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Regulatory aspects of esterase 6 activity variation in sibling *Drosophila* species

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Esterase 6 in *Drosophila melanogaster, Drosophila simulans* and *Drosophila mauritiana* is produced in several life stages and diverse tissues, but the major pulse of expression is in the sperm ejaculatory duct of adult males. Comparison of EST6 activity levels among several lines of *D. melanogaster, D. simulans* and *D. mauritiana* reveals two major quantitative differences among the species. First, newly eclosed females of both *D. simulans* and *D. mauritiana* show significantly higher EST6 activity than those of *D. melanogaster.* Secondly, 5-day-old adult *D. simulans* have significantly higher activities than *D. mauritiana* in both sexes and significantly higher activity than *D. melanogaster* in males. The genetic bases of the differences between *D. melanogaster* and the other species are investigated through germ line transfer of the *D. simulans* and *D. mauritiana Est-6* genes plus 1.2 kb of 5′ and 0.2 kb of their 3′ flanking sequences into *D. melanogaster.* The newly eclosed female activities of the transformants resemble those of the two donor species, suggesting that the interspecific differences in this aspect of expression are due to *cis*-inherited factors contained within the transferred DNA. In contrast, the 5-day adult activity of the *D. simulans* transgene resembles the recipient species, *D. melanogaster*, suggesting that the difference between *D. simulans* and *D. melanogaster* in this aspect of expression is due to *trans*-acting factors. We also find that third instar larval activities of the *D. simulans* transgene and 5-day male activities of the *D. mauritiana* transgene are lower than those of either parental species, suggesting that not all the promoter elements relevant to these aspects of expression are included in the transferred DNA.

Keywords: activity variation, *Drosophila*, esterase 6, gene regulation, germ line transformation, interspecific comparisons.

Introduction

Evidence is accumulating that evolutionary change in gene expression can result from two types of regulatory mutation. One involves *cis*-inherited mutations in the gene's promoter and the other involves *trans*-inherited mutations in protein(s) that directly or indirectly affect the functioning of the promoter. While analyses of gene expression in interspecific hybrids and interspecific tissue transplants have provided evidence of both types of change (e.g. Aronshtam & Kuzin, 1974; Cavener, 1985; Kuhn & Sprey, 1987), a more direct test is now available. Specifically, interspecific gene transfer experiments can test whether species-specific differences in the expression of a particular transgene are due to *cis*-acting elements within the introduced DNA, or to *trans*-acting factors in the host genome, or to a combination of both.

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Most gene transfer experiments among *Drosophila* species have used *D. melanogaster* as a host and several have demonstrated that observed interspecific differences are due to *cis*-acting elements directing donor species-specific patterns of expression (reviewed in Dickinson, 1991). Relatively few studies have yielded evidence for *trans*-acting control over interspecific differences in expression (*Ddc*, Bray & Hirsh, 1986; *Adh*, Fischer & Maniatis, 1986 and Wu *et al.*, 1990; urate oxidase, Wallrath & Friedman, 1991; *Gld*, Cavener, 1992 and references therein). It is notable that all four systems in which *trans*-effects have been demonstrated are responsive to juvenile hormone (JH) and/or ecdysone (Riddiford, 1992).

Here we explore the contribution of *cis*- and *trans*-inherited changes to quantitative differences in esterase 6 (*Est-6*/EST6) expression among three sibling species of *Drosophila*. The three species are *D. melanogaster, D. simulans* and *D. mauritiana*, which all show significantly higher EST6 expression in mature

adult males than females, due to a major pulse of EST6 production in the anterior sperm ejaculatory duct (Stein *et al.*, 1984; Morton & Singh, 1985; Uspenskii *et al.*, 1988). Although little else is known about the tissue distribution of EST6 expression in *D. mauritiana*, the other two species share many other sites of expression, including substantial activity in haemolymph throughout development (Aronshtam & Kuzin, 1974). In *D. melanogaster* at least, the control of EST6 expression in ejaculatory ducts and possibly other tissues is modulated by both JH and ecdysone (Richmond & Tepper, 1983; Stein *et al.*, 1984). The overall structure of the *Est-6* gene and promoter region is conserved among the three species; two large, perfectly conserved regions in the 350 bp immediately 5′ of the gene are thought to be responsible for basal levels of *Est-6* expression while numerous nucleotide substitutions and small insertions/deletions over the next 700 bp 5′ provide the scope for regulatory divergence (Karotam *et al.*, 1993).

In this study we have undertaken a quantitative comparison of EST6 expression in several lines of *D. melanogaster, D. simulans* and *D. mauritiana* across three stages of development and both sexes. We have then tested the genetic basis for the observed interspecific differences, using germ line transformation to introduce two *D. simulans* and one *D. mauritiana Est-6* alleles and their flanking regions into the same *D. melanogaster* background.

Materials and methods

Fly stocks

The interspecific activity comparisons were based on 10 field-derived isofemale lines and seven other laboratory stocks of unknown origin. The five *D. melanogaster* stocks used were all isofemale lines: mel-1 to mel-4 were derived from Coffs Harbour, Australia by Cooke *et al.* (1987) and mel-5 (the source of the *Est-6* genomic sequence of Collet *et al.*, 1990) was isolated from Indiana, U.S.A. (stock Dm145 of R.C. Richmond, University S. Florida). The seven *D. simulans* lines comprised: five isofemale lines (sim-1 to sim-5), which were isolated from Coffs Harbour, Australia; and two laboratory stocks, sim-6, which was the *D. simulans* stock of A.R. Lohe (Case West. University, Ohio) and sim-7 which was the C135.20 stock from the Bowling Green Stock Center. The five *D. mauritiana* stocks comprised: mau-1 and mau-2, which were isolated from the G72 strain of D. L. Hartl (Washington University, St. Louis); mau-3, which was the G122 strain of D. L. Hartl; mau-4, which was the *D. mauritiana* stock of A. R. Lohe; and mau-5, which was the Cambridge stock of the Bowling Green Stock Center. The *D. simulans* strains isolated from the Coffs Harbour population were made homozygous for EST6 by repeated sib-mating. These and all other strains mentioned above were confirmed as pure breeding for EST6 allozyme status by high resolution electrophoretic analyses (methods of Cooke *et al.*, 1987).

Strains of *D. melanogaster* used in germ line transformation experiments were *w*; Δ2-3(99B), *w*; TM3/TM6B and *w*; EST6^{null} (Sheehan *et al.*, 1979; Robertson *et al.*, 1988). All stocks were maintained at 18°C on standard yeast–treacle–cornmeal media (Healy *et al.*, 1991).

Biochemical analyses

EST6 activity was measured on homogenates of wandering third instar larvae (3IL), newly eclosed (≤2 h old) adult males (NE♂) and females (NE♀) and 5-day-old virgin males (5D♂) and females (5D♀). Separate homogenates from triplicate cultures were assayed for each wild type and transformant strain. All organisms collected for assays were snap frozen in liquid nitrogen and stored at −80°C. Homogenates were prepared by the methods of Healy *et al.* (1991), divided into aliquots and stored at −20°C. Two replicates of each homogenate were assayed spectrophotometrically for β-naphthyl acetate hydrolysing activity in the presence of eserine sulfate and *p*-chloromercuribenzoate acid (Healy *et al.*, 1991), an assay shown to be specific for EST6 in these species (Sheehan *et al.*, 1979; Healy *et al.*, 1991; J. K., M. J. Healy & J. G. O., unpublished data). Replicate determinations of the protein concentration of each homogenate were obtained by the method of Bradford (1976) using the BioRad protein assay kit II. EST6 specific activities were calculated as μmol β-naphthol produced per 30 min per mg protein and logarithmic transformations of these values were analysed using Genstat 5 Release 2.1 (Lane *et al.*, 1987; Digby *et al.*, 1989).

Germ line transformation

Three genomic clones of *Est-6* which had been sequenced previously (Karotam *et al.*, 1993; J. K., T. M. Boyce & J. G. O., unpublished data) were used as donor DNA in the transformation experiments. The two *D. simulans* clones both comprised a 3.00 kb *Hin*dIII/*Sca*I fragment including the 1.68 kb *Est-6* gene, 1.18 kb of 5′ flanking sequence and 0.14 kb of 3′ flanking sequence. The 3.10 kb *Hin*dIII/*Sca*I fragment from *D. mauritiana* was homologous to those from *D. simulans* but slightly larger, due to a 102 bp insertion

in the 5′ flanking region 1.14 kb 5′ of the start of translation (J. K. & J. G. O., unpublished data). One of the *D. simulans* clones (hereafter denoted simE6A) had been isolated from the sim-1 stock above but the strains from which the other two clones (simE6B and mauE6) were derived were no longer available. Plasmid DNA was amplified, prepared and digested by methods described in Karotam *et al.* (1993). All three fragments were subcloned into the *Bam*HI site of the pCaSpeR transformation vector (Robertson *et al.*, 1988) using *Bcl*I linkers (BRL) and the methods of Rusche & Howard-Flanders (1985) and Karotam *et al.* (1993). All pCaSpeR clones chosen for injection had the *Est-6* insert in the same orientation, such that the *Est-6* and *white* genes would be convergently transcribed.

DNA from these clones was purified through two rounds of $CsCl_2$ gradient centrifugation and then micro-injected into embryos produced by the *w*; Δ2-3(99B) × *w*; TM3/TM6B cross, as described by Zachar *et al.* (1987). Transformants were identified by the rescue of the white eye colour and those in which eye colour assorted independently of the third chromosome markers were crossed into an *Est-6* null background by replacement of their third chromosomes with those from the *D. melanogaster* EST6^{null} stock. After establishing homozygous stocks, each integrated construct was confirmed as a single copy by Southern blot hybridization (methods as in Karotam *et al.*, 1993) using the *D. melanogaster Est-6* genomic clone as a probe (Collet *et al.*, 1990). Four independent transformants of each *D. simulans* subclone (denoted mel^{simE6A} lines 1 to 4 and mel^{simE6B} lines 1 to 4) and five of the *D. mauritiana* subclone (mel^{mauE6} lines 1 to 5) were chosen for further analyses. The inserted DNA was inherited on the X chromosome of mel^{simE6A} lines 3 and 4, mel^{simE6B} line 3 and mel^{mauE6} lines 1, 2 and 3, and on the autosomes of the remainder.

Results

The patterns of EST6 expression in the seventeen lines of *D. simulans*, *D. mauritiana* and *D. melanogaster* are broadly similar (Fig. 1). In all three species third instar larval activity tends to be lower than that in adults, newly eclosed adults show lower activity than 5-day adults and adult females show lower activity than males. The latter difference is much less pronounced in newly eclosed flies (generally less than two-fold) than 5-day flies (up to 26-fold, depending on the line).

Table 1 summarizes the analysis of the data from the 17 lines and three species for each of the five activity measures. For most measures there is significant variation (up to six-fold) among lines within species (see Fig.

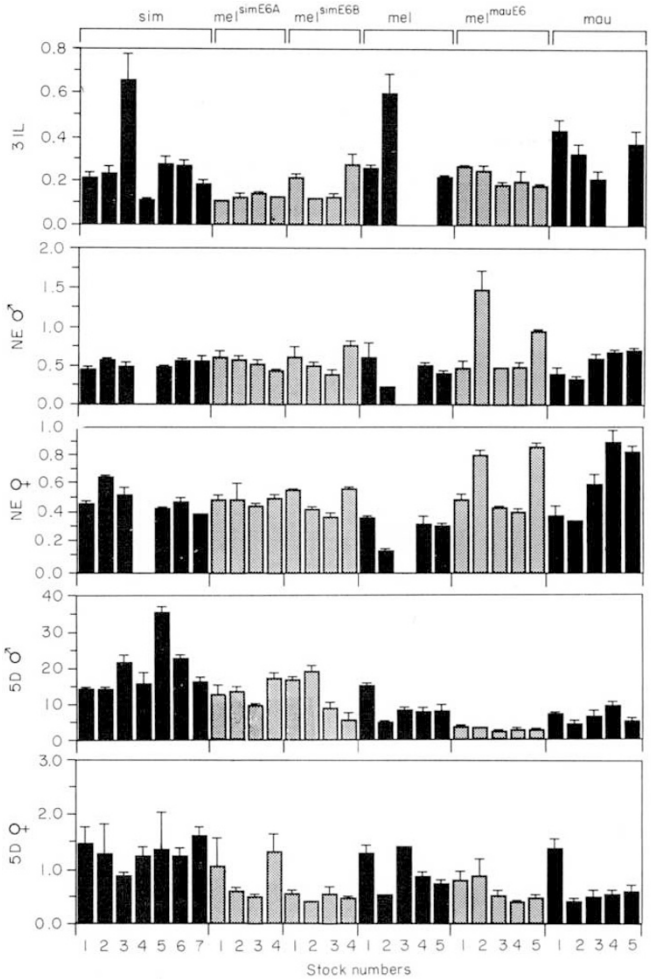

Fig. 1 Mean EST6 specific activities (± S.E.) of wild type *D. melanogaster* (mel), *D. simulans* (sim) and *D. mauritiana* (mau) lines (solid bars) and the mel^{simE6A}, mel^{simE6B} and mel^{mauE6} transformant lines (stippled bars) for three developmental stages and both sexes, expressed as μmol β-naphthol produced/30 min/mg protein. Note that the mean 5-day male activity of the five *D. melanogaster* lines was 8.26 ± 1.70, significantly higher than the comparable value from the 42 lines tested by Game & Oakeshott, 1989; 5.27 ± 0.19, after adjusting their values to equivalent units. However, our mean 5-day female activity (0.95 ± 0.17) was not significantly different to the corresponding (adjusted) value from Game & Oakeshott, 1989 (0.90 ± 0.03). The difference in 5-day male activities suggests that the present study will underestimate interspecific differences involving *D. melanogaster*, which is as low or lower than either of the other two species for this measure.

1), but two major differences also distinguish the three species, the first of which is in newly eclosed female activities. Pairwise comparisons show this to be due to the relatively low (two-fold lower on average) EST6 activities of *D. melanogaster* females. The second major difference among the species is in 5-day adults,

Table 1 Analyses of variance (F ratios with degrees of freedom in brackets) for each of the five EST6 activity measures and the two canonical variates assessing differences among the three species (mel/sim/mau) and among lines within the species (Lines). Significant effects from the three-way comparisons among the species are decomposed into the contributions from the three pairwise comparisons. Note that only cultures for which data were available for all five activity measures were used in the canonical variate analyses

Source of variation	3rd instar larvae	Newly eclosed adults		5-day adults		Canonical variates	
		Males	Females	Males	Females	CV1	CV2
mel/sim/mau	0.75 (2,11)	1.13 (2,12)	5.98* (2,12)	17.41*** (2,14)	7.00** (2,14)	16.00** (2,9)	11.98** (2,9)
Lines	12.71*** (11,27)	6.21*** (12,29)	19.49*** (12,27)	8.28*** (14,33)	1.91 (14,25)	6.53** (9,8)	3.68* (9,8)
mel/sim			9.46* (1,8)	17.37** (1,10)	3.23 (1,10)	6.00* (1,6)	18.32** (1,6)
Lines	17.12*** (8,20)	4.67** (8,19)	19.55*** (8,18)	10.74*** (10,23)		19.08** (6,5)	3.50 (6,5)
mel/mau			7.32* (1,7)	0.89 (1,8)	2.62 (1,8)	0.28 (1,4)	29.82** (1,4)
Lines	10.13*** (5,13)	7.80*** (7,17)	22.67*** (7,16)	6.54*** (8,20)		3.27 (4,3)	3.03 (4,3)
sim/mau			0.89 (1,9)	37.31*** (1,10)	12.96** (1,10)	39.66*** (1,8)	1.70 (1,8)
Lines	10.47*** (9,21)	5.93*** (9,22)	16.20*** (9,20)	8.44*** (10,23)		4.85* (8,8)	4.14* (8,8)

*$P<0.05$, **$P<0.01$, ***$P<0.001$.

where activity in *D. simulans* is significantly higher (two-fold higher on average) than *D. mauritiana* in both sexes and significantly higher than *D. melanogaster* in males.

Canonical variate analyses (Digby *et al.*, 1989) were carried out on the five activity measures in order to derive two independent composite variables which best represent the differences between the species. The two derived variables are related to the five original variables as follows:

$$CV1 = -0.64\ \ln(3IL) - 0.58\ \ln(NE\sigma) + 0.26\ \ln(NE\varphi) + 2.58\ \ln(5D\sigma) - 0.09\ \ln(5D\varphi) - 7.10$$

$$CV2 = -1.14\ \ln(3IL) - 3.87\ \ln(NE\sigma) + 5.13\ \ln(NE\varphi) + 0.02\ \ln(5D\sigma) - 0.31\ \ln(5D\varphi) - 0.26.$$

CV1 is mainly weighted on 5-day male activity (with a weighting at least four-fold larger than that for any other activity measure) and distinguishes *D. simulans* from both *D. melanogaster* and *D. mauritiana* but does not separate the latter two species. CV2 shows a strong negative contribution from newly eclosed males and a strong positive one from newly eclosed females (the weightings for both being at least three-fold larger than those for the other measures) and distinguishes *D. melanogaster* from both *D. mauritiana* and *D. simulans*, but does not separate *D. simulans* and *D. mauritiana*. Both canonical variates contribute fairly evenly to the total variation, CV1 accounting for 59 per cent and CV2 for the remainder. Values for the two canonical variates for each line and species are represented graphically in Fig. 2 while Table 1 shows the corresponding analyses of variance.

Like the wild type lines from all three species, the *D. melanogaster* lines transformed with the two *D. simulans Est-6* genes also show higher activities in adults than in larvae, in 5-day than newly eclosed adults, and in males than females (Fig. 1). Some significant differences were found among lines within the two types of transformant (mel^{simE6A} and mel^{simE6B}) for several activity measures ($F_{6,16}=8.27$ for third instar larvae; $F_{6,16}=3.83$ for newly eclosed males; $F_{6,15}=7.67$ for 5-day males, $P<0.05$ in all cases). These differences presumably reflect effects on the expression of the transgenes due to differences in the position of integration, an effect generally observed in *Drosophila* transformation studies (e.g. Wu *et al.*, 1990; Kirkpatrick & Martin, 1992). Significantly, however, there were no overall differences between mel^{simE6A} and mel^{simE6B} lines for any activity measure (the largest $F_{1,6}=4.64$, $P>0.05$ in all cases), so these data have been pooled for all subsequent analyses. Although the

endogenous activity of the simE6B gene is not known, nucleotide sequence analysis of the flanking regions of this and the simE6A gene revealed only eight single base pair differences and one single base pair insertion/deletion in the 1.18 kb of transformed DNA 5′ of the gene, and five single base pair differences in the 0.14 kb of 3′ untranslated sequence included in the transformed DNA (J. K., T. M. Boyce & J. G. O., unpublished data), so the two sets of transformants might be expected to show similar activities.

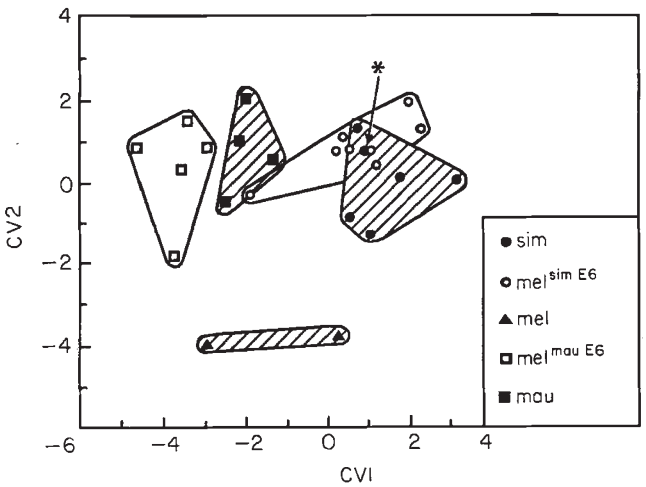

Fig. 2 Distribution of wild type and transformant lines for the two canonical variates CV1 and CV2. Wild type *D. melanogaster* (mel), *D. simulans* (sim) and *D. mauritiana* (mau) lines are enclosed in the three shaded regions, the mel^{simE6} and mel^{mauE6} transformants in two unshaded regions. The *D. simulans* line (sim-1) from which one of the transgenes was derived is denoted by an asterisk.

The eight mel^{simE6} lines differ from the wild type lines of either the donor (*D. simulans*) or recipient (*D. melanogaster*) species in several activity measures (Table 2). The third instar larval activities of the transformants are lower than those of both parental species ($P < 0.05$ in both cases). The transformants also differ from *D. melanogaster* ($P < 0.01$) but not *D. simulans* for newly eclosed female activities, although there are no differences from either parental species in newly eclosed male activities. Finally, the transformants do not differ significantly from *D. melanogaster* in 5-day adult male or female activities but their values for both these activities are lower than wild type *D. simulans* ($P < 0.05$ for males, $P < 0.001$ for females). Thus for those measures in which the transformants differ from one or the other parental species, the expression of the transgene resembles the donor rather than the recipient species for newly eclosed female activities but resembles the recipient rather than the donor for 5-day adult male and female activities, while for larval activities it differs from both parental species.

The analyses above involve comparison of the mel^{simE6} transformants to several lines from both the donor and recipient species. However, one of the lines, sim-1, from which the transgenes had been isolated had been available for inclusion in the activity assays. Therefore the eight transformant lines (the two sets not differing in any activity measure, see above) could be compared with sim-1 for a more specific test of differences from the donor species. (Note that the activities of the transgenes were assayed in a recipient *D. melanogaster* line homozygous for an endogenous $EST6^{null}$ allele, so the equivalent comparison of the transformants to the recipient line was not mean-

Table 2 Analyses of variance (F ratios with degrees of freedom in brackets) for each of the five EST6 activity measures and the two canonical variates assessing differences between the mel^{simE6} transformants and each of the two parental species (mel and sim), as well as differences among lines within these groups (Lines). Note that only cultures for which data were available for all five activity measures were used in the canonical variate analyses

Source of variation	3rd instar larvae	Newly eclosed adults		5-day adults		Canonical variates	
		Males	Females	Males	Females	CV1	CV2
mel/mel^{simE6}	9.54*	3.25	12.74**	3.52	4.00	1.92	34.90***
	(1,9)	(1,10)	(1,10)	(1,11)	(1,11)	(1,8)	(1,8)
Lines	14.19***	4.71**	8.92***	7.29***	2.86*	5.14*	0.49
	(9,22)	(10,23)	(10,23)	(11,25)	(11,21)	(8,8)	(8,8)
sim/mel^{simE6}	5.41*	0.17	0.10	5.25*	20.57***	1.37	2.58
	(1,13)	(1,12)	(1,12)	(1,13)	(1,13)	(1,12)	(1,12)
Lines	12.54***	2.69*	3.86**	7.61***	1.38	5.65**	1.13
	(13,30)	(12,28)	(12,27)	(13,28)	(13,27)	(12,13)	(12,13)

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

ingful.) Only one of the three differences between the transformants and the donor seen in the full analyses above is found to recur in the sim-1 analysis. This is for 5-day female activities ($F_{1,3} = 5.08$, $P < 0.05$ on a one-tailed test), which had been highly significant ($P < 0.001$) in the full analyses. The less statistically robust differences from *D. simulans* ($P < 0.05$) that the full analyses identified for third instar larvae and 5-day males were not significant in the comparisons to sim-1. Thus the relatively large differences from the donor species in 5-day female activity emerge clearly in both analyses, whereas the smaller differences in third instar larval and 5-day male activities are only significant in the full analyses.

Analyses of the EST6 activities of the mel^{mauE6} transformants in relation to all the available wild type *D. melanogaster* and *D. mauritiana* lines are shown in Table 3. The transformants have significantly ($P < 0.05$) lower third instar larval activities than wild type *D. mauritiana* but do not differ from *D. melanogaster*. There are no significant activity differences among newly eclosed males and, while the newly eclosed female activities of *D. mauritiana* and the transformants do not differ, both are significantly lower than those of *D. melanogaster* ($P < 0.05$). The 5-day female activities of the transformants are similar to those of *D. mauritiana* but significantly lower than those of *D. melanogaster* ($P < 0.05$), whereas the 5-day male activities of the transformants are significantly lower than those of both parental species ($P < 0.01$ in both cases). In summary, the transgene carried by the mel^{mauE6} transformants resembles the recipient species for third instar larval activities, and the donor species for newly eclosed and 5-day female activities; however their 5-day male activities are significantly lower than those of both parental species.

Since the *D. mauritiana* line from which the donor DNA was isolated was not available, no parallel of the 'sim-1 analyses' above could be carried out for *D. mauritiana*. In its absence we note that the only differences that were significant below $P < 0.01$ in the full *D. mauritiana* analyses were the low 5-day male activities of the transformants relative to both parental species.

Comparison of all the transformants to their respective parental species for the canonical variates (Fig. 2, Tables 1–3) shows that for CV2 the transformants resemble the donor species and differ from the recipient. Thus CV2 (with opposing contributions from newly eclosed male and female activities) distinguishes wild type lines of *D. melanogaster* from both *D. simulans* and *D. mauritiana* and the transformants overlap the respective donor species (*D. simulans* and *D. mauritiana*) but are clearly distinguished from the recipient (*D. melanogaster*; $P < 0.001$ and $P < 0.05$, respectively). Although CV1 (mainly weighted for 5-day male activities) clearly distinguishes the wild type *D. melanogaster* and *D. simulans* lines, the values of CV1 for the mel^{simE6} transformants lie between those of both parental species and do not differ significantly from either. CV1 does not show a statistically significant difference between *D. melanogaster* and *D. mauritiana* either, but in this case the mel^{mauE6} transformants differ from the donor, *D. mauritiana* ($P < 0.01$), but not the recipient, *D. melanogaster*.

The canonical analysis is thus consistent with the univariate analyses in terms of differences from the recipient species. These differences are clearly evident for the transgenes from both species for CV2, which is

Table 3 Analyses of variance (F ratios with degrees of freedom in brackets) for each of the five EST6 activity measures and the two canonical variates assessing differences between the mel^{mauE6} transformants and each of the two parental species (mel and mau), as well as differences among lines within these groups (Lines). Note that only cultures for which data were available for all five activity measures were used in the canonical variate analyses

Source of variation	3rd instar larvae	Newly eclosed adults		5-day adults		Canonical variates	
		Males	Females	Males	Females	CV1	CV2
mel/mel^{mauE6}	2.53	2.82	8.84*	23.63**	6.87*	4.90	13.22*
	(1,6)	(1,7)	(1,7)	(1,8)	(1,8)	(1,5)	(1,5)
Lines	10.54***	12.16***	37.25***	3.88**	2.37	2.11	2.14
	(6,16)	(7,17)	(7,17)	(8,20)	(8,15)	(5,7)	(5,7)
mau/mel^{mauE6}	9.22*	1.09	0.00	24.82**	0.14	18.64**	0.40
	(1,7)	(1,8)	(1,8)	(1,8)	(1,8)	(1,7)	(1,7)
Lines	3.03*	14.56***	27.30***	2.39	3.56*	0.96	2.65
	(7,17)	(8,20)	(8,19)	(8,20)	(8,19)	(7,10)	(7,10)

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

heavily weighted for the variable (newly eclosed female activity) that showed the clearest differences from the recipient for both donor species in the univariate analyses. On the other hand, the only differences from the donor species revealed by the canonical variate analysis is for the mauE6 transgene for CV1, which is mainly weighted for 5-day male activity. Four other differences from the donor species had also emerged from the univariate analyses, notably for 5-day males and females for *D. simulans* and third instar larvae for both these species, but clearly none of these differences would contribute to the CV1 effect for the mauE6 transgene.

Discussion

A systematic comparison of EST6 activity levels in *D. melanogaster, D. simulans* and *D. mauritiana* reveals broadly similar stage- and sex-specific patterns of EST6 expression. Nevertheless, there are also significant intraspecific as well as interspecific differences in EST6 activity levels in the three developmental stages and in both sexes.

Significant activity variation is found among lines within all three species in almost all measures, with up to six-fold variation in individual measures. Thus the levels of intraspecific variation in EST6 activity reported previously for *D. melanogaster* lines (Game & Oakeshott, 1989) recur among our lines of this species and are also evident in small random samples of *D. simulans* and *D. mauritiana*. Game & Oakeshott (1989) found that the variation they detected in EST6 activity among *D. melanogaster* lines reflects differences in the amount of enzyme and that the variation in 4–5-day adult male and female activities is only weakly correlated. This suggests that the variation is due to regulatory rather than structural differences in EST6, and that different regulatory polymorphisms affect activity in the two sexes. This is perhaps not surprising given the differences in the tissue distribution of EST6 between the sexes in these species (Aronshtam & Kuzin, 1974; Healy *et al.*, 1991). For example, in 5-day adults the majority of male activity is in ejaculatory ducts while in females the majority of activity is in haemolymph (Aronshtam & Kuzin, 1974; Morton & Singh, 1985; Uspenskii *et al.*, 1988; Healy *et al.*, 1991).

Physiological and genetic data identify several levels at which EST6 expression is regulated and all are potentially subject to intraspecific polymorphism, either in *cis*- or *trans*-acting factors. The physiological data come from transplants of male reproductive organs into female abdomens (Aronshtam & Kuzin, 1974) and from exposure of dissected abdomens and explants of ejaculatory ducts to JH and ecdysone (Richmond & Tepper, 1983; Stein *et al.*, 1984). These data indicate that both hormones contribute to the induction of EST6 in adults of *D. melanogaster*, although the effect in males may be at least partly mediated by an intermediary signal associated with the accessory glands. Genetic data pertaining to EST6 regulation implicate both *cis*- and *trans*-control of activity variation, at least within *D. melanogaster*. Significant activity variation due to *trans*-acting polymorphisms on the X chromosome has been described among both laboratory and field derived strains (Aronshtam & Korochkin, 1975; Richmond & Tepper, 1983; Tepper *et al.*, 1984), while *cis*-acting effects are implicated from correlations between restriction fragment length polymorphisms in the *Est-6* promoter and activity variation in field derived isochromosomal lines (Game & Oakeshott, 1990).

The two major interspecific differences in EST6 expression we have found are the relatively low newly eclosed female activities of *D. melanogaster* and the relatively high 5-day adult activities of *D. simulans*, these differences also being reflected in the canonical variate analyses. Although we have no direct causal evidence, we favour regulatory rather than structural differences as an explanation for these interspecific differences. Structural differences in the EST6 protein would be more likely to produce systemic effects on EST6 activity levels rather than the temporal- and sex-specific effects seen here. Further evidence that these differences are not systemic is that reproductive tract activity accounts for differing proportions of total male activity among single lines of these species, being a higher proportion of male activity in *D. simulans* than in either *D. melanogaster* or *D. mauritiana* (70 vs. 47 and 38 per cent respectively, Morton & Singh, 1985). (Note however that we find none of the three species express EST6 in testes, whereas Morton & Singh (1985) reported that *D. mauritiana*, albeit not the other two species, does show EST6 activity in this tissue; J. K. & J. G. O., unpublished data.)

The aims of our germ line transformation of *D. melanogaster* with *Est-6* genes from *D. simulans* and *D. mauritiana* have been to distinguish between *cis*- and *trans*-acting effects as the causes of the differences in EST6 expression among the three species. The restriction fragments from *D. simulans* and *D. mauritiana* used in our interspecific gene transfer experiments are precisely homologous to those used previously for similar experiments within *D. melanogaster* (Oakeshott *et al.*, 1990; M. J. Healy, M. M. Dumancic & J. G. O., unpublished data). In general, the EST6 activities of the interspecific transformants overlap the range of values for at least one parental species in almost all measures, confirming previous evidence from *D. melanogaster*

that 1.2 kb of 5′ and 0.2 kb of 3′ flanking sequences are generally sufficient for most qualitative and quantitative aspects of EST6 expression (Oakeshott *et al.*, 1990; M. J. Healy, M. M. Dumancic & J. G. O., unpublished data).

However, two exceptions are that third instar larvae of the *D. simulans* transformants and 5-day males of the *D. mauritiana* transformants show significantly lower activity than either parental species, suggesting that some species-specific promoter elements affecting these aspects of expression may lie outside the flanking sequences included with the transgenes. It is interesting in this respect that the next 350 bp beyond the 5′ boundary of the transgene constructs show a similar level of divergence to that in the adjacent 350 bp within the constructs, at least between *D. melanogaster* and *D. simulans* (J. K., T. M. Boyce and J. G. O., unpublished data).

As is the case for the majority of interspecific transfer experiments involving *Drosophila* genes (Dickinson, 1991; Cavener, 1992), the expression of *D. simulans* and *D. mauritiana* EST6 in the *D. melanogaster* background more closely resembles the donor species pattern than that of the recipient. Donor-specific levels of EST6 expression indicative of *cis*-inherited controls are clearly seen for both the *D. simulans* and *D. mauritiana* transgenes in newly eclosed female activities and the canonical variate, CV2, which carries strong but opposing weightings from newly eclosed male and female activities. Similar but smaller donor effects are also apparent for the 5-day female activity of the *D. mauritiana* transgene. All these differences from the pattern of expression of the recipient species presumably reflect interspecific differences in promoter sequences that affect the level and/or tissue-specificity of EST6 expression.

Although the overall structure of the promoter region is conserved across the three species, there are many base substitutions and small insertions/deletions among them that could cause these effects (Karotam *et al.*, 1993). The levels of divergence of *D. melanogaster* from the other two species are low ($\leq$2.3 per cent) in the 350 bp immediately 5′ of the *Est-6* coding region but significantly higher in the next 700 bp of the promoter region ($\geq$9.8 per cent). The 0.14 kb of 3′ untranslated sequence included in the transformed constructs are also highly divergent ($\geq$10.1 per cent, Karotam *et al.*, 1993).

Evidence for predominant *cis*-control of interspecific differences in EST6 expression was also apparent after germ line transformation of the *D. pseudoobscura* homologue of *Est-6* (termed *Est5B*) into *D. melanogaster* (Brady & Richmond, 1990). The major pulse of EST5B expression in *D. pseudoobscura* is in the adult eye, not the ejaculatory duct as in the case of *D. melanogaster*, and it is notable that detectable sequence similarity between the promoter regions of *Est-6* and *Est5B* is confined to the first 174 bp 5′ of the gene (Brady *et al.*, 1990). Although only 450 bp of 5′, but 1.1 kb of 3′ flanking sequence, were included with the *Est5B* transgene, the major pulse of expression occurred in the adult eye of the transformants, therefore resembling the donor species' pattern of expression.

Unlike the *D. pseudoobscura Est-6* homologue, we do find some evidence for *trans*-acting control of the *Est-6* transgenes from *D. simulans* and *D. mauritiana*. Thus, the mel^{simE6} transformants have significantly lower 5-day female activities than the wild type *D. simulans* lines but are not significantly different from *D. melanogaster* wild type. (Note that we cannot distinguish between *cis*- and *trans*-effects for 5-day males, since the donor line (sim-1) and recipient species show similar activity levels; see Fig. 1.) There is also some evidence for *trans*-effects on the mauE6 transgene in third instar larvae and CV1.

Such *trans*-effects could be artefacts of our experiment if some of the *D. simulans Est-6* promoter was omitted from the transformed sequences, element(s) in the omitted region being absent from the wild type *D. melanogaster* promoter but being responsive to *trans*-acting factor(s) found in both species. We cannot discount this possibility, but would note that our evidence for *trans*-acting effects is consistent with evidence from analysis of *D. melanogaster*/*D. simulans* hybrids which suggests the existence of species-specific *trans*-acting modifiers of *Est-6* expression on the X chromosome (Aronshtam *et al.*, 1975; Tepper *et al.*, 1982). Hybrid males bearing a *D. simulans* X chromosome show reduced levels of *D. melanogaster*-derived EST6 activity in all tissues and stages examined (albeit most pronounced in the ejaculatory duct), while *D. simulans*-derived EST6 activity (distinguished by its different electrophoretic mobility) is unaffected (Korochkin *et al.*, 1974; Aronshtam & Korochkin, 1975). Similar but smaller effects of the *D. simulans*-derived X chromosome are sometimes seen when a *D. melanogaster* X chromosome is also present in hybrid females, depending on the parental strains used (Korochkin *et al.*, 1974).

It is an intriguing feature of our data that while *cis*-controls are clearly evident for the interspecific activity differences in newly eclosed flies (namely the newly eclosed female and CV2 results for both the *D. simulans* and *D. mauritiana* transgenes), *trans*-controls are more in evidence for the 5-day adult activities (namely 5-day female activity for the *D. simulans* transgene and CV1 for the *D. mauritiana* transgene). Our

current knowledge of EST6 physiology is insufficient to interpret this pattern in any detail although it is clear that induction by JH and/or ecdysone contributes to the increase in EST6 activity post-eclosion (Richmond & Tepper, 1983; Stein *et al.*, 1984). This, together with the fact that both variation in ecdysone titre and the gene for a putative cytosolic JH binding protein map to the X chromosome in *D. melanogaster* (Kiss *et al.*, 1978; Klose *et al.*, 1980; Shemshedini & Wilson, 1990), suggests that these hormones may be involved in the interspecific *trans*-effects on EST6 expression documented herein. For ecdysone at least, specific promoter elements that respond to the hormone have now been identified in a number of genes (Andres & Thummel, 1992) and we have identified three putative consensus ecdysone response elements (AG(C/T)G(C/T)A; Pongs, 1988) in the 1.18 kb of 5′ *Est-6* flanking sequences in all three species (starting at positions −269, −443 and −662 in the *D. melanogaster* sequence; Karotam *et al.*, 1993). Some ecdysone-inducible genes (e.g. *P1*, Maschat *et al.*, 1991) are tissue-specific in their response to the hormone and may act as tissue-specific transcription factors (Andres & Thummel, 1992).

Four other *Drosophila* genes for which interspecific germ line transfer experiments have previously shown some *trans*-control of interspecific expression differences (*Ddc, Adh*, urate oxidase and *Gld*, see Introduction) are all also responsive to changing JH and/or ecdysone titres (Riddiford, 1992, and references therein). This emerging pattern suggests that while *cis*-acting changes will be powerful agents for evolutionary changes in the expression of individual genes, evolutionary changes in the titres of key hormones could affect the expression of whole suites of genes.

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