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## Is Esterase-P Encoded by a Cryptic Pseudogene In *Drosophila melanogaster*?

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### ABSTRACT

We have amplified and sequenced the gene encoding Esterase-P (*Est-P*) in 10 strains of *Drosophila melanogaster*. Three premature termination codons occur in the coding region of the gene in two strains. This observation, together with other indirect evidence, leads us to propose that *Est-P* may be a pseudogene in *D. melanogaster*. *Est-P* would be a "cryptic" pseudogene, in the sense that it retains intact the coding sequence (without stop codons and other alterations usually observed in pseudogenes) in most *D. melanogaster* strains. We conjecture that the  $\beta$ -esterase cluster may consist in other *Drosophila* species of functional and nonfunctional genes. We also conjecture that the rarity of detected pseudogenes in *Drosophila* may be due to the difficulty of discovering them, because most of them are cryptic.

EUKARYOTIC genomes contain, in addition to functional genes, sequences that are related to particular genes but exhibit changes that inactivate their expression. Such sequences have been designated "pseudogenes" (reviewed by VANIN 1985; WEINER *et al.* 1986; WILDE 1986). Since they were first described (JACQ *et al.* 1977; FEDOROFF and BROWN 1978), pseudogenes have been identified in a variety of gene families. Some are unprocessed pseudogenes that have introns and are usually chromosomally linked to their functional counterparts, suggesting that they arise by tandem gene duplication, followed by divergence and inactivation. Processed pseudogenes lack introns and are believed to arise from reverse transcription of processed mRNA, followed by integration of the resulting DNA into a new chromosomal location in the genome. Most pseudogenes are presumably not expressed, although several are transcribed into RNA molecules (FISCHER and MANIATIS 1985; SORGE *et al.* 1990; AUBERT *et al.* 1992; CURRIE and SULLIVAN 1994; FÜRBAH and VANSELOW 1995), and processed pseudogenes that are translated into protein molecules have been discovered (MCCARREY and THOMAS 1987).

Pseudogenes along with their functional counterparts provide useful models for understanding gene evolutionary processes, since they allow the comparison of mode and rate of molecular changes in sequences with and without functional constraints (LI *et al.* 1981, 1985b; MIYATA and YASUNAGA 1981; MIYATA and HAYASHIDA 1981; GOJOBORI *et al.* 1982; LI 1982, 1983).

Only a few examples of pseudogenes are known in *Drosophila*. A transcriptionally active (unprocessed) alcohol dehydrogenase (*Adh*) pseudogene, closely linked

to one or two functional counterparts, has been described in *D. mulleri* (FISCHER and MANIATIS 1985), *D. mojavensis* (ATKINSON *et al.* 1988), *D. mettleri* (YUM *et al.* 1991), *D. hydei* (MENOTTI-RAYMOND *et al.* 1991), *D. buzzatii* (SCHAFFER 1992), *D. peninsularis*, and *D. mercatorum* (SULLIVAN *et al.* 1994). In all these species, the reading frames of the *Adh* pseudogenes contain premature termination codons, resulting from one or more frameshifts, which along with indels prevent productive translation. KYLSTEN *et al.* (1990) have found two unprocessed pseudogenes, included in the cecropin locus of *D. melanogaster*. Processed pseudogenes of alcohol dehydrogenase have been found in *D. yakuba* and *D. teissieri* (JEFFS and ASHBURNER 1991; JEFFS *et al.* 1994) and phosphoglyceromytase (*Pglum*) in *D. melanogaster* (CURRIE and SULLIVAN 1994). The *Adh* and *Pglum* processed pseudogenes lack introns and are located on different chromosomes (*Adh*) or different arms of chromosome (*Pglum*) from their functional counterparts.

COLLET *et al.* (1990) have detected a tandem duplication of the *Est-6* gene in *D. melanogaster*, denoted as *Est-P*. Both genes belong to the  $\beta$ -esterase gene cluster (KOROCHKIN *et al.* 1987) and are located within subsection 69 A1-A3 on the left arm of polytene chromosome 3 (PROCUNIER *et al.* 1991), with the same 5' to 3' orientation (*Est-P* is downstream from *Est-6*). Both genes comprise two exons, with length of 1387 and 248 bp, separated by a short intron, which is 51 and 56 bp long in *Est-6* and *Est-P*, respectively. The coding regions of the genes are separated by only 197 bp. The two genes show 64 and 60% similarity in the DNA and protein sequence, respectively (COLLET *et al.* 1990).

In *Drosophila* species, the  $\beta$ -esterase gene cluster includes two or (only in *D. pseudoobscura*) three known closely linked genes (OAKESHOTT *et al.* 1993, 1995), which have the same direction of transcription and similar exon/intron structure (YENIKOLOPOV *et al.* 1983,

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1989a; BRADY *et al.* 1990; COLLET *et al.* 1990; EAST *et al.* 1990). The distance between the coding sequences of different members of the gene cluster ranges from 197 bp (between *Est-6* and *Est-P*) in *D. melanogaster* (COLLET *et al.* 1990) and 740 bp (between *Est-A* and *Est-B*) in *D. buzzatii* (EAST *et al.* 1990), to at least 1000 bp (between *Est-S* and *Est-X*) in *D. virilis* (YENIKOLOPOV *et al.* 1989b) and ~1700 bp (between *Est-5A* and *Est-5B*) in *D. pseudoobscura* (BRADY *et al.* 1990).

There is an extensive literature devoted to different aspects of the molecular population and evolutionary genetics of *D. melanogaster Est-6* (reviewed by OAKES-HOTT *et al.* 1993, 1995). *EST-6* plays an important role in the reproductive biology of *D. melanogaster*. A high level of the enzyme has been detected in the ejaculatory duct of adult males (ARONSHTAM and KUZIN 1974; KORCHKIN *et al.* 1974; KUZIN *et al.* 1975; SHEEHAN *et al.* 1979; RICHMOND *et al.* 1980; USPENSKY *et al.* 1988; KORCHKIN *et al.* 1990). RICHMOND *et al.* (1980) have shown that *EST-6* is transferred to females as a component of the seminal fluid during copulation. The adaptive significance of *Est-6* allozyme variation has been demonstrated (see reviews in RICHMOND *et al.* 1990; OAKES-HOTT *et al.* 1993, 1995).

Significantly less information is available about *Est-P*. COLLET *et al.* (1990) have detected *Est-P* transcripts in *D. melanogaster* late larvae and adults of each sex, whereas the *Est-6* transcripts are found mainly in adult males. They have, accordingly, proposed distinct physiological functions for the products of the two genes.

We have amplified and sequenced *Est-P* in 10 strains of *D. melanogaster* and found three premature termination codons in two of the 10 strains, which should prevent productive translation of the gene in those strains. We propose that *Est-P* may be a pseudogene in *D. melanogaster*, which is not essential although it retains a potentially functional coding sequence in a majority of the *D. melanogaster* strains we have studied.

## MATERIALS AND METHODS

**Drosophila strains:** The *D. melanogaster* strains were derived from wild flies collected by F. J. AYALA (October 1991) in El Rio Vineyard, Lockeford, California. The strains were made fully homozygous for the third chromosome by means of crosses with balancer stocks, as described by SEAGER and AYALA (1982). The strains were named in accordance with the superoxide dismutase electrophoretic alleles they carry, Fast (F) or Slow (S), as follows: 255S, 510S, 521S, 94F, 174F, 357F, 377F, 521F, 581F, 968F.

**PCR primers, amplification, and sequencing:** Total genomic DNA was extracted using the procedure described by PALUMBI *et al.* (1991). The *Est-6* and *Est-P* sequences, previously published by COLLET *et al.* (1990), were used for designing PCR and sequence primers. The amplified fragments (2.5 kb long) encompassed part of *Est-6* exon II, the intergenic region between *Est-6* and *Est-P*, the whole *Est-P* gene, and 360 bp of the 3'-untranslated region. The two primers used for the PCR amplification reactions were as follows: 5'-gataatgtaggtagtaggagaaattc-3' (forward primer), and 5'-ctatgtagctttgtggagcagattgtgt-3' (reverse primer).

All PCR reactions were carried out, as described by KWI-

TOWSKI *et al.* (1991), in final volumes of 100  $\mu$ l containing 40  $\mu$ M each of dNTP, 2.5 U AmpliTaq DNA Polymerase (Perkin-Elmer), 0.2  $\mu$ M each of forward and reverse primers, buffer (Perkin-Elmer) at a final concentration of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, and ~50 ng of template (total genomic) DNA. The mixtures were overlaid with mineral oil, placed in a DNA thermal cycler (Perkin Elmer Cetus), incubated 5 min at 95° and subjected to multiple cycles of denaturation, annealing, and extension under the following conditions: 95° for 1.0 min, 56° for 1.0 min, and 72° for 2.0 min (for the first cycle and progressively adding 3 sec at 72° for every subsequent cycle). After 30 cycles, a final 7-min extension period at 72° concluded each amplification reaction. Samples were stored at 4° for several hours or at -20° for up to 2 weeks.

One-tenth of each reaction volume was assayed on a 0.8% agarose gel. If the desired PCR product was detected, the remainder of the reaction was purified with Wizard PCR preps DNA purification system (Promega corporation). The purified PCR product was directly cloned using the TA cloning kit (Invitrogen, San Diego, CA). Cloned DNA was purified using the QIAprep plasmid preparation system (QIAGEN Inc., CA).

DNA sequencing was done by the dideoxy chain-termination technique (SANGER *et al.* 1977) with Sequenase Version 2.0 T7 DNA polymerase (Amersham Life Science Inc., USA) using <sup>32</sup>P-labeled dATP. Five internal primers (a-e) were used for sequencing (see Figure 1): (a) 5'-tacagctacgaatcgattcc-3', (b) 5'-ggaaactatgggtctaaaggat-3', (c) 5'-tggtgtggagccgctcagatgacac-3', (d) 5'-tagatctccaccgaaagtatg-3', (e) 5'-ttgtaaaacgaggaatgac-3'.

**DNA sequence analysis:** All primers were designed using the computer program DNASIS for Windows (1994, Hitachi Software Engineering Co., Ltd.). This program allowed us also to check the secondary structure of primers. Multiple alignment was carried out manually, using the program DARNWIN (elaborated by Dr. ROBERT TYLER from our laboratory) and automatically, using the program CLUSTAL W (THOMPSON *et al.* 1994). Additional DNA sequences of the *Drosophila*  $\beta$ -esterase genes were obtained from the GenBank database, with the accession numbers *D. melanogaster*, *Est-6* and *Est-P*, M33780, M33781; *D. mauritiana*, *Est-6*, L10671; *D. simulans*, *Est-6*, L34263; *D. pseudoobscura*, *Est-5C* and *Est-5B*, M55907; *Est-5A*, M55908; *D. virilis*, *Est-S*, X70351. The *D. buzzatii Est-A* and *Est-B* sequences were obtained from EAST *et al.* (1990). The phylogenetic analysis of the esterase sequences was performed with the computer program MEGA (KUMAR *et al.* 1993). The pattern of synonymous and nonsynonymous substitutions was estimated by the method of LI (1993; see LI *et al.* 1985a), but synonymous sites were counted according to SATTA (1993).

## RESULTS

Figure 1 shows the organization of the duplicated  $\beta$ -esterase genes in *D. melanogaster* and our sequencing strategy. The polymorphic nucleotide sites in the *Est-P* coding sequence of the 10 strains are shown in Figure 2; the polymorphic amino acid sites are shown in Figure 3. We detected three premature termination codons and one short insertion in two of the 10 *D. melanogaster* strains. One premature termination codon (TGA) occurs in the 357F strain and two tandemly located termination codons (both TGA) in the 510S strain, where they are associated with a 9-bp-long insertion. We have confirmed the presence of the stop codons in second clones, obtained from separate PCR amplifications, in-



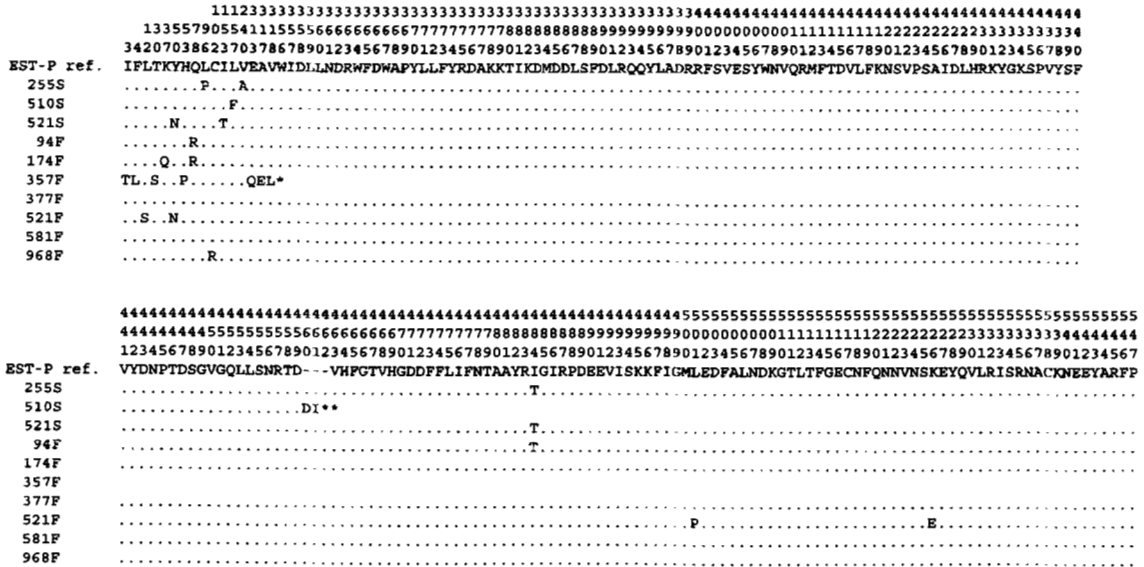


FIGURE 3.—Polymorphic sites in the inferred *EST-P* amino acid sequence of 10 strains of *D. melanogaster*. The three stop codons are indicated by \* (356 in 357F; 462 and 463 in 510S). Other conventions as in Figure 2.

acids in the complete sequence). As noted above, the deleted polypeptide fragments include two cysteine residues, the catalytic histidine, and one (for 510S strain), or three (for 357F strain) potential N-linked glycosylation sites, all of which are important for maintaining esterase function. The catalytic histidine and the cysteine sites are strictly conserved in all *Drosophila*'s  $\beta$ -esterase sequences already published (data not shown). The catalytic histidine is one of three key residues in the esterase molecule that directly effect the esterase reaction (CARTER and WELLS 1988; MYERS *et al.* 1988; SCHRAG *et al.* 1991; SUSSMAN *et al.* 1991). The cysteine residues are involved in the formation of disulfide bridges, which support the three-dimensional molecular structure, and are crucial for molecular conformational stability. It is known that the active sites of other serine hydrolases with similar catalytic mechanisms are stabilized by disulfide bridges (MYERS *et al.* 1988). *Est-6* is a glycoprotein (MANE *et al.* 1983) and contains four

potential N-linked glycosylation sites, three (strain 357F) or two (strain 510S) of which are lost in *Est-P* sequences. We can assume that protein molecules lacking those sites are not functionally efficient.

The fact that two perfectly viable strains lack functional *Est-P* genes suggests that *Est-P* is not an essential gene and may be a pseudogene. There are also indirect sources of evidence that support the hypothesis that *Est-P* is a pseudogene.

1. The *Est-6* promoter has four regulatory elements and the most important one, which controls gene expression in the ejaculatory duct, is located in the region from  $-511$  to  $-1132$  bp, upstream from the initiation codon (LUDWIG *et al.* 1993). The intergenic region between *Est-6* and *Est-P* is only 197 bp, and thus there is not enough sequence to account for an *Est-P* promoter, if we extrapolate from *Est-6* that the *Est-P* native promoter should be longer than 197 bp. Some significant part of the promoter was lost during or after duplication. This event destroyed normal gene regulation in one of the duplicated products and determined which

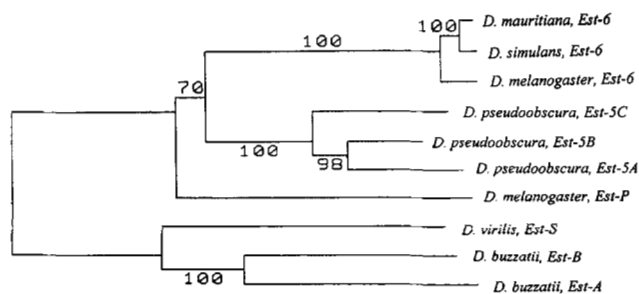


FIGURE 4.—Phylogenetic relationships of  $\beta$ -esterase genes in *Drosophila*. The sequences of *Est-A* and *Est-B* of *D. buzzatii* are from EAST *et al.* (1990). Other sequences are from GenBank (see MATERIALS AND METHODS for the accession numbers). The unrooted phylogenetic tree has been obtained by the neighbor-joining method (SAITOU and NEI 1987) using Jukes-Cantor distances for exon I. The numbers are bootstrap probability values based on 1000 replications.

TABLE 1

The rate of synonymous and nonsynonymous substitutions, and the  $\gamma$  parameter for intra- and interspecific comparisons between *Est-P* and *Est-6*

| Comparison                            | $K_s$ | $K_n$ | $\gamma$ |
|---------------------------------------|-------|-------|----------|
| <i>mel Est-P</i> vs. <i>mel Est-6</i> | 0.241 | 0.572 | 0.422    |
| <i>mel Est-P</i> vs. <i>sim Est-6</i> | 0.237 | 0.576 | 0.411    |
| <i>mel Est-P</i> vs. <i>mau Est-6</i> | 0.234 | 0.580 | 0.403    |
| <i>mel Est-6</i> vs. <i>sim Est-6</i> | 0.022 | 0.109 | 0.203    |
| <i>mel Est-6</i> vs. <i>mau Est-6</i> | 0.025 | 0.097 | 0.259    |
| <i>sim Est-6</i> vs. <i>mau Est-6</i> | 0.009 | 0.041 | 0.212    |

The species abbreviations are as follows: *mel*, *D. melanogaster*; *sim*, *D. simulans*; *mau*, *D. mauritiana*.  $K_s$ , rate of synonymous;  $K_n$ , rate of nonsynonymous; rate of  $\gamma = K_n/K_s$ .

TABLE 2

Total number of synonymous and nonsynonymous substitutions among 10 alleles of *Est-P* and *Est-6* in *D. melanogaster*

| Gene         | $N_a$ | $N_s$ | $K_a$ | $K_s$ | $\gamma$ |
|--------------|-------|-------|-------|-------|----------|
| <i>Est-P</i> | 34    | 22    | 0.029 | 0.047 | 0.624    |
| <i>Est-6</i> | 17    | 28    | 0.015 | 0.060 | 0.250    |

$K_a$ ,  $K_s$ , and  $\gamma$  as in Table 1.  $N_s$ , number of synonymous;  $N_a$ , number of nonsynonymous.

of the two *Est-6* or *Est-P* remained an essential gene. Termination codons may then have appeared later by chance, since there were no functional constraints that would strongly select against them. Pseudogenes have been detected that have no obvious defects (such as frame-shift mutations and/or premature termination codons) and retain high sequence similarity to their functional counterparts, but lack promoter elements. The deletion of promoter parts completely inactivates the expression of these genes (SIBBALD and BLENCOWE 1990; MATTERS and GOODENOUGH 1992). A possible escape of the argument we have just advanced is to assume that a segment of the *Est-6* gene may have been hijacked into becoming the promoter of *Est-P* (see COLLET *et al.* 1990). This seems unlikely but is not, of course, impossible.

2. Members of a multigene family often share an evolutionary process called concerted or coincidental evolution (ARNHEIM 1983). Several molecular mechanisms and theoretical models have been proposed to explain patterns of concerted evolution (NAGYLAKI 1984; OHTA 1984). Both nonreciprocal (gene conversion) and reciprocal (unequal sister chromatid exchange) recombination of DNA among members of gene families contribute toward their concerted evolution, so that the members of the family retain much greater similarity than expected in terms of the time since their independent origin. The phylogenetic tree of the *Drosophila*  $\beta$ -esterase genes suggest that a gene duplication may have occurred previous to the divergence of the subgenera *Sophophora* (which includes *D. melanogaster* and *D. pseudoobscura*) and *Drosophila* (*D. buzzatii*). If so, concerted evolution might account for the greater similarity between the two (or three) paralogous genes of each species than between the orthologous genes from different species. This is the situation observed in *D. pseudoobscura* and *D. buzzatii*, whereas the divergence between the two paralogous genes is much greater in *D. melanogaster* (Figure 4). This greater divergence could, however, be accounted for if *Est-P* would have acquired a new function. We submit, however, based on the overall of evidence, that it is rather the loss of function of *Est-P* that accounts for its extensive divergence from *Est-6*.

3. COLLET *et al.* (1990) detected *Est-P* transcripts in late larvae and adults of each sex. However HEALY *et al.*

TABLE 3

The rate of synonymous and nonsynonymous substitutions, and the  $\gamma$  parameter between three genes of *D. pseudoobscura*

| Comparison           | $K_a$ | $K_s$ | $\gamma$ |
|----------------------|-------|-------|----------|
| <i>Est-5A:Est-5B</i> | 0.133 | 0.304 | 0.438    |
| <i>Est-5A:Est-5C</i> | 0.161 | 0.397 | 0.406    |
| <i>Est-5B:Est-5C</i> | 0.092 | 0.311 | 0.288    |

Abbreviations as in Table 1.

(1991) failed to find the *Est-P* protein product among 22 esterases in *D. melanogaster*, assayed by polyacrylamide gel electrophoresis and isoelectric focusing. As we have mentioned above, some pseudogenes can be transcriptionally active. Thus, the presence of *Est-P* transcripts is not definitive proof of functionality, while the absence of the protein product in a functional organism is indirect evidence of lack of (at least, essential) function.

4. In functional genes most nonsynonymous substitutions are deleterious and are eliminated from a population by negative selection. As a consequence, in functional genes the rate of nonsynonymous substitutions is generally significantly lower than the rate of synonymous substitutions. Pseudogenes are not subject to functional constraints, and thus synonymous and nonsynonymous substitutions rates are expected to be the same, and the expected ratio of the rates,  $\gamma = K_a/K_s$ , should gradually become one (KIMURA 1983). However, the  $\gamma$  values of a pseudogene and a paralogous functional gene may be fairly similar, if the duplication occurred recently, or if the pseudogene became nonfunctional recently. The possibility of gene conversion between the pseudogene and its functional counterpart also has to be taken into account, since it will contribute to conserve the similarity of the  $\gamma$  values.

Be that as it may, if we observe that  $\gamma$  is greater between a putative pseudogene and its functional counterpart than between two corresponding functional genes, this will be evidence favoring the interpretation that the putative pseudogene is indeed a pseudogene. This will be the case whether the comparison is made between species or between alleles of the same species. Both tests, as summarized in Tables 1 and 2, favor the hypothesis that *Est-P* is a pseudogene.

If we assume that the rate of nonsynonymous substitutions is constant, the  $K_s$  values in Table 1 conform to the accepted phylogeny of the *melanogaster* group species, in which *D. simulans* and *D. mauritiana* are sister species, and *D. melanogaster* is sister to the cluster of the other two (*e.g.*, POWELL and DESALLE 1995, Figure 4). The average  $K_s$  for the three *Est-P:Est-6* comparisons in Table 1 is 0.576, or 5.6 times the average  $K_s$  between *D. melanogaster* and the other two species. If we assume that the *melanogaster* and (*simulans* + *mauritiana*) lineages diverged 2.5 million years ago (POWELL and DESALLE

1995, Table IV), the *Est-P:Est-6* duplication would have occurred 14 million years ago. This inference must, however, be received with considerable caution for reasons mentioned above, particularly the possibility of gene conversion between *Est-P* and *Est-6*. If we accept the duplication date of 14 million years ago, it follows that the phylogeny based on nucleotide distances (Figures 4) is in error (introduced by different rates of nucleotide evolution in different genes), since the divergence between the *obscura* and *melanogaster* lineages occurred about 40 million years ago (see, e.g., Table IV in POWELL and DESALLE 1995).

An *Est-6* null allele has been found in the carnation eye color (*car*, X-linked, recessive) laboratory stock of *D. melanogaster* [sc ec ev et<sup>6</sup> v g<sup>2</sup> f/FM<sub>3</sub> y<sup>31d</sup> sc<sup>8</sup> dm B 1] (JOHNSON *et al.* 1966). If null alleles were common for *Est-6*, we might propose that this gene also is a pseudogene. However no *Est-6* null alleles have been observed in natural populations (VOELKER *et al.* 1980; LANGLEY *et al.* 1981; OAKESHOTT *et al.* 1989). Moreover, GILBERT and RICHMOND (1982) have shown that *Est-6* null-allele males have significantly lower reproductive fitness than typical (wild-type) *Est-6* males. SAAD *et al.* (1994) have, moreover, found strong association between the activity level of EST-6 and the reproductive fitness in *D. melanogaster* wild-derived lines. These results (see extensive discussion in OAKESHOTT *et al.* 1993, 1995) strongly suggest that *Est-6* in *D. melanogaster* is engaged in adaptively significant physiological function and therefore cannot be a pseudogene. The null allele observed in a laboratory stock (JOHNSON *et al.* 1966) may represent a spontaneous null mutation that would have very low probability to survive in nature. It may be likely that this spontaneous null mutation arose as a consequence of hybrid dysgenesis (SVED 1979; PRUDHOMMEAU and PROUST 1990; GEORGIEV and YELAGIN 1992), which increases mutation rates in specific crosses that involve strains long cultured in the laboratory. In addition to the *Est-6* null allele, JOHNSON *et al.* (1966) found another null allele for a separate esterase locus, *Est-C* in the same *D. melanogaster* stock. An abundance of null alleles certainly may occur in hybrid dysgenesis crosses. Because of the crosses used in our study to generate homozygous strains, the question arises whether our *Est-P* "null" alleles may have arisen by hybrid dysgenesis. This hypothesis seems unlikely, given that no other null alleles have been found in the numerous El Rio strains of *D. melanogaster* assayed in our laboratory for *Sod*, *Xdh*, *Gpdh* and other genes, even though all strains were made homozygous by the same extraction and maintenance procedures (HUDSON *et al.* 1994; F. J. AYALA and collaborators, unpublished results).

The original loss of function of *Est-P* in *D. melanogaster* may have happened owing to the deletion of part of the promoter region when the *Est-6* duplication occurred. After that, due to lack of functional constraints, null mutations, leading to stop codons would be tolerated and persist in some individuals. A relative abundance

of premature stop codons is likely to arise only as a result of frameshift mutation. Nevertheless, stop codons are likely to be rare even in pseudogenes or nonessential genes. First, we note that the rate at which a null mutation becomes fixed in a population is highly dependent on population size and mutation rate (LI 1980, 1983). In a very large population, a null mutation may never become fixed (FISHER 1935; NEI and ROYCHOUDHURY 1973; LI 1980, 1983). Moreover, MODIANO *et al.* (1981) have shown that the pattern of codon usage in human globin genes is such that codons that can mutate by single nucleotide substitution to a termination codon are never used whenever the corresponding amino acid is specified also by other triplets. Thus, the number of opportunities to mutate to an untranslatable codon is reduced to the minimum compatible with the amino acid composition of a gene. They propose that living organisms have adopted mechanisms that considerably reduce mutations with especially severe consequences (MODIANO *et al.* 1981). The combination of these two factors (*i.e.*, fixation rate of null mutations and pattern of codon usage) may explain why the *Est-P* gene lacks premature stop codons in most individuals, despite the fact that the *Est-6/Est-P* duplication event is probably as old as the divergence of the *repleta* and *melanogaster* group (COLLET *et al.* 1990), which has been estimated to have occurred 44 million years ago by some authors (KWIATOWSKI *et al.* 1994), but 60–80 million years ago by others (*e.g.*, THROCKMORTON 1975). We may consider *Est-P* as a "cryptic" pseudogene, because only two strains out of 10 studied strains exhibit stop codons. In all other strains the coding sequence of *Est-P* is intact, without premature stop codons or other alterations typically observed in pseudogenes.

In spite of extensive molecular genetic studies, few pseudogenes have been found in *Drosophila* (see Introduction). This might be because many of them are cryptic, for similar reasons as we have just pointed out for *Est-P* in *D. melanogaster*. Particularly, the  $\beta$ -esterase gene cluster in other *Drosophila* species may also consist of functional and nonfunctional genes. The results displayed in Table 3, for pairwise comparisons between the  $\beta$ -esterase genes of *D. pseudoobscura*, suggest that *Est-5A* may be a pseudogene. Negative evidence supporting this conjecture is that translational products have not been identified for this gene (BRADY *et al.* 1990). The similarity of  $K_i$  values in Table 3 suggests that the two gene duplications giving rise to *Est-5A*, *Est-5B*, and *Est-5C* in *D. pseudoobscura* may have occurred in rapid succession, with the *Est-5A:Est-5B* being the most recent. If we assume that  $K_i$  is constant between *D. melanogaster* and *D. pseudoobscura*, the  $\beta$ -esterase duplications would have occurred ~8 million years ago (assuming again 2.5 million years for the divergence between *D. melanogaster* and *D. simulans*). The same reservations pointed out above, particularly the possibility of gene conversion, indicate that the inferred time of divergence between the *D. pseudoobscura*  $\beta$ -esterase

genes should be received with much caution. Indeed, BRADY *et al.* (1992; see also OAKESHOTT *et al.* 1995) have proposed that a gene duplication occurred before the divergence of *D. melanogaster* and *D. pseudoobscura*, so that *D. melanogaster Est-P* and *D. pseudoobscura Est-5A* are orthologous to each other, whereas *D. melanogaster Est-6* is orthologous to *D. pseudoobscura Est-5B* (and *Est-5C*).

Pseudogenes are named using the designation of the functional counterpart with additional prefix " $\psi$ ". We propose that the *Est-P* pseudogene in *D. melanogaster* be designated as  $\psi$ *Est-6*. To prevent confusion in the *Drosophila*  $\beta$ -esterase nomenclature, we prefer to maintain the suffix "6" in the pseudogene abbreviation, indicating its likely origin as a duplicated product of the gene designated *Est-6* in *D. melanogaster*.

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