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Evolution

Molecular analysis of evolutionary changes in the expression of *Drosophila* esterases

(esterase 5/esterase 6/gene regulation)

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ABSTRACT ***Drosophila melanogaster* transformed with the esterase 5 (*Est-5*) gene from *Drosophila pseudoobscura* were used to assess the evolutionary basis for differences in the sex- and tissue-specific expression of the esterase 5 (EST 5) enzyme in *D. pseudoobscura* relative to its homologue in *D. melanogaster*, EST 6. EST 5 is expressed in the eyes and hemolymph of transformed *D. melanogaster* just as it is in *D. pseudoobscura*, but it is not detectable in the ejaculatory duct, where the homologous enzyme, EST 6, is most abundant. EST 5 also occurs at equal levels in both sexes of the transformants and *D. pseudoobscura*, whereas EST 6 is more abundant in male than in female *D. melanogaster*. Northern analysis of transformed and untransformed flies indicates that the expression patterns of EST 5 and EST 6 are controlled at the level of transcription and suggests that regulatory differences between *Est-6* and *Est-5* have evolved mainly through cis-acting regulatory changes in the two loci rather than through alterations in trans-acting factors. Equal expression of EST 5 in male and female transformants also indicates that the X-chromosome-linked *Est-5* gene of *D. pseudoobscura*, when isolated as a 4.5-kilobase restriction fragment, is not dosage compensated after integration into an autosome of *D. melanogaster*.**

Alterations in gene regulation are considered to be a prerequisite for macroevolutionary change and species divergence (1–3). We have undertaken a comparative molecular analysis of the esterase 6 (EST 6; carboxylic-ester hydrolase, EC 3.1.1.1) enzyme in *Drosophila melanogaster* and its homologue in *Drosophila pseudoobscura*, EST 5, to investigate the mechanisms that underlie changes in gene expression. Previously, we described cloning of both the *Est-6* and *Est-5* genes (4, 5). Those studies revealed similarities in the protein products, transcripts, and DNA sequences of the two genes that strongly corroborated previous biochemical and genetic evidence for their homology (5). Despite their common evolutionary origin, EST 6 and EST 5 exhibit remarkable differences in their tissue- and sex-specific expression. EST 5 is found in the eyes and hemolymph of adult *D. pseudoobscura* of both sexes (6). EST 6, on the other hand, occurs primarily in adult male *D. melanogaster*, and its activity is mainly in the anterior ejaculatory duct. Lower levels of EST 6 are also found in the hemolymph of both sexes (7).

We have investigated the mechanistic basis for the different expression patterns of EST 6 and EST 5 by examining the tissue and sex localization of EST 5 protein and *Est-5* transcripts after introducing this gene into *D. melanogaster* by *P*-element transformation. Our data reveal that patterns of EST 5 activity in the transformants match those in *D. pseudoobscura* and that the different expression profiles of EST 6 and EST 5 reflect variation in transcriptional regulation that appears to result from differences in cis-acting regulatory sequences in these two species.

MATERIALS AND METHODS

Esterase activity of whole fly or tissue homogenates was measured on nondenaturing 10% polyacrylamide gels according to the methods of Vernick *et al.* (8). Esterases were detected with α- and β-naphthyl acetate substrates and fast garnet salt (Sigma) (9). Tissues were hand dissected and thoroughly rinsed in homogenization buffer before grinding and loading onto gels. Hemolymph was isolated from adult flies according to Kambysellis (10) and from larvae by the methods of Singh and Coulthart (11). Protein levels were measured according to Bradford (12). Protein band intensities were determined by using a Quick Scan R & D densitometer (Helena Laboratories) at a wavelength of 525 nm.

D. melanogaster homozygous for both the slow electrophoretic variant of EST 6 (13) and the ry^{506} mutation were used as untransformed controls. The same line served as the recipient strain for transformation with the *Est-5* gene from *D. pseudoobscura* (5). Flies homozygous for the EST $5^{1.00}$ allele (14) were employed as *D. pseudoobscura* controls for protein and RNA analysis.

RNA was isolated from adult flies or fly parts by the methods of Collet *et al.* (15). Total RNA was separated in formaldehyde/agarose gels and transferred to nylon membranes (Amersham) by standard procedures (16). Hybridizations were done according to Brady *et al.* (5), using single-stranded RNA probes transcribed from the phage T7 promoter in the vectors pGEM-1 or pGEM-3 (Promega Biotec).

RESULTS

We have previously described the cloning of the *Est-5* locus from *D. pseudoobscura* by screening a genomic library with the cloned *Est-6* gene of *D. melanogaster* (5). Three separate genes, *Est-5A*, *Est-5B*, and *Est-5C*, that share sequence similarity to *Est-6* are contained within a 12-kilobase (kb) interval (5). When each of these genes was introduced into *D. melanogaster* by *P*-element transformation, it was revealed that the middle gene, *Est-5B*, encodes the EST 5 protein (5). Hereafter, we use *Est-5* as a synonym for *Est-5B*. Fig. 1 is a restriction map of the *Est-5* gene cluster of *D. pseudoobscura*. The *Hin*dIII/*Eco*RI fragment containing the coding region of *Est-5B* along with approximately 450 base pairs (bp) of 5′ and 1200 bp of 3′ flanking DNA (Fig. 1) was subcloned and injected into embryos of *D. melanogaster*. Four homozygous

Abbreviations: EST 5 and 6, esterase 5 and 6 enzymes.

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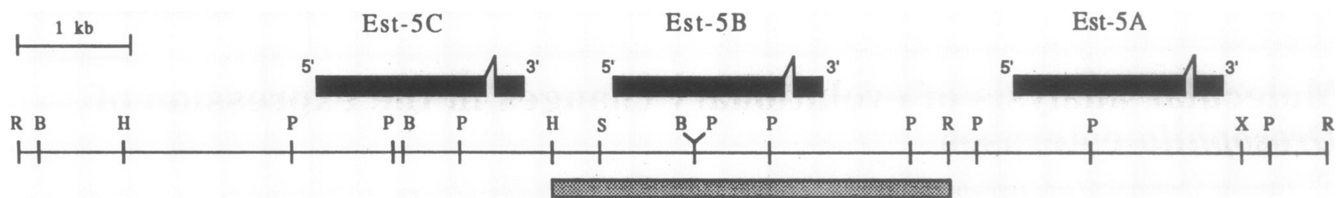

FIG. 1. Restriction map of the *Est-5* locus of *D. pseudoobscura* and surrounding DNA. *Est-5A*, *Est-5B*, and *Est-5C* are three related loci. The *Hin*dIII/*Eco*RI fragment indicated by the shaded bar below the map was introduced into *D. melanogaster* by *P*-element transformation. Restriction sites: B, *Bam*HI; H, *Hin*dIII; P, *Pst* I; R, *Eco*RI; S, *Sal* I; X, *Xba* I.

lines of transformed flies were established (5). All four transformed lines expressed EST 5 protein with the same relative tissue- and sex-specific activity levels (data not shown). One of the lines, containing a single insertion in chromosome III, was randomly selected for further study as described below.

Tissue Localization of EST 5 in Transformed *D. melanogaster*. In *D. pseudoobscura*, it has been estimated that 40% of EST 5 activity appears in the eyes, while the remainder occurs in the hemolymph (6). In contrast, EST 6 activity in *D. melanogaster* is primarily found in the anterior ejaculatory duct, with lower levels also present in the hemolymph of both sexes (9). Therefore, we began by examining the levels of EST 5 and EST 6 in male reproductive tracts, hemolymph, and eyes of untransformed adult flies of both species and in the transformed *D. melanogaster*.

Fig. 2 (lanes 1–3) shows esterase activity in adult male reproductive systems. EST 6 is abundant in the reproductive tracts of untransformed and transformed male *D. melanogaster*. EST 5, on the other hand, is absent from the reproductive systems of *D. pseudoobscura* and *D. melanogaster* transformed with *Est-5*. Lanes 4–6 of Fig. 2 reveal the presence of EST 6 and EST 5 in the hemolymph of their respective species and show that EST 5 is also abundant in the hemolymph of the transformants.

Eyes were dissected from *D. pseudoobscura* and from transformed and untransformed *D. melanogaster*, and the relative amounts of EST 5 and EST 6 in the separated eyes as well as in the eyeless heads were determined. We find that EST 6 and EST 5 are present in the eyeless heads of the appropriate species (Fig. 3). However, in the isolated eyes, EST 6 is present in only low amounts, while EST 5 occurs at levels equivalent to or greater than those seen in the heads. EST 6 and EST 5 in lanes 4–6 probably result from residual hemolymph in the eyeless heads, as it is difficult to remove all of the hemolymph from these tissues. In the eyes, however, the low level of EST 6 suggests that hemolymph contamination is minimal, and in fact, this band may actually represent previously undetected EST 6 expression in these tissues (see below). The large amounts of EST 5 present in the eyes of *D. pseudoobscura* and transformed *D. melanogaster* agree with previous reports that the eyes are a major site of activity for this enzyme in *D. pseudoobscura*.

The above data show that EST 5 activity in the transformants occurs in the same tissues known to be sites of EST 5 activity in *D. pseudoobscura*, namely the eyes and the hemolymph. Furthermore, the absence of EST 5 from the male reproductive systems of the transformed flies indicates that EST 5 accumulation in these flies is not being regulated in the same manner as the endogenous EST 6 protein. These findings are consistent with the results of other experiments that have shown that genes transferred between species of *Drosophila* (17, 18) or between *Bombyx mori* and *D. melanogaster* (19) retain the tissue-specific expression patterns that were observed in the original species. However, in some cases, genes that are introduced into a new species may also be expressed in tissues where they are not normally observed (20). We therefore looked for EST 5 activity in a number of other adult and larval tissues in transformed flies and in *D. pseudoobscura* to assay for ectopic expression of this enzyme in the transformants. The results of those studies are presented in Table 1. EST 5 and EST 6 are expressed at low levels in most of the adult tissues we examined; larval expression is primarily in the hemolymph. None of the

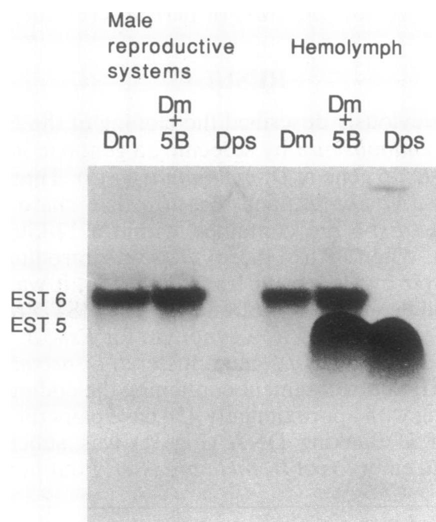

FIG. 2. Nondenaturing polyacrylamide gel stained for esterase activity, showing levels of EST 6 and EST 5 activity in isolated male reproductive systems and hemolymph. Dm, untransformed *D. melanogaster*; Dm + 5B, *D. melanogaster* transformed with *Est-5*; Dps, *D. pseudoobscura*. Each lane contains tissue from 12 adult male flies.

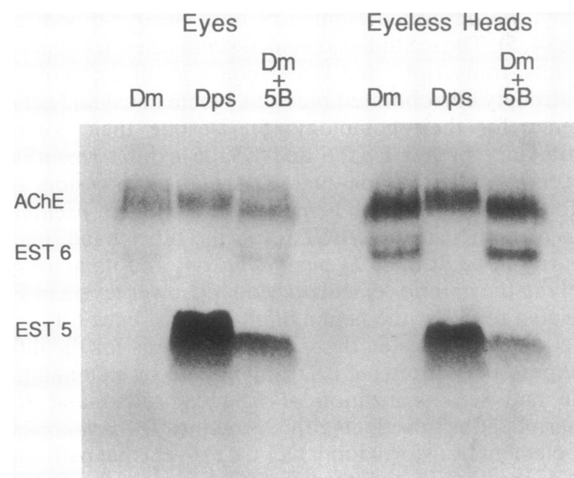

FIG. 3. Nondenaturing polyacrylamide gel stained for esterase activity, showing levels of EST 6 and EST 5 activity in dissected eyes and in corresponding eyeless heads. Dm, untransformed *D. melanogaster*; Dm + 5B, *D. melanogaster* transformed with *Est-5*; Dps, *D. pseudoobscura*; AChE, acetylcholinesterase. Each lane contains homogenates from 20 eyes or 10 heads.

Table 1. Relative activities of EST 6 and EST 5 in dissected tissues from 12 third-instar larvae or adult flies

Tissue	Isozyme	Esterase activity		
		Dm	Dm + 5B	Dps
Adults				
Total gut	EST 5		+	+
	EST 6	+	+	
Crop	EST 5		+	+
	EST 6	+	+	
Malpighian tubules	EST 5		+	+
	EST 6	+	+	
Ovaries	EST 5		++	++
	EST 6	++	++	
Body wall	EST 5		+	+
	EST 6	+	+	
Larvae				
Brain	EST 5		−	−
	EST 6	−	−	
Midgut	EST 5		−	−
	EST 6	−	−	
Hindgut	EST 5		−	−
	EST 6	−	−	
Malpighian tubules	EST 5		−	−
	EST 6	−	−	
Salivary glands	EST 5		−	−
	EST 6	−	−	
Fat body	EST 5		−	−
	EST 6	−	−	
Tracheae	EST 5		−	−
	EST 6	−	−	
Hemolymph	EST 5		+++	+++
	EST 6	+++	+++	
Carcass	EST 5		+	+
	EST 6	+	+	

Activities were measured by analysis of nondenaturing polyacrylamide gels. −, No activity; +, low activity; ++, moderate activity; +++, high activity; Dm, untransformed *D. melanogaster*; Dm + 5B, *D. melanogaster* transformed with *Est-5*; and Dps, *D. pseudoobscura*.

tissues that we looked at in the transformants showed significantly higher or lower levels of EST 5 expression than did the corresponding tissues in *D. pseudoobscura*.

Sex Localization of EST 5 in Transformed *D. melanogaster*. We examined the relative levels of EST 5 activity in whole male and female transformed *D. melanogaster* to determine whether equal amounts of this enzyme are present in both sexes as is observed in *D. pseudoobscura* or whether there is male-dominant expression of EST 5 as is observed for EST 6 in *D. melanogaster*. Fig. 4 shows the amounts of EST 5 and EST 6 activity in equal quantities of male and female protein from the three strains. Densitometric analysis of the band intensities indicates that there is approximately a 2-fold difference in the amount of EST 6 activity between male and female *D. melanogaster* seen in lanes 1 vs. 2 and 3 vs. 4. In contrast, EST 5 shows no significant difference in activity between males and females of either *D. pseudoobscura* or the transformants (lanes 3 vs. 4 and 5 vs. 6).

RNA Analysis. The above data suggest that both tissue and sex localization of EST 5 in the transformed flies are identical to the patterns observed for this enzyme in *D. pseudoobscura*. However, the localization of activity may not reflect the actual patterns of *Est-5* gene expression. It is conceivable that EST 5 is synthesized elsewhere, secreted into the hemolymph, and then transported to the eyes. Indeed, the presence of EST 5 in the hemolymph of both *D. pseudoobscura* and the transformants is consistent with such a model. Moreover, such a scheme would not be unique to EST 5; eye pigments in *D. melanogaster* are known to be synthesized in

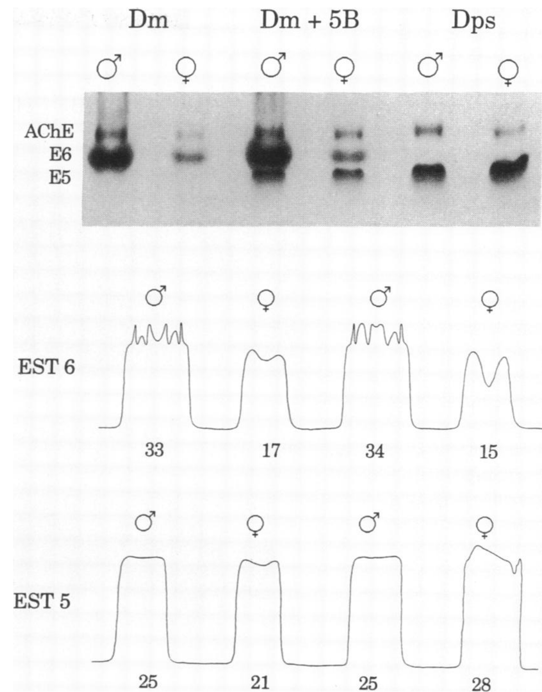

FIG. 4. Nondenaturing polyacrylamide gel showing relative EST 6 and EST 5 activity in the two sexes. Each lane contains equal amounts of total protein from 4- to 5-day-old virgin flies. Dm, untransformed *D. melanogaster*; Dm + 5B, *D. melanogaster* transformed with *Est-5*; Dps, *D. pseudoobscura*; AChE, acetylcholinesterase; E6, EST 6; E5, EST 5. Densitometric traces across the EST 6 and EST 5 bands are shown below the gels. (Note that there are no EST 6 bands for Dps and no EST 5 bands for Dm.) Numbers indicate the relative area under each peak expressed as a percentage of the total area under all four peaks for that isozyme.

the body and then transported to the eyes (21). Any inferences about the evolution of gene regulation in this system must be based on a knowledge of the sites of *Est-5* and *Est-6* transcription as well as the sites of enzyme activity. We therefore examined the relative amounts of *Est-5* and *Est-6* transcripts in the heads and bodies of male and female flies to determine whether the distribution of *Est-5* transcripts coincided with the sites of enzyme activity.

Fig. 5 shows a Northern blot containing equal amounts of total RNA from heads and bodies of male and female flies. In Fig. 5*A*, the blot has been hybridized with the *Est-5* gene of *D. pseudoobscura* (Fig. 1). The two transcripts produced by this gene (5) are detected in the heads and bodies of both sexes of *D. pseudoobscura* and the transformed *D. melanogaster*. The relative amounts of these transcripts appear to be higher in the heads than in the bodies, but no difference is evident between males and females in either strain. In contrast, *Est-6* transcript levels in *D. melanogaster* (Fig. 5*B*) are highest in male bodies. However, it is also evident that *Est-6* transcripts are present in the heads of both sexes as well as in female bodies. Bands in the last four lanes of Fig. 5*B* represent residual *Est-5* signal that was not removed prior to hybridization with *Est-6*. These results indicate that the patterns of *Est-5* and *Est-6* transcription correspond to the enzyme activity patterns revealed by the native protein gel analyses.

DISCUSSION

Tissue- and sex-specific activity levels of the EST 5 enzyme of *D. pseudoobscura* in transformed *D. melanogaster* coincide with those in *D. pseudoobscura* and are distinctly different from the activity patterns observed for EST 6, the

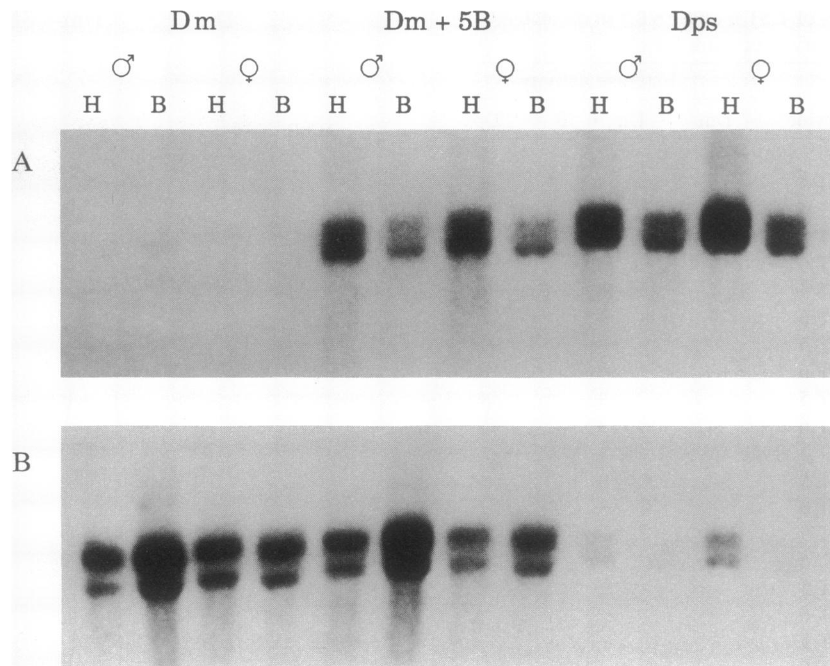

FIG. 5. Northern blot with equal amounts of total RNA from heads (H) and bodies (B) of male and female flies from untransformed *D. melanogaster* (Dm), *D. melanogaster* transformed with *Est-5* (Dm + 5B), and *D. pseudoobscura* (Dps). (*A*) Hybridized with a single-stranded RNA probe corresponding to the *Hin*dIII/*Eco*RI fragment containing *Est-5B* shown in Fig. 1. (*B*) Same blot probed with a single-stranded RNA probe corresponding to the *Est-6* cDNA clone (4).

homologous enzyme in *D. melanogaster*. Our observations concerning the abundance of *Est-5* transcripts in the heads of both sexes, the equal levels of *Est-5* transcripts in male and female *D. pseudoobscura* and transformed *D. melanogaster*, and the preferential accumulation of *Est-6* transcripts in male bodies all imply that differences in these expression patterns are controlled at the level of transcription. *Est-6* transcription in the heads of *D. melanogaster* was unexpected and suggests that the low levels of EST 6 activity detected in the eyes of *D. melanogaster* may represent actual EST 6 expression in those tissues rather than an artifact of hemolymph contamination. We previously reported that the promoters for *Est-5* and *Est-6* include four small regions of conserved sequence; one of these, a 12-bp palindrome, is also found in regulatory sequences of the *per* locus (5, 22). *per* also is expressed in the eyes of *D. melanogaster* (23). Therefore, this particular palindromic sequence might be a tissue-specific enhancer (24) responsible for directing the transcription of *Est-5* and *Est-6* in the eyes of both species.

Regulatory differences that have evolved between *Est-6* and *Est-5* appear to represent cis-acting alterations. If the different expression patterns resulted from the actions of different trans-acting regulatory factors in each species, we would have expected *Est-5* to be expressed in the transformants in a manner identical to *Est-6*; i.e., we would have expected to see EST 5 activity in the ejaculatory duct. Moreover, these results imply that the transcription factors that recognize the cis-acting regulatory sequences in EST 5 have been conserved since the divergence of the two species 20–46 million years ago (25, 26). Dickinson (27) concluded that cis-acting changes are the predominant evolutionary mechanism for altering gene regulation because they allow the expression of one structural locus to be modulated independently of other genes. Trans-acting regulatory factors, on the other hand, are less likely to be altered because such changes will often have widespread ramifications involving multiple structural loci (28) and are expected to be deleterious.

The presence of *Est-5* and *Est-6* transcripts in the heads and bodies of male and female *D. pseudoobscura* and *D. melanogaster* leads us to postulate that the EST 5/6 enzyme in the common ancestor of these species exhibited a more ubiquitous expression pattern than is currently observed for the two proteins. After *D. pseudoobscura* and *D. melanogaster* diverged, regulatory mutations may have occurred that enhanced EST 5 expression in the eyes, while similar mutations in *Est-6* led to increased ejaculatory duct expression. Increased expression in one tissue appears to have been accompanied by decreased expression in other tissues as is reflected by the apparent lack of EST 5 activity in male reproductive systems. The creation of these specialized expression patterns may have been facilitated by the presence of paralogous genes in each species that might have been able to compensate for the absence of EST 6 or EST 5 activity in a particular tissue or stage of development (5, 15).

The significance of the different patterns of expression of EST 5 and EST 6 is unclear. At this point, we can only speculate why EST 5 and EST 6 exhibit the observed specificities. EST 6 is transferred from males to females during mating (29). Once inside the females, EST 6 is rapidly transferred to the hemolymph and has been shown to affect sperm utilization and remating behavior (30). However, these observed effects may not reflect the primary functions of this enzyme. We have shown that EST 6 may act as a protease (31), as related esterases do in several species (32).

EST 5 expression in the eyes of *D. pseudoobscura* is even more puzzling. It has been reported that crystallins, the major soluble proteins of the vertebrate eye lens, are, in some cases, common metabolic enzymes that have been recruited to fulfill a structural role in the eye (33). An analogous situation may exist for EST 5. EST 5 in the body of *D. pseudoobscura* may have a catalytic function, while in the eyes it may serve a completely different purpose.

Tissue-specific gene expression may not be indicative of an adaptive role for the product of a gene (34). Regulatory mechanisms for many gene systems are likely to have arisen through a trial and error pathway by the accumulation of

random mutations and may be far from optimal (35). It is therefore conceivable that expression of EST 5 and EST 6 in the eyes and ejaculatory ducts, respectively, is not an adaptation. Further proof that these diverse expression patterns reflect the work of selective processes may depend on a knowledge of the natural substrates of these enzymes.

Our observation that *Est-5* transcripts and EST 5 enzyme occur in equal amounts in male and female transformants is surprising in light of the fact that *Est-5* is X-chromosome-linked in *D. pseudoobscura* (36). Others have found that X-linked genes transferred to autosomes by *P*-element transformation are at least partially dosage compensated, as seen by higher product or transcript levels in male transformants (37–39). Our results suggest that the transduced *Est-5* gene is not dosage compensated at this particular insertion site on chromosome III, probably because the transformation construct did not contain one of the X chromosome sequence elements that are hypothesized to cause dosage compensation of X-linked genes (39).

Changes in gene regulation are widely believed to have an important role in speciation and macroevolution (1–3). While we have no evidence that the EST 5 and EST 6 proteins directly contribute to species differences, the different expression patterns of these enzymes support the contentions that extreme changes in gene regulation can evolve over a relatively short period of time and that patterns of gene expression may be unique characteristics of individual species.

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