original 41 male lines. EST 6 activity was examined over a nine-month period at four different times in order to demonstrate that EST 6 activities are relatively stable through time. Rank (see text) is shown as a function of assay number. Assay number one represents the original data set (see Table 1A). This experiment involves male lines 3, 5, 6, 9 and 30.

Comparison of the sublines (Fig. 2) shows similar but not identical results. Because of the significant between-replicate variance previously described, EST 6 activity for these lines has been arranged in rank order, in which a rank of one corresponds to the highest activity line and a rank of five corresponds to the lowest activity line. Assay number one is the initial enzyme analysis found in Table 1A. Assays numbered 2–4 represent separate analyses of EST 6 activity separated by three-month intervals. With the exception of line 30 in the second assay, enzyme activities are relatively stable through time; this indicates that *X* chromosome effects are genetic and are not artifacts of homogenization or assay procedures. These results confirm our previous findings (Tepper *et al.* 1982) that the structural locus coding for EST 6 is regulated by loci which may be genetically variable.

(iii) *Distribution of activity differences among co-isogenic male lines*

Fig. 3 shows the distribution of EST 6 activities among the co-isogenic male lines (Table 1A). The majority of classes (28/41) fall into the activity range of 19–27 OD units/ml. If the regulatory element(s) that controls EST 6 activity is

polymorphic, we would expect the activity distribution to deviate significantly from a normal distribution depending on the number of regulatory loci involved. Therefore this distribution was tested to determine whether it departed significantly from a normal distribution (Sokal & Rohlf, 1969). The test revealed that the distribution of EST 6 activities among co-isogenic male lines was skewed to the right ($g_1 = 0{\cdot}888$, $P < 0{\cdot}02$) and had a slight tendency to platykurtosis ($g_2 = -0{\cdot}149$, $P > 0{\cdot}05$). Thus the distribution is asymmetric. One possible interpretation of these

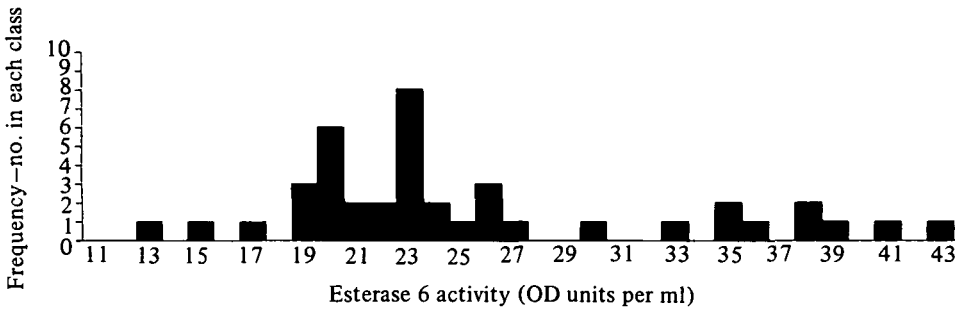


Fig. 3. A histogram showing the distribution of EST 6 activities among the co-isogenic male lines (see Table 1 A). A test to determine if the distribution was normal revealed that the distribution of EST 6 activities was skewed to the right ($g_1 = 0{\cdot}888$, $P < 0{\cdot}02$).

data is that a sex-linked, EST 6 regulatory locus is polymorphic in natural populations.

(iv) *EST 6 activity in co-isogenic lines determined by CRM levels*

In order to determine whether activity variation was due to differences in enzyme protein levels, we analysed a subset of our male co-isogenic lines using Laurell rocket electrophoresis.

A linear relationship was always observed between rocket height and the amount of EST 6 activity. This relationship was linear for both crude and purified preparations of EST 6. A significant correlation ($r = 0{\cdot}923$, $P < 0{\cdot}01$) was found for EST 6 activity (OD units/ml) measured by our spectrophotometric assay and Laurell rocket peak height from the means of 12 different sublines of flies. Therefore, the amount of EST 6 CRM (measured by rocket height) is directly proportional to enzyme activity, indicating that most of the variation in EST 6 activity in our lines is accounted for by variation in the quantity of EST 6 present. The CRM and activity differences found are probably the result of differential rates of enzyme synthesis or degradation.

## 4. DISCUSSION

We have presented a variety of data which support the hypothesis that loci other than the structural gene for EST 6 affect enzyme activity in adult males. These results confirm our preliminary experiments (Tepper *et al.* 1982) and shed some light on the extent of genetic variation in enzyme activities in natural populations,

the relative importance of regulatory genes in causing this variation, and the possibility that modifier loci may be polymorphic in natural populations. Data from the *X* chromosome isolation lines suggest that some of these regulatory loci act in trans. The Laurell rocket electrophoresis data indicate that the regulatory locus (loci) controls the amount of EST 6 present. The stability data provide evidence that enzyme activity differences are stable through time. A critical question now under investigation is whether this regulatory element(s) maps to a single location on the *X* chromosome.

Most studies that explore the interactions between regulatory elements and their corresponding structural genes are done by extracting chromosomes from natural populations. The crosses used to create male lines (Fig. 1) are identical to the types of crosses that induce hybrid dysgenic events (Engels, 1979; Engels & Preston, 1979; Kidwell, 1981). Such events can result in a syndrome of effects including male and female sterility, male recombination, and contamination of homologous and non-homologous chromosomes by mobile genetic elements known as P factors (Engels, 1980). Although this type of analysis may induce hybrid dysgenic events, none of the previous studies has considered the possible effects of dysgenesis on their results (e.g. Finnerty & Johnson, 1979; and Laurie-Ahlberg *et al.* 1981). Preliminary results using hybrid female sterility as an indication of hybrid dysgenesis (Engels, 1979; Engels & Preston, 1979) suggest that some of the variability found in our *X* chromosome extraction lines may be a result of hybrid dysgenesis.

There is clearly a great deal of genetic variation affecting enzyme activity among chromosomes derived from natural populations. The present results suggest that variants of modified loci may contribute substantially to the total variation of enzyme activity in natural populations. These results are just a beginning. Specific loci must be mapped and their allelic contents quantified. A complete analysis of the developmental, physiological, biochemical and genetic components of our high and low EST 6 activity lines may aid in understanding the role that regulatory variability plays in evolution.

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