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Studies of esterase 6 in *Drosophila melanogaster*.

XI. Modification of esterase 6 activity by unlinked genes

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SUMMARY

The often remarkable similarity in structural gene products among related species has led to the hypothesis that species differences may reside largely in changes at regulatory gene loci. This hypothesis assumes that groups capable of speciating have allelic variation at regulatory loci in their natural populations. We have undertaken an analysis of the mode of regulation of the *esterase 6* (*Est 6*) locus in *Drosophila melanogaster* to determine the nature and extent of regulatory gene variation in natural populations. Analyses of esterase 6 (EST 6) activity among strains carrying the same thermostability variants reveal that significant, specific-activity differences are present. Reciprocal crosses between lines having high and low EST 6 activity show that loci other than the *Est 6* structural gene influence EST 6 activity. Analyses of male hybrids from crosses between *D. melanogaster* and *simulans* indicate that the *X* chromosome of these flies affects the expression of the *Est 6* locus, resulting in unequal levels of enzyme activity from the two alleles. The effect is sex and tissue specific. Female hybrids carrying the *X* chromosomes of both species exhibit equal expression of the two *Est 6* alleles. We have determined whether natural populations are polymorphic for X chromosomes which affect EST 6 activity by extracting single X chromosomes from wild-collected males and placing these chromosomes in identical genetic backgrounds. Stocks which are otherwise genetically identical but carry independently derived *X* chromosomes show significant differences in the activity of EST 6. These data suggest that regulatory loci may be commonly polymorphic in natural populations.

1. INTRODUCTION

Recent evidence suggests that genetic variation in structural genes (i.e. those coding for structural proteins and enzymes) may not be the primary substrate for adaptation and speciation (Wallace, 1963; Stebbins, 1969; Carson, 1975; Vallentine & Campbell, 1975; Wilson, 1975; McDonald *et al.* 1977; Hedrick & McDonald, 1980). Regulatory loci which control the integration of developmental, physiological, and behavioural traits may be more important than structural loci in the

speciation process (Templeton, 1980*a*, *b*). If adaptation and speciation are to be fully understood, evolutionary biologists must explore the genome for non-structural loci segregating for allelic variants which result in fitness differences between genotypes. Such loci are considered to be regulatory for our purposes if they control the timing or expression of other genes (Hedrick & McDonald, 1980).

Many genes in *Drosophila* are known to be regulated (Korochkin, Evgeniev & Matveeva, 1972; Pipkin & Hewitt, 1972; Dickinson, 1975; Chovnick *et al.* 1976; Korochkin *et al.* 1976; Abraham & Doane, 1978; Anderson & McDonald, 1981) and several studies provide evidence that these regulatory genes may be polymorphic. McDonald *et al.* (1977) demonstrated that an adaptive change in alcohol tolerance in *D. melanogaster* was a result of changes in one or more genes regulating the amount of alcohol dehydrogenase. Similar regulatory variation for other enzyme systems in *Drosophila* has also been reported (Ward, 1975; Barnes & Birley, 1978; McDonald & Ayala, 1978; Finnerty, McCarron & Johnson, 1979; Finnerty & Johnson, 1979; Laurie-Ahlberg *et al.* 1980; Norman & Prakash, 1980). We report here an analysis of the genetic basis for variation in EST 6 activity in *D. melanogaster*.

The *Est 6* locus in *D. melanogaster* is the structural gene for a carboxylesterase (E.C. 3.1.1.1) which is polymorphic for two major electromorphs (slow and fast) in all natural populations investigated (Wright, 1963; Girard, Palabost & Petit, 1977; Cavener & Clegg, 1981). Structural variants of EST 6 map to position 36.8 on the third chromosome (Wright, 1963; Cochrane & Richmond, 1979). EST 6 is synthesized in the anterior ejaculatory duct of the adult males' reproductive system and is transferred to females prior to sperm transfer as a component of the seminal fluid (Aronshtam & Kuzin, 1974; Sheehan, Richmond & Cochrane, 1979; Richmond *et al.* 1980). Male, seminal-fluid EST 6 influences female productivity and the timing of subsequent remating (Gilbert, Richmond & Sheehan, 1981; Gilbert & Richmond, 1981).

Using a variety of procedures, we show that EST 6 activity is regulated by a locus (or loci) not linked to the structural gene. Experiments employing strains differing only in *X* chromosomes carried by males provide insight into the probable polymorphic nature of the regulatory loci.

2. MATERIALS AND METHODS

(i) *Culture conditions*

Stocks of both *D. melanogaster* and *D. simulans* were utilized in these experiments. All stocks were maintained on standard cornmeal–agar–molasses medium at 25 °C and on a 12:12 light–dark cycle.

(ii) *Sample homogenization*

Flies to be analysed on polyacrylamide disc gels were individually homogenized in 40 μl of 0·1 M sodium phosphate buffer (pH 6·8) containing 0·3 M sucrose using a Kontes Glass Duall 20 homogenizer and a motor-driven Teflon pestle.

For spectrophotometric assays, 30 flies were homogenized in 1·0 ml of 0·1 M sodium phosphate buffer (pH 6·8) using the same procedure as above. Crude homogenates were centrifuged at 15600 ***g*** for 5 min and the supernatant stored at −25 °C.

(iii) *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).

(iv) *Electrophoretic analysis*

Disc-gel electrophoresis was carried out at 4 °C for 2½ h at 100 V and 25 mA in 3 × 80 mm cylindrical gels using the buffer system of Davis (1964). Following electrophoresis, the gels were soaked in 0·1 M sodium phosphate buffer (pH 6·8) containing 1×10^{-3} M eserine sulphate and 5×10^{-4} M *p*-chloromercuribenzoate to inhibit esterases other than EST 6 (Sheehan *et al.* 1979). Gels were stained for EST 6 activity using α-napthyl propionate as a substrate and Fast Blue RR as the coupling dye. Portions of the gel showing EST 6 activity were scanned in a Perkin-Elmer Model 52, UV-Vis spectrophotometer employing an integrating gel scanner. EST 6 activity is given below in arbitrary units of area measured by the integrating densitometer. Calibration with a dilution series of a single EST 6 homogenate subjected to electrophoresis and stained in a standard manner confirmed that dye intensity is linear with enzyme concentration and staining time under the conditions used.

(v) *Comparison of EST 6 activites in thermostability lines*

Three strains each homozygous for the thermostability allele, *Est* $6^{1\cdot00-2}$ (slow mobility), and three strains each homozygous for the thermostability allele, *Est* $6^{1\cdot10-2}$ (fast mobility) (Cochrane & Richmond, 1979) were analysed for protein content and EST 6 activity as described. Each strain was grown as two replicate cultures. Two replicate homogenates of each sex were prepared from each culture yielding a total of four homogenates per strain and an overall total of 48 homogenates (Fig. 1). EST 6 activity and protein content were determined in duplicate for each homogenate. Activity is expressed in specific activity units (μmoles of β-napthol produced/30 min/mg protein at 27 °C). An analysis of

variance was performed on the data following the methods of Lindman (1974) for a mixed factor, nested design (Fig. 1).

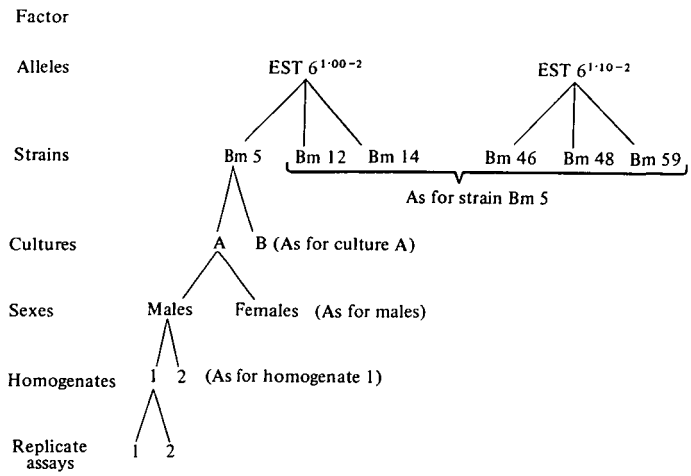

Fig. 1. Experimental design used to determine the effects of various factors on the activity of EST 6. Summary of design:

Factor	Notation	No. of levels	Nature of factor
Alleles	A	2	Fixed
Strains	S	3	Fixed, nested in A
Cultures	C	2	Random, nested in S and A
Sexes	X	2	Fixed
Homogenates	H	2	Random, nested in X, C, S & A
Replicates	R	2	Random, nested in H, X, C, S & A

(vi) *EST 6 activity in high and low hybrid lines*

Two strains of *D. melanogaster* were made homozygous for wild-extracted third chromosomes using the TM3 balancer (Lindsley & Grell, 1968; Cochrane & Richmond, 1979). Strain Dm 48, a high EST 6 activity line, carries the slow (S) allele, and Dm 49, a low EST 6 activity line, carries the fast (F) allele. Reciprocal crosses between these two lines were made. Both parental males and F_1 males from reciprocal crosses were analysed for EST 6 activity using our spectrophotometric and densitometric assays.

(vii) *Interspecific hybrid crosses*

Reciprocal crosses (three males and three females per vial) were carried out between strains of *D. melanogaster* and *D. simulans* homozygous for alternate *Est 6* alleles (Fig. 2). Crosses between *D. melanogaster* and *D. simulans* were successful only when the hybrid offspring inherited an *X* chromosome from the *D. simulans*

parent (Sturtevant, 1920; Aronshtam & Korochkin, 1975). Three different interspecific crosses were made (Fig. 2) and hybrid offspring were collected. The first cross consisted of *D. simulans* females from a yellow body and white eye line (*yw*) carrying an *Est* 6^S allele. The *D. melanogaster* males for this cross were from a

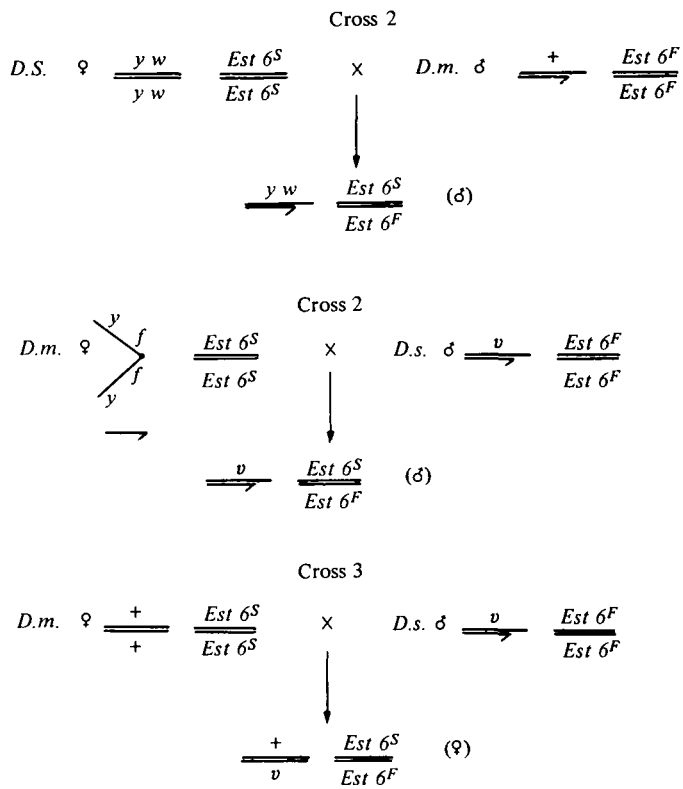

Fig. 2. Crosses made between *Drosophila melanogaster* (*D.m.*) and *simulans* (*D.s.*) to examine the relative activity of EST 6 allozymes. Genotypes of the X and III chromosomes are shown. Genetic nomenclature follows Lindsley and Grell (1968). *y* = yellow body, *w* = white eyes, *v* = vermilion eyes, *f* = forked bristles. All markers are sex-linked. The females in cross 2 carried an attached-*X* chromosome as well as a free *Y* chromosome. *Est 6* is on chromosome III.

wild-type stock carrying an *Est* 6^F allele. Offspring from these crosses were heterozygous (*Est* $6^{S/F}$), *yw* sterile males. In the second cross, *D. simulans* males carrying the *x*-linked marker vermilion eyes (*v*) and homozygous for the *Est* 6^F allele were crossed to *D. melanogaster* females homozygous for the *Est* 6^S allele carrying an attached *X* chromosome marked with yellow body and forked bristles (*yf*:). Offspring from this cross were represented by vermilion eyed males that were *Est* $6^{S/F}$. No triplo *X* females were found. In the third cross, *D. simulans* males marked with *v* and *Est* 6^F were crossed to *D. melanogaster* females which carried

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a wild-type *X* chromosome and were *Est 6*^{S}. This cross produced only females since males die in the larval stages of development (Aronshtam & Korochkin, 1975).

Parents and offspring from all crosses were analysed for EST 6 activity by disk-gel electrophoresis followed by quantitative densitometry. Homogenates of dissected

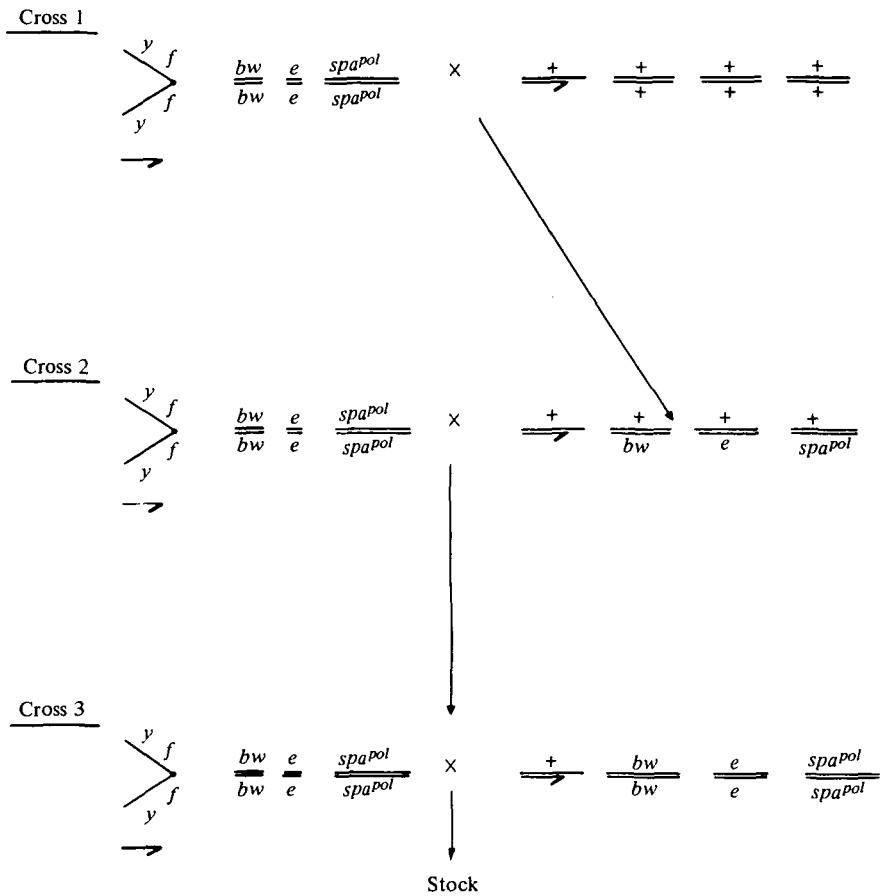

Fig. 3. Genetic scheme used to place *X* chromosomes derived from wild-collected males on a standard genetic background. Genotypes of the *X*, II, III and IV chromosomes are shown. Genetic nomenclature follows Lindsley & Grell (1968). The attached-*X*, free *Y* stock is marked with: *y* = yellow body, *f* = forked bristles, both *X*-linked loci; *bw* = brown eye colour, 2nd chromosome; *e* = ebony body, 3rd chromosome; *spa*^{pol} = sparkling poliert eye colour, 4th chromosome. A separate replicate experiment employed a similar attached-*X* stock marked with *ci* (cubitus interruptus, interruped cubital vein of wing) instead of *spa*^{pol}.

anterior ejaculatory ducts (AED) from hybrid males were also analyzed to determine if EST 6 activity differences observed in whole fly homogenates were tissue specific.

(viii) *Effect of extracted* X *chromosmes on EST 6 activity*

Wild-caught *D. melanogaster* males (collected in Bloomington, Indiana, in September of 1979) were crossed to attached *X*, free *Y* females (*yf*:) homozygous for recessive markers on all autosomes (Fig. 3). Two different experiments were completed. In the first experiment, a *yf*:, *bw*, *e*, spa^{pol} stock (see Fig. 3) was inbred for seven generations in our laboratory. Each of five wild-caught males was crossed sequentially to five females to yield a total of five separate male lines with five replicate cultures for each line (i.e. original wild male). Replicate cultures derived from a single parental male but five different and presumably isogenic females allow a control for environmental affects on EST 6 activity and possible residual genetic variation remaining within the inbred *yf*:, *bw*, *e*, spa^{pol} stock. F_1 males from the initial cross (Fig. 3) were then backcrossed to females from the inbred line to obtain a strain that was identical to the inbred *yf*:, *bw*, *e*, spa^{pol} stock except that males carried an *X* chromosome derived from a wild male. Spectrophotometric assays for EST 6 activity and total protein concentration were run on multiple-male fly homogenates. A second experiment employed a *yf*:, *bw*, *e*, *ci* stock which had been inbred for 15 generations in our laboratory.

Data from both experiments consisted of parallel measurements of EST 6 activity and total protein concentration from each of two homogenates from each culture. A nested analysis of the variance in protein concentration revealed that there were significant differences in averages between male groups and among replicate cultures within male groups. A similar analysis performed on specific activity data (optical density units per mg protein per 30 min at 27 °C) showed significant differences between male groups and among replicate cultures within male groups. We have examined the possibility that differences in EST 6 specific activity due to the source of the *X* chromosome (i.e. male groups) may be accounted for solely by the effect of *X* chromosomes on total protein level by completing nested analyses of covariance (Bliss, 1970; Steel & Torrie, 1970). In these analyses protein concentration is treated as the independent variable and EST 6 activity as the dependent variable. Such an analysis is preferable to using specific activity data since it corrects for differences in protein concentration among homogenates by using the regression of EST 6 activity on protein levels.

3. RESULTS

(i) *Comparison of EST 6 activity levels in thermostability lines*

Cochrane & Richmond (1979) analysed a series of isogenic, third-chromosome lines carrying the same electrophoretic variant for differences in the thermostability of EST 6. Several such variants were detected and found to map at or very close to the structural locus. We used six of these strains to ask whether differences in EST 6 activity might be found between strains which presumably share the same structural allele for EST 6 but have different genetic backgrounds. The experimental

design used (Fig. 1) also allowed assessment (by ANOVA) of the effects of various environmental and experimental factors on EST 6 specific activity. The results (Table 1) confirm previous findings (Sheehan *et al.* 1979) that EST 6 activity in males greatly exceeds that in females and show that strains carrying the same thermostability variant have highly significant differences in EST 6 activity. No

Table 1. *Mean specific activities* (±S.E.M.) *of two different Est 6 thermostability variants.*

(Specific activity is expressed in μ-moles of β-napthol produced/30 min/mg protein at 27 °C. Also presented is an analysis of variance of specific activities. See Fig. 1 for experimental design.)

		Mean specific activity of *Est 6*	
Allele	Strain	Female	Male
Est-$6^{1.00-2}$	Bm 5	0·297±0·050	0·672±0·048
Est-$6^{1.00-2}$	Bm 12	0·247±0·010	0·452±0·039
Est-$6^{1.00-2}$	Bm 14	0·363±0·012	1·747±0·116
Est-$6^{1.10-2}$	Bm 46	0·280±0·018	0·359±0·009
Est-$6^{1.10-2}$	Bm 48	0·401±0·047	1·491±0·263
Est-$6^{1.10-2}$	Bm 59	0·332±0·014	1·279±0·101

Analysis of Variance

Source	D.F.	Mean square	*F*	*P*
Alleles	1	0·04	1·1	> 0·05
Strains	4	1·00	24·2	< 0·001
Cultures	6	0·04	1·6	> 0·05
Sexes	1	5·54	90·0	< 0·001
Sexes × alleles	1	0·01	0·1	> 0·05
Sexes × strains	4	0·71	11·5	< 0·01
Sexes × cultures	6	0·06	2·4	> 0·05
Homogenates	24	0·03		

significant differences were found beween the two *Est 6* alleles represented. The presence of a significant sexes × strains interaction term in the ANOVA indicates that EST 6 activity in the two sexes responds differentially over a range of genetic backgrounds. These results suggest that either the strains carrying the same thermostability variant are still genetically heterogeneous at the *Est 6* locus, or genetic background differences between strains carrying the same allele result in differences in EST 6 activity. It is the latter possibility which we explore further below.

(ii) *EST 6 activity in hybrids between high- and low-activity strains*

Reciprocal crosses between a high-activity, *Est* 6^S line (Dm 48) and a low-activity, *Est* 6^F line (DM 49) produced male progenies whose specific activities were indistinguishable from the average of the two parental lines (Table 2). However, quantitative densitometric analyses of EST 6 activity in gels of the parents and

progeny revealed that the intermediate level of EST 6 activity in the male progeny did not result from simple additive expression of the two *Est 6* alleles (Table 2). Rather, in the hybrid, the activity of the EST 6^S enzyme appeared to be less than the activity of EST 6^F, contradicting the expected results (Fig. 4 and Table 2). These data do not distinguish among transcriptional, post-transcriptional or translational effects which are allele-specific (Cochrane & Richmond, 1979), but do support the hypothesis that EST 6 activity may be affected by genes other than the structural locus.

Table 2. *An analysis of EST 6 activity in high and low hybrid lines*

(Dm 48 is a high-activity line carrying *Est* 6^S and DM 49 is a low-activity line carrying *Est* 6^F. Both strains were made homozygous for their third chromosome using the TM3 balancer (see Materials and Methods). Specific activities are given for the male parents and offspring of the reciprocal crosses. Activities were measured in duplicate on each of three separate homogenates (10 ♂♂/homogenate) for each strain. Specific activity is expressed in μmoles of β-Napthol produced/30 min/mg protein at 27 °C. Densitometric analyses of EST 6 activity for male parents and offspring are expressed as the ratio of activity of the *EST* 6^S to the *EST* 6^F allozyme.)

Strain	Specific activity ±S.E.M.	*N**	Densitometric ratio ±S.E.M. (*EST* 6^S/*EST* 6^F)
DM 48 (*Est* 6^S)	2·57±0·12	12	
	avg. = 1·92		2·03±0·174
DM 49 (*Est* 6^F)	1·26±0·06	5	
F_1 Dm 48 × Dm 49	1·86±0·16	9	1·06±0·041
F_1 Dm 49 × Dm 48	1·95±0·15	8	1·04±0·052

* Sample sizes are the number of separate gels scanned for each strain. For the parental lines (DM 48, 49) the densitometric ratio was determined as the average ratio of areas under a scan (see for example Fig. 4) for 12 and 5 gels of DM 48 and DM 49 respectively.

(iii) *EST 6 activity in hybrids between* D. melanogaster *and* D. simulans

Analysis of the expression of alternative alleles in species hybrids is a valuable means of examining the regulation of the locus in question (Davidson, 1976; Dickinson, 1980). Aronshtam & Korochkin (1975) studied the relative expression of the *Est 6* alleles in hybrids between *D. melanogaster* and *D. simulans*. Their results suggested that elements on the *X* chromosome regulate the expression of the structural gene for EST 6. We have completed similar experiments using different stocks and carefully controlled densitometric analyses of activity-stained gels.

The interspecific crosses employed are shown in Fig. 2. In cross 1, *D. simulans* females homozygous for the *X*-linked markers, yellow body (*y*) and white eyes (*w*) and the *Est* 6^S allele were crossed to wild-type *D. melanogaster* males homozygous for *Est* 6^F allele. The progeny of this cross were all sterile males hemizygous for

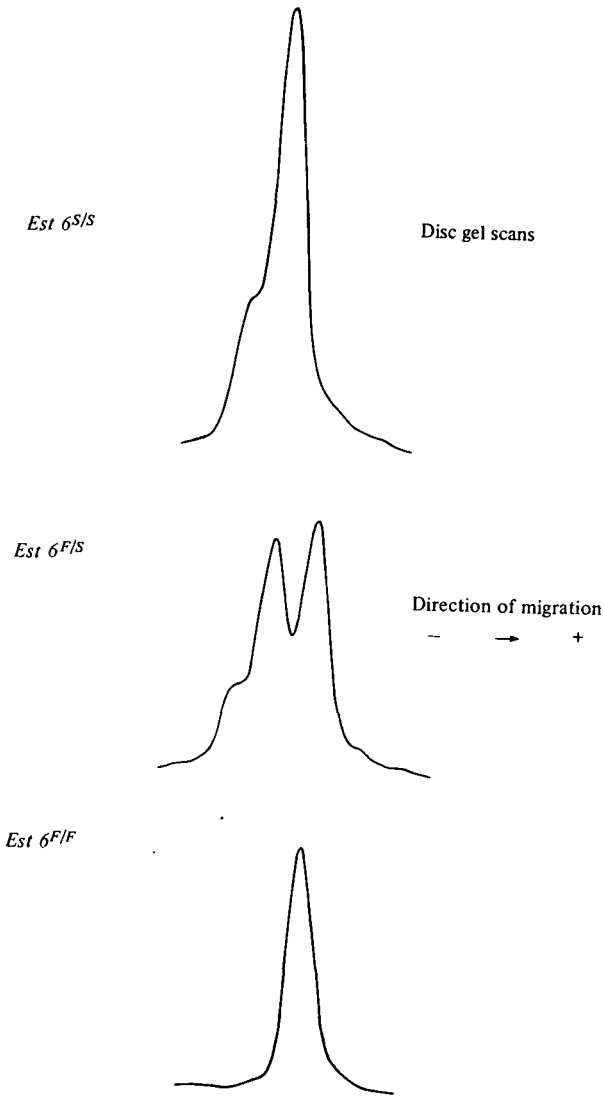

Fig. 4. Densitometric scans of gels stained for EST 6 activity (see text). The ordinate is an arbitrary scale and is identical for each scan. Analyses of males from the parental strains are on the top and bottom and male progeny in the middle. Electrophoretic migration was from left to right.

y and *w* and heterozygous for *Est* 6^S/*Est* 6^F as expected (Sturtevant 1920). These hybrid males were homogenized as described and subjected to disk, acrylamide gel electrophoresis. The gels were stained for esterase activity and scanned with an integrating densitometer. In these hybrids, EST 6^S activity exceeds that of EST 6^F by a factor of 1·94 (Table 3). As a control for the possibility that these results were due to an alteration in the tissue-specific expression of EST 6 in interspecific

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hybrids, we analysed EST 6 activity in dissected anterior ejaculatory ducts from hybrid males (Table 3, row 2). Unequal EST 6 activity persists even in this single tissue.

The second cross (Fig. 2) was designed to produce hybrid males whose *X* chromosome derived from the *simulans* father rather than from the *simulans* mother as in cross 1. Crosses between *D. simulans* males and *melanogaster* females

Table 3. *Densitometric analyses of relative EST 6 activity in interspecific hybrids between* D. melanogaster (D.m.) *and* simulans (D.s.) *and parental stocks*

(See Fig. 2 for the derivation of hybrid flies. EST 6 activity is expressed as the ratio of the activity of the EST 6^S to the EST 6^F allozyme as measured in the same gel. Unless otherwise indicated homogenates of whole flies were analysed.)

Row	Cross no.*	Source of *X* chromosome	No. of flies analysed	Ratio ± S.E.M. (EST 6^S/EST 6^F)
1	1	*D.s.*	42	1·94 ± 0·07
2	1†	*D.s.*	10	1·93 ± 0·01
3	2	*D.s.*	25	0·55 ± 0·06
4	3	*D.s.* + *D.m.*	25	1·02 ± 0·03
5	1, 2, 3‡	—	10	1·07 ± 0·30

* See Fig. 2.
† Anterior ejaculatory ducts analysed.
‡ Homogenates of parents from all three crosses, see text.

normally produce only sterile females (see Cross 3, Fig. 2). The *melanogaster* female used in cross 2 carried an attached-*X* chromosome and a free *Y* chromosome which results in the production of hybrid males whose *X* chromosome derives from the *simulans* male parent and the *Y* from the *melanogaster* parent. In these hybrid males, *EST* 6^F activity predominates over *EST* 6^S by a factor of 1·82. In both crosses 1 and 2 (Fig. 2), enzyme activity coded by the structural gene transmitted by the gamete also carrying the *X* chromosome predominates over the activity of the allozyme coded by the structural gene transmitted by the other parent. A maternal effect is ruled out since the egg cytoplasm in crosses 1 and 2 is of different origin than the structural gene coding for the enzyme whose activity is greater. These results confirm the findings of Aronshtam and Korochkin (1975) and support the hypothesis that *X*-linked factors regulate the expression of EST 6 activity.

If this hypothesis holds, female hybrids should show equivalent levels of EST 6 activity. Cross 3 (Fig. 2) was performed to test this possibility. Although EST 6 activity in females is reduced over that found in males, the activity of both allozymes is the same in female hybrids (Table 3, row 4). Control gels containing homogenates from both parents in each of the three crosses shown in Fig. 2 also showed approximately equivalent levels of activity for both enzymes (Table 3, row 5).

There is an alternative explanation of these data (Dr J. J. Bonner, personal communication) which we believe to be unlikely, but which cannot be excluded by our results. Suppose the structural alleles of *Est 6* carry species-specific

promoters which are autogenously regulated by EST 6 protein. Since the hybrid males tested always carry an *X* chromosome derived from their *simulans* parent, we are unable to separate the effects of *X* chromosome regulation from autogenous regulation resulting from elements at the structural locus. Equivalent expression in female hybrids could arise from a difference in the mechanism of regulation in the two sexes. However, the expression of acid phosphatase and aldehyde oxidase activity (both have third chromosome structural loci) in *simulans–melanogaster*, male hybrids apparently results from equivalent expression of the structural genes from each species (MacIntyre, 1966; Courtright, 1967). Pipkin & Hewitt (1972) found that alcohol dehydrogenase expression in *simulans–melanogaster*, male hybrids deviated more from expectation than in *melanogaster–simulans*, female hybrids. Since this enzyme is not sex or tissue specific, they attribute their findings to an imbalance between the *X* and the autosomes in male hybrids.

The analysis of EST 6 activity in hybrid flies suggests that the differences in EST 6 activities found among strains of the same thermostability variant, and our failure to find additive EST 6 expression in progeny of some high- and low-activity lines may be due to heterogeneity in the *X* chromosomes carried by various stocks. We have examined this possibility by extracting *X* chromosomes from wild-collected males and determining their effect on EST 6 activity in a standard genetic background. These experiments are described below.

(iv) *Est 6 activity in coisogenic lines differing only in their* X *chromosomes*

We have used two attached *X*, free-*Y* stocks ($\hat{XX}Y$) in which all autosomes are marked with recessive morphological genes to place *X* chromosomes derived from wild-collected males on a standard, coisogenic genetic background (see Fig. 3 and Materials and Methods). Each $\hat{XX}Y$ stock was used to isolate five wild-collected *X* chromosomes to give a total of ten independently derived *X* chromosome lines. In most cases, five replicates of each line were generated by crossing the original wild-collected male to five individual females from an $\hat{XX}Y$ stock (Fig. 3).

Preliminary statistical analyses of the data from these two experiments revealed that there were significant differences in total protein concentration between lines differing in their *X* chromosome and between replicate cultures within the same line. Accordingly we have used analysis of covariance methods to adjust the mean EST 6 activities of each line to an equivalent concentration of total protein (Tables 4 and 5). This method is preferable to the standard specific activity expression since it utilizes the actual relationship between protein concentration and enzyme activity to adjust activity levels rather than assuming a perfect correlation between the variables.

The mean adjusted EST 6 activities given in Tables 4A and 5A differ by 240% for the two experiments. Recent reanalysis of some of these lines has confirmed this difference. This result suggests that the genetic backgrounds (i.e. 2nd, 3rd, 4th chromosomes) of the two $\hat{XX}Y$ stocks are significantly different. This is expected as they have been maintained as separate stocks in the Mid-America Drosophila

Stock Center (Bowling Green State University) and for more than 3 years in our laboratory. Although both $X\hat{X}Y$ stocks carry the EST 6^S electromorph, we do not know if the structural loci in these stocks are identical. Thus the differences in EST 6 activities between the two experiments is not surprising.

Table 4

((A) Protein concentration (mean mg/ml ± S.E.M.) and EST 6 activity (mean OD units/ml ± S.E.M.) for five lines differing only in their *X* chromosomes. The lines were isolated using the *yf*::; *bw*; *e*; spa^{pol} 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	Protein	EST 6 activity	Adjusted EST 6 activity
7–1	1·427 ± 0·023	15·29 ± 0·13	15·30
7–2	1·244 ± 0·047	15·13 ± 0·29	15·17
7–4	1·494 ± 0·063	15·18 ± 0·05	15·18
7–5	1·407 ± 0·058	17·24 ± 0·91	17·25
7–9	1·753 ± 0·049	21·12 ± 0·37	21·07

(B) *Nested analysis of covariance*

		Sums of squares and products			Adjusted mean squares for EST 6 activity			
Source	D.F.	P^2*	$P \times E$†	E^2‡	D.F.	*MS*	*F*	*P*
Among lines	4	1·098	12·478	202·30	4	20·60	4·14	0·02
Among replicates within lines	14	0·719	3·539	85·56	14	4·98	88·9	< 0·001
Error	19	0·076	0·013	1·00	18	0·056		
Total	37	1·893	16·030	288·86	36			

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

The nested analyses of covariance given in Tables 4 B and 5 B test the hypothesis that there are no significant differences in EST 6 activity between lines of flies which differ in the *X* chromosome which they carry. This hypothesis is clearly rejected for the data of Table 4 and is at the borderline of statistical significance for the data of Table 5. We conclude that natural populations of *D. melanogaster* are genetically variable for factors carried by the *X* chromosome which influence the level of EST 6 activity. For the experiment summarized in Table 4, different *X* chromosomes have resulted in a 39% difference in EST 6 activity between the highest and lowest lines. In both experiments, there was significant within line, between replicate variance. This result suggests that either there were large environmental differences between cultures or that different females from the same inbred $X\hat{X}Y$ stock were not genetically identical or some combination of both factors contributed to 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.

The crosses used to place wild-derived *X* chromosomes on a standard background are exactly those which should result in the induction of hybrid dysgenic events (Engels, 1980). Such events can result in a syndrome of effects including sterility,

Table 5

((A) Protein concentration (mean mg/ml ± S.E.M.) and EST 6 activity (mean OD units/ml ± S.E.M.) for five 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	Protein	EST 6 activity	Adjusted EST 6 activity
15–1	1·344 ± 0·047	42·17 ± 1·14	44·69
15–2	1·684 ± 0·025	41·95 ± 0·49	39·57
15–4	1·533 ± 0·096	37·75 ± 1·24	37·59
15–5	1·474 ± 0·067	41·24 ± 1·09	41·95
15–6	1·574 ± 0·038	41·97 ± 1·21	41·21

(B) *Nested analysis of covariance*

		Sums of squares and products				Adjusted mean squares for EST 6 activity		
Source	D.F.	P^2*	$P \times E$†	E^2‡	D.F.	*MS*	*F*	*P*
Among lines	4	0·563	−0·182	133·42	4	42·25	2·85	0·054
Among replicates within lines	18	1·378	11·738	363·32	18	14·85	10·03	< 0·001
Error	23	0·116	1·707	57·66	22	1·48		
Total	45	2·057	13·263	554·40	44			

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

male recombination and the contamination of homologous or non-homologous chromosomes by mobile genetic elements. In our crossing scheme (Fig. 3), male sterility would have prevented the establishment of a stock. Male recombination could have moved genes, possibly influencing EST 6 activity, from the wild-type chromosomes to the marked autosomes in the progeny of cross 2. This possibility is an unlikely explanation for our results since males carrying wild-derived *X* chromosomes were repeatedly backcrossed to $X\hat{X}Y$ stock. This procedure would result in the substitution of autosomes from the $X\hat{X}Y$ stock for possible recombinant autosomes in the progeny of the male in cross 2. We cannot exclude the possibility that the structural locus for *Est 6* has been contaminated by a mobile element, but the number of lines found which exhibit different levels of EST 6 activity would

seem to mitigate against a presumably rare contaminant event for a given locus. Many of these presumed dysgenic effects in our analyses are susceptible to investigation and such experiments are currently in progress.

4. DISCUSSION

We have presented four independent sets of data which support the hypothesis that loci other than the structural gene for EST 6 affect its activity in adult males. Data from the *D. melanogaster–simulans* hybrids and the *X* chromosome isolation lines suggest that some of these regulatory loci act in trans. The species hybrid data further suggest that there is a species difference in the response to whatever regulatory mechanism is activated by the *X* chromosome in hybrids and provides support for the hypothesis that species differ in this regulatory gene (Wallace, 1963; Carson, 1975; Wilson, 1975).

If regulatory gene differences are fundamental to the speciation process and contribute to interspecific genetic differentiation, then it is reasonable to suppose that such loci will be polymorphic within natural populations thus forming the substrate from which species are elaborated. Our results using isolated *X* chromosome lines confirm the findings of others (Barnes & Birley, 1978; McDonald & Ayala, 1978; Laurie-Ahlberg, 1980; Wilson & McDonald, 1981) that structural loci coding for enzymes are regulated by loci which are themselves genetically variable. These results, however, are just a beginning. The theories of population genetics and the newly presented theories of speciation (Templeton, 1980*a*, *b*) are formulated in terms of gene and genotype frequencies. Just as information about the frequency of lethal-bearing chromosomes was not sufficient to determine the levels of genetic variation in natural populations (Lewontin, 1974), so too will data on the incidence of chromosomes influencing enzyme activity be unsatisfactory. Specific loci must be mapped and their allelic content quantitated. The EST 6 system in *D. melanogaster* provides the necessary tools to achieve this aim.

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REFERENCES

Abraham, I. & Doane, W. (1978). Genetic regulation of tissue-specific expression of amylase structural genes in *Drosophila melanogaster*. *Proceedings National Academy of Science* (*U.S.A.*) **75**, 4446–4450.

Anderson, S. & McDonald, J. F. (1981). Effect of environmental alcohol on *in vivo* properties of *Drosophila* alcohol dehydrogenase. *Biochemical Genetics* **19**, 421–430.

Aronshtam, A. A. & Kuzin, B. A. (1974). Investigation of the activity of esterase 6 in ontogenesis in *Drosophila melanogaster* and *Drosophila simulans*. *Zhurnal Obshei Biologii* (*USSR*) **35**, 926–933 (in Russian).

Aronshtam, A. A. & Korochkin, L. I. (1975). Expression of allelomorphs of the locus Est 6 in hybrids between *Drosophila melanogaster* and *Drosophila simulans*. *Genetika* **11**, 87–91.

BARNES, B. W. & BIRLEY, A. J. (1978). Genetic variation for enzyme activity in a population of *Drosophila melanogaster*. IV. Analysis of alcohol dehydrogenase activity in chromosome substitution lines. *Heredity* **40**, 51–57.

BLISS, C. I. (1970). *Statistics in Biology*, vol. 2. New York: McGraw-Hill.

CARSON, H. L. (1975). The genetics of speciation at the diploid level. *American Naturalist* **109**, 83–92.

CAVENER, D. R. & CLEGG, M. T. (1981). Temporal stability of allozyme frequencies in a natural population of *Drosophila melanogaster*. *Genetics* (submitted).

CHOVNICK, A., GELBART, W., MCCARRON, M., OSMOND, B., CANDIDO, E. P. M. & BALLIE, D. L. (1976). Organization of the rosy locus in *Drosophila melanogaster*: Evidence for a control element adjacent to the xanthine dehydrogenase structural element. *Genetics* **84**, 233–255.

COCHRANE, B. & RICHMOND, R. C. (1979). Studies of esterase 6 in *Drosophila melanogaster*. I. The genetics of a posttranslational modification. *Biochemical Genetics* **17**, 167–183.

COURTRIGHT, J. B. (1967). Polygenic control of aldehyde oxidase in *Drosophila*. *Genetics* **57**, 25–38.

DAVIDSON, E. H. (1976). *Gene Activity in Early Development*. New York: Academic Press.

DAVIS, B. J. (1964). Disc electrophoresis. II. Methods and applications to human serum proteins. *Annual New York Academia of Science* **121**, 404–422.

DICKINSON, W. J. (1975). A genetic locus affecting the developmental expression of an enzyme in *Drosophila melanogaster*. *Developmental Biology* **42**, 131–140.

DICKINSON, W. J. (1980). Tissue specificity of enzyme expression regulated by diffusable factors: Evidence in *Drosophila* hybrids. *Science* **207**, 995–997.

ENGELS, W. R. (1980). Hybrid dysgenesis in *Drosophila* and the stochastic loss hypothesis. *Cold Spring Harbor Symposia on Quantitative Biology* **20**, 561–565.

FINNERTY, V. & JOHNSON, G. (1979). Post-translational modification as a potential explanation of high levels of enzyme polymorphism: Xanthine dehydrogenase and aldehyde oxidase in *Drosophila melanogaster*. *Genetics* **91**, 695–722.

FINNERTY, V., MCCARRON, M. & JOHNSON, G. (1979). Gene expression in *Drosophila*: post-translational modification of aldehyde oxidase and xanthine dehydrogenase. *Molecular General Genetics* **172**, 37–43.

GILBERT, D. G. & RICHMOND, R. C. (1981). Esterase 6 in *Drosophila melanogaster*: reproductive function of active and null males at low temperature. *Proceedings National Academy of Science* (*U.S.A.*) (in the Press, May).

GILBERT, D. G., RICHMOND, R. C. & SHEEHAN, K. B. (1981). Studies of Esterase 6 in *Drosophila melanogaster*. VII. The timing of remating in females inseminated by males having active or null alleles. *Behavioral Genetics* **11**, 195–208.

GIRARD, P., PALABOST, L. & PETIT, C. (1977). Enzyme variation at seven loci in nine natural populations of *Drosophila melanogaster*. *Biochemical Genetics* **15**, 589–599.

HEDRICK, P. W. & MCDONALD, J. F. (1980). Regulatory gene adaptation: An evolutionary model. *Heredity* **45**, 83–97.

KOROCHKIN, L. I., EVGENIEV, M. & MATVEEVA, N. (1972). The influence of genotype on phenotype expression of esterase in Drosophila of the *virilis* group. *Drosophila Information Service* **49**, 103.

KOROCHKIN, L. I., BELYAEVA, E. S., MATVEEVA, N. M., KUZIN, B. A., & SEROV, O. L. (1976). Genetics of esterases in *Drosophila*. IV. Slow-migrating S-esterase in *Drosophila* of the *virilis* group. *Biochemical Genetics* **14**, 161–182.

LAURIE-AHLBERG, C. C., MARONI, G. P., BRAWLEY, G. C., LUCCHESI, J. C. & WEIR, B. S. (1980). Quantitative genetic variation of enzyme activities in natural populations of *Drosophila melanogaster*. *Proceedings National Academy of Science* (*U.S.A.*) **77**, 1073–1077.

LEWONTIN, R. C. (1974). *Genetic Basis of Evolutionary Change*. New York: Columbia Press.

LINDMAN, H. R. (1974). *Analysis of Variance in Complex Experimental Designs*. San Francisco: W. H. Freeman.

LINDSLEY, D. L. & GRELL, E. H. (1968). *The Genetic Variations of* Drosophila melanogaster. Carnegie Institution, Washington, D.C.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the folin phenol reagent. *Journal Biological Chemistry* **193**. 265–275.

MacIntyre, R. (1966). The genetics of acid phosphatase in *Drosophila melanogaster* and *Drosophila simulans*. *Genetics* **53**, 461–474.

McDonald, J. F., Chambers, G. K., David, J. & Ayala, F. J. (1977). Adaptive response due to changes in gene regulation: A study with *Drosophila*. *Proceedings National Academy of Science (U.S.A.)* **74**, 4562–4566.

McDonald, J. F. & Ayala, F. J. (1978). Genetic and biochemical basis of enzyme activity in natural populations. I. Alcohol dehydrogenase in *Drosophila melanogaster*. *Genetics* **89**, 371–388.

Norman, R. A. & Prakash, S. (1980). Activity variants of acid phosphatase-3 among chromosome 3 inversions of *Drosophila pseudoobscura*. *Genetics* **96**, 743–755.

Pipkin, S. B. & Hewitt, N. E. (1972). Variation of alcohol dehydrogenase levels in *Drosophila* species hybrids. *Journal of Heredity* **63**, 267–270.

Richmond, R. C., Gilbert, D. G., Sheehan, K. S., Gromko, M. H., & Butterworth, F. W. (1980). Esterase 6 and reproduction in *Drosophila melanogaster*. *Science* **207**, 1483–1485.

Sheehan, K., Richmond, R. C., & Cochrane, B. J. (1979). Studies of esterase 6 in *Drosophila melanogaster*. III. The developmental pattern and tissue distribution. *Insect Biochemistry* **9**, 443–450.

Stebbins, G. L. (1969). *The Basis of Progressive Evolution*. Chapel Hill: University of North Carolina Press.

Steel, R. G. D. & Torrie, J. H. (1970). *Principles and Procedures in Statistics*. New York: McGraw-Hill.

Sturtevent, A. H. (1920). *Genetic Studies on Drosophila simulans*. I. Introduction. Hybrids with *Drosophila melanogaster*. *Genetics* **5**, 488–500.

Templeton, A. (1980*a*). *The theory of speciation via the founder principle*. *Genetics* **94**, 1011–1038.

Templeton, A. (1980*b*). Modes of speciation and inferences based on genetic distance. *Evolution* **34**, 719–729.

Vallentine, J. W. & Campbell, C. A. (1975). Genetic regulation and the fossil record. *American Scientist* **63**, 673–680.

Wallace, B. (1963). Genetic diversity, genetic uniformity and heterosis. *Canadian Journal of Genetic Cytology* **5**, 239–253.

Ward, R. (1975). Alcohol dehydrogenase activity in *Drosophila melanogaster*: A quantitative character. *Genetical Research* **26**, 81–93.

Wilson, A. C. (1975). Evolutionary importance of gene regulation. *Stadler Symposia* **7**, 117–133.

Wilson, P. G. & McDonald, J. F. (1981). A comparative study of enzyme activity variation between α glycerophosphate and alcohol dehydrogeneses in *Drosophila melanogaster*. *Genetics* **55**, 75–79.

Wright, T. R. F. (1963). The genetics of an esterase in *Drosophila melanogaster*. *Genetics* **48**, 787–801.