Localization of Sequences Regulating Ancestral and Acquired Sites of Esterase6 Activity in Drosophila melanogaster

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We have broadly defined the DNA regions regulating esterase6 activity in several life stages and tissue types of D. melanogaster using P-element-mediated transformation of constructs that contain the esterase6 coding region and deletions or substitutions in 5' or 3' flanking DNA. Hemolymph is a conserved ancestral site of EST6 activity in Drosophila and the primary sequences regulating its activity lie between −171 and −25 bp relative to the translation initiation site: deletion of these sequences decreases activity approximately 20-fold. Hemolymph activity is also modulated by four other DNA regions, three of which lie 5' and one of which lies 3' of the coding region. Of these, two have positive and two have negative effects, each of approximately twofold. Esterase6 activity is present also in two male reproductive tract tissues; the ejaculatory bulb, which is another ancestral activity site, and the ejaculatory duct, which is a recently acquired site within the melanogaster species subgroup. Activities in these tissues are at least in part independently regulated: activity in the ejaculatory bulb is conferred by sequences between −273 and −172 bp (threefold decrease when deleted), while activity in the ejaculatory duct is conferred by more distal sequences between −844 and −614 bp (fourfold decrease when deleted). The reproductive tract activity is further modulated by two additional DNA regions, one in 5' DNA (−613 to −284 bp; threefold decrease when deleted) and the other in 3' DNA (+1860 to +2731 bp; threefold decrease when deleted) that probably overlaps the adjacent esteraseP gene. Collating these data with previous studies suggests that expression of EST6 in the ancestral sites is mainly regulated by conserved proximal sequences while more variable distal sequences regulate expression in the acquired ejaculatory duct site.

Introduction

Although the cis-acting regulatory sequences that control temporally and spatially specific expression have been characterized in many genes, relatively little information is available on regulatory sequences responsible for conservation and changes in expression patterns across species. In several genes, including alcohol dehydrogenase (Adh) and two chorion gene clusters, conserved sequences that regulate conserved aspects of expression have been identified. Adh expression in the fat body of larvae and adults is regulated by elements with enhancer properties that are conserved in D. melanogaster and D. mulleri (Abel, Bhatt, and Maniatis 1992; Falb and Maniatis 1992a) and the interaction of these elements with defined transcription factors is also conserved (Falb and Maniatis 1992b). Within the autosomal chorion gene cluster, at least some of the sequences controlling amplification are conserved between D. melanogaster and D. grimshawi (Orr-Weaver 1991). Moreover, the sequences controlling temporal and spatial expression in follicle cells of the chorion genes in both clusters are conserved across species as diverse as Bombyx mori, Ceratitis capitata, and D. melanogaster (Swinnen et al. 1992; Tullius et al. 1993). However, less is known about the changes in regulatory sequences that lead to changes in the expression profile. We are addressing this question through analysis of the esterase6 (E.C. 3.1.1.1) gene/enzyme (Est6/EST6) system. The genes encoding EST6 and its homologues have been isolated and characterized in at least seven Drosophila species from the subgenera Drosophila and Sophophora (Oakeshott et al. 1993; Van Papenrecht 1995). This extensive cross-species molecular analysis of Est6 now provides the framework for identifying the regulatory sequence variation underlying the evolutionary changes in the EST6 expression pattern.

The spatial expression pattern of EST6 and its homologues generally has been described in adults. The predominant sites of adult activity are the hemolymph and the male reproductive tract, although intensive analysis of a few species also reveals small amounts of activity in other tissues such as the gut, eye, proboscis, and antennae (Brady and Richmond 1990; Oakeshott, Healy, and Game 1990; Healy, Dumancic, and Oakeshott 1991). Hemolymph activity is conserved across the subgenera Drosophila and Sophophora, with all 25 species from 9 subgroups examined showing relatively high levels of activity. This suggests that the hemolymph is an ancestral activity site. In contrast, activity in the male reproductive tract is variable and two significant differences are apparent between species of the subgenus Drosophila and Sophophora. First, ejaculatory bulb activity is relatively high in Drosophila species, but low to nonexistent in Sophophora species. Second, only Sophophora species show activity in the ejaculatory duct, although there is considerable variation in the level of activity within the subgenus.

Our long-term aim is to understand the molecular basis for this variability of EST6 activity in the male reproductive tract in Sophophora species. These species have variable levels of EST6 activity in the testes and accessory gland and activity in the ejaculatory bulb has recently been detected in D. melanogaster (Oakeshott, Healy, and Game 1990; Ludwig, Tumariina, and Richmond 1993). However, it is the anterior ejaculatory duct activity that shows the greatest variation. EST6 activities...
are relatively high in the anterior ejaculatory ducts of three species in the melanogaster subgroup, *D. simulans*, *D. mauritiana*, and *D. melanogaster*, while very low levels of activity are found in this tissue in the more distantly related species *D. pseudoobscura*, *D. yakuba*, and *D. erecta* (Oakeshott, Healy, and Game 1990).

Three lines of evidence suggest that the major aspects of *Est6* expression, including differences in ejaculatory duct activities, are mainly due to cis-inherited sequences flanking the *Est6* coding region rather than to trans-acting factors. First, interspecific transformations using the *Est6* genes from *D. simulans* and *D. mauritiana* (Karotam and Oakeshott 1993) and the *Est6* homologue from *D. pseudoobscura* (*EstSB*; Brady and Richmond 1990) revealed that, in general, the pattern of EST6 activity resembles the donor rather than the recipient species, *D. melanogaster*. Second, RFLP and sequencing studies have identified sequences associated with male-specific activity in the 5′ flanking sequences of *D. melanogaster* (Game and Oakeshott 1990; Odgers, Healy, and Oakeshott 1995). Finally, deletion analyses of the *D. melanogaster* 5′ flanking sequences linked to a reporter gene have identified two regions that direct expression in the ejaculatory duct or bulb (Ludwig, Tamarina, and Richmond 1993).

While it is likely that the variability within the male reproductive tract is at least in part due to changes in the flanking sequences, the capacity of these sequences to change may be constrained by the proximity of *EstP*, a tandem duplication of *Est6* which lies less than 0.2 kb 3′ of *Est6* (Collet et al. 1990). The *Est6* and *EstP* genes show distinct expression profiles, with *EstP* expressed predominantly in late third-instar larvae, suggesting that the gene products are functionally distinct (Collet et al. 1990).

In this paper we explore the regulatory mechanisms by which the *Est6* gene of *D. melanogaster* has maintained its ancestral expression profile and yet accommodated an acquired male-specific activity site. Specifically, we have used P-element-mediated transformation to introduce into the *D. melanogaster* genome constructs carrying the *Est6* coding and intronic DNA linked to deleted or mutated 5′ and 3′ flanking sequences. Quantitative analyses of the resultant lines have defined the broad locations of the *D. melanogaster* sequences regulating EST6 activity in the hemolymph at three life stages and in the adult male ejaculatory bulb and anterior ejaculatory duct. We demonstrate that ancestral activities in the hemolymph and ejaculatory bulb are directed by conserved sequences close to the transcription initiation site, while the more recently acquired site of activity, the anterior ejaculatory duct, is directed by more distal sequences lying both 5′ and 3′ of the *Est6* coding region that exhibit greater nucleotide variation between species. The 3′ sequences appear to lie within the transcription unit of the adjacent *EstP* gene.

### Materials and Methods

#### Construction of Modified *Est6* Genes

Ten in vitro modified *Est6* genes were generated using genomic DNA isolated from the wild-type strain Dm145 (fig. 1; Collet et al. 1990; Karotam, Delves, and Oakeshott 1993). All constructs contained the *Est6* coding region and intron but had variable amounts of 3′ and 5′ flanking DNA.

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**Fig. 1.**—Organization of the *Est6* and *EstP* genes, showing the translated DNA (hatched) and the position of the *Est6* TATA box in the wild-type Dm145 strain (A) and in lines with modified genes carrying 3′ and/or 5′ deletions (B) or TATA box mutations (C). The number of independent lines carrying each modified gene is indicated.
Three constructs carried 1,174 bp of 5' DNA (relative to the translation site start) but variable amounts of 3' DNA. They were generated by digestion with HindIII and Sea I (construct -1174/+1859) or HindIII alone (construct -1174/+2731) to form the ligation of HindIII/Nru I- and Nru V/Hae II-digested fragments (construct -1174/+4174). Four other constructs that shared a 3' endpoint of +1859 carried successively less 5' DNA. Their 3' endpoints were generated by digestion with Sea I and their 5' endpoints were generated by using the nested deletion method (constructs -844/+1859, -613/+1859, -283/+1859, and -171/+1859). The endpoint of each deletion was determined by DNA sequencing using Est6-specific primers. Another construct with unique 5' and 3' endpoints (construct -24/+4252) was generated by digestion with EcoRI.

Constructs T/-68 and C/-68 were variants of -1174/+1859 that each carried a point mutation at +68 bp in the putative TATA box. They were generated by site-directed mutagenesis as described in Myers, Healy, and Oakeshott (1993) using 21-mer oligonucleotides carrying the required substitutions (AATAAA to TA-). Care was taken to incorporate errors (TAAA or CATAAA, respectively).

Where necessary, DNA fragments were purified and end-filled using DNA polymerase I prior to insertion into the transformation vector. Constructs -1174/+1859, -844/+1859, -283/+1859, T/-68, and C/-68 were cloned directly into the Hpa I site of Carnegie 20 while constructs -1174/+4174, -613/+1859, and -171/+1859 were ligated to Bcl I linkers and cloned into the BamHI site of CaSpeR (Robinson et al. 1988). Construct -1174/+12731 was inserted into both vectors using these procedures and as there were no significant differences in EST6 activities between the two classes of constructs (data not shown), the data for them have been combined. Construct -24/+4252 was cloned directly into the EcoRI site of CaSpeR. All Carnegie 20 and CaSpeR constructs carried the Est6 gene in the same orientation, so that it was transcribed in the same direction as the marker gene (royal+ or white+).

DNA preparation, Southern blot hybridization, DNA sequencing, and other recombinant DNA techniques were performed as described previously (Sambrook, Fritsch, and Maniatis 1989; Game and Oakeshott 1990, Karotam and Oakeshott 1993). DNA sequencing analyses were performed using the DNAid+ package for Macintosh (version 1.8).

Generation of Transformed *Drosophila* Lines

P-element vectors containing the modified Est6 genes were microinjected into embryos using the procedures of Zachar et al. (1987) with the 2-3(9B) strain as the transposase source (Roberson et al. 1988). Adults from the microinjected embryos were mated to Est6mull or Est6mull flies (w;Est6mull or Est6mull, Sheehan, Richmond, and Cochrane 1979) and stocks were generated that were homozygous for both Est6mull and the inserted modified Est6 gene. The chromosomal position of which each modified gene integrated was determined using classical genetic methods and insertions were verified as single-copy events in Southern blot hybridization experiments by screening for diagnostic restriction fragments. All *Drosophila* stocks were cultured on agar-cornmeal-treacle media at 25°C in a 12:12 light/dark cycle.

Esterase6 Assays

Quantitative spectrophotometric or densitometric data were obtained for 10 EST6 activity measures: whole postfeeding third-instar larvae, whole virgin 1- and 5-day-old adult males and females, hemolymph from postfeeding third-instar larvae and virgin 1-day-old adult males and females and reproductive tracts and ejaculatory duct/bulb complexes from virgin 1-day-old adult males. Groups of 20 virgin 1- and 5-day-old adult males and females and postfeeding third-instar larvae were collected, frozen in liquid nitrogen, and stored at -80°C. Tissue samples were collected and homogenized as described by Healy, Dumanic, and Oakeshott (1991). Protein concentrations were determined by the method of Bradford (1976) using the BioRad Protein Assay Kit and bovine serum albumin as the standard.

EST6 activities in all tissue and whole-adult samples were assayed spectrophotometrically with β-naphthylacetate as the substrate and in the presence of eserine sulfate (10-5 M) and p-chloromercuribenzoate (pCMB; 10-4 M) after modifications to the previously described technique (Sheehan, Richmond, and Cochrane 1979). Briefly, the incubation volumes were reduced to 100 μl, the reactions were performed in a 96-well microtiter plate, and the amount of naphthol produced was measured in a BioRad microplate reader (model 3550). EST6-specific activity was expressed as mmol β-naphthol/30 min/mg protein.

EST6 activities in postfeeding third-instar larval samples were determined after fractionation by native polyacrylamide gel electrophoresis (PAGE) rather than spectrophotometrically because other esterases present in these samples hydrolyzed β-naphthylacetate and their activities could not be inhibited by pCMB or eserine sulfate. PAGE was performed as described by Healy, Dumanic, and Oakeshott (1991) except that Triton X-100 was omitted. Following electrophoresis, gels were treated as described by Healy, Dumanic, and Oakeshott (1991). Briefly, the gels were soaked in 0.1 M sodium phosphate buffer, pH 6.6, containing 2 × 10-5 M eserine sulfate and 5 × 10-4 M pCMB prior to visualisation of EST6 activity. The gel regions showing EST6 activity were subjected to densitometry using a Shimadzu dual-wavelength TLC scanner model (CS-930) and EST6-specific activity was expressed in arbitrary units of signal intensity/mg protein.

All 10 EST6 activity measures were estimated from two to six independent transformed lines for each construct and the wild-type line Dm145. Samples from three independent cultures of each line were assayed. Significant effects conferred by each construct were identified by performing an analysis of variance which compared the variation between constructs with the variation between the lines carrying each construct that arises because of different chromosomal sites of insertion. These
comparing the activities of the three constructs and comparisons of effects of successive deletions using the three constructs for six activity measures (table 1) both the larval and adult stages.

The role of 3' flanking sequences was assessed by productive tracts from 1-day-old adult virgin males were obtained histochemically. The tissues were dissected in Ringer's buffer and fixed for 1 h in 4% paraformaldehyde (pH 6.8). Tissues were then incubated for 2 min at room temperature in the esterase substrate solutions described by Healy, Dumancic, and Oakeshott (1991), except that they were buffered with PBS (pH 6.8) and contained 3% Triton X-100 and 3 × 10^{-4} M eserine sulfate, and examined by light microscopy. For each construct, at least four individuals were analyzed from the construct with degrees of freedom of 1, 2, 5, 17-19, 3, 5-10, and 2, 7 or 8, respectively, for the four analyses.

and other statistical tests were performed using the Statview package.

Qualitative data on EST6 activities in whole reproductive tracts from 1-day-old adult virgin males were contained 3% Triton X-100 and 3 × 10^{-4} M eserine sulfate, and examined by light microscopy. For each construct, at least four individuals were analyzed from the one line whose EST6 activity was closest to the mean for the construct.

Results

The first objective was to test if the longest construct in our study was sufficient to confer wild-type levels of EST6 activity. Lines carrying this construct (~1174/4174), with 1,174 bp of 5' and 2,489 bp of 3' flanking DNA, were compared to the wild-type strain Dm145. No differences were found for any of the 10 activity measures (table 1 and fig. 2), suggesting that the ~1174/4174 construct contains the majority of regulatory elements required for wild-type expression in both the larval and adult stages.

Organization of Est6 Regulatory Sequences

The role of 3' flanking sequences was assessed by comparing the activities of the three constructs ~1174/+4174, ~1174/+2731, and ~1174/+1859. An analysis of variance revealed significant variation among the three constructs for six activity measures (table 1) and comparisons of effects of successive deletions using Fisher's PLSD test revealed that the variation was largely attributable to the +1860- to +2731-bp region. Deletion of +2732 to +4174 bp had no effect on any of the activity measures while deletion of +1860 to +2731 bp reduced activity two- to severalfold in whole larvae and larval hemolymph, male and female hemolymph, 1 day old adult females, and the ejaculatory duct/bulb complex (figs. 2 and 3). Thus, our data indicate that the +1860- to +2731-bp region confers a single positive regulatory effect in several, but not all, tissues and life stages (fig. 4).

The roles of sequences 5' of the translation start site were first analyzed using the six constructs ~1174/+1859, ~1174/+1859, ~1174/+1859, ~171/+1859, ~283/+1859, ~24/+4252, in which the amount of 5' DNA varied from 24 to 1,174 bp. An analysis of variance comparing the effects of these constructs showed significant differences for all activity measures (table 1) and comparisons of the effects of adjacent constructs localized significant effects to specific 5' regions.

Comparison of the ~1174/+1859 and ~1174/+1859 constructs showed that deletion of ~1174 to ~845 bp increased EST6 activity in both whole larvae and larval hemolymph. Similar increases in male and female hemolymph activities also approached significance (P < 0.1; figs. 2 and 3). Comparison of the confidence limits on the fold increases suggests that this region confers a single negative regulatory effect of two- to fivefold in hemolymph across both juvenile and adult stages (figs. 3 and 4).

Comparison of the ~844/+1859 and ~613/+1859 constructs showed effects of the ~844- to ~614-bp region on EST6 activity in six of the eight adult measures but in neither of the larval measures (figs. 2 and 3). Deletion of this region reduced EST6 activities approximately fourfold in both the ejaculatory duct/bulb complex and the whole-male reproductive tract but increased activities by two- to fivefold in male hemolymph, 1-day-old adult males, and 1- and 5-day-old adult females (fig. 3). This suggests that the ~844- to ~614-bp region confers a positive regulatory effect in male reproductive tissue and a negative regulatory effect in some other adult tissues, including male hemolymph (fig. 4).

Activities were significantly reduced in 9 of the 10 activity measures after deletion of ~613 to ~284 bp and a similar reduction in the 10th measure, whole male reproductive tract, also approached significance (P < 0.1; figs. 2 and 3). Comparison of the confidence limits on the fold activity differences indicated that the effects varied from two- to sixfold with no clear subdivision within this range. These data suggest that the ~614- to ~284-bp region confers a single positive regulatory effect that operates widely across life stages and tissues (fig. 4).

Deletion of ~283 to ~172 bp increased EST6 activities in whole larvae, larval hemolymph, and 1-day-old adult males (figs. 2 and 3), and an increase in female hemolymph activity also approached significance (P < 0.1). The sizes of these increases in activities fell into two classes: larval measures increased five- to
ninefold and adult measures increased approximately twofold. In contrast, activity in the ejaculatory duct/bulb complex was reduced approximately threefold. As the ejaculatory duct/bulb activity comprises less than 10% of the whole-male activity in these constructs (data not shown), the decrease in ejaculatory duct activity has little effect on the increase in whole-male activity. We conclude that the region confers two negative regulatory effects in the hemolymph and some other tissue(s), which differ quantitatively between developmental stages, plus a positive effect in the ejaculatory duct/bulb complex (fig. 4).

Deletion of -171 to -25 bp is associated with reduced EST6 activities in nine measures, the exception being the ejaculatory duct/bulb complex for which activities are negligible in lines with either the
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**Fig. 3.** The effect of deleting or mutating each DNA region of the Est6 flanking DNA on EST6 activities in whole organisms of five life stages and in five tissue types. Means of the fold change in (untransformed) EST6 activities are shown with their confidence limits in parentheses. For each change in EST6 activity, the direction of the change (increase, $\Uparrow$; decrease, $\Downarrow$; no change, $\Uparrow$) and its statistical significance (no asterisk, $P < 0.1$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) are shown.
FIG. 4—Summary of DNA sequences which affect EST6 activities in whole organisms of five life stages and in five tissue types. The organisms of the Est6 and Est6 genes with the endpoints of each deletion and the sites of mutated bases are shown. Note that the 5' DNA is illustrated on an expanded scale relative to the structural and 3' flanking DNA. Activity measures for which sequences have a positive regulatory effect are shown above the gene and those for which there was a negative regulatory effect are shown below. WL = whole larvae, LH = larval hemolymph, IdF = 1-day-old adult females, 5dF = 5-day-old adult females, FH = female hemolymph, I dM = 1-day-old adult males, 5dM = 5-day-old adult males, MH = male hemolymph, RT = male reproductive tract, ED/B = ejaculatory duct/bulb complex.
-171/+1859 or -24/+4252 construct. Although the constructs with these 5’ endpoints also had different 3’ endpoints, the construct with the least amount of 5’ DNA had the most 3’ DNA. Analysis of the effect of the 3’ sequences (above) indicates that the -171/+1859 and -24/+4252 construct effects described here cannot be attributed to these extra 3’ sequences. Thus, most of the activity differences observed between constructs -171/+1859 and -24/+4252 are due to sequences in the -171- to -25-bp region.

Within this region the sizes of the effects on activities and their confidence limits divide the measures into four groups: whole larvae (370-fold), larval and adult hemolymph (20-fold), 1- and 5-day-old adult males (7- to 10-fold), and 1- and 5-day-old adult females and male reproductive tracts (3- to 4-fold; figs. 2 and 3). The substantially larger effect in whole larvae compared to larval hemolymph implies an even greater difference in some other larval hemolymph which also show EST6 activity, such as fat body, imaginal discs, and body wall (Healy, Dumanic, and Oaksheil 1991). Also note that male hemolymph and male reproductive tract activity account for approximately 20% and 3%, respectively, of whole-male activity in lines with the -171/+1859 construct (data not shown). Thus, the 7- to 10-fold change in whole-male activity cannot be attributed to the average of the 20-fold hemolymph and 3- to 4-fold reproductive tract activity changes. We conclude that the -171- to -25-bp region confers at least four classes of positive regulatory effects, including a very large effect (>370 fold) in unidentified larval tissue(s), a 20-fold effect in both larval and adult hemolymph, about an 8-fold effect in unidentified male tissue(s), and about 3-fold effects in unidentified female tissue(s) and the male reproductive tract (fig. 4).

The role, if any, of the nonconsensus first nucleotide in the putative TATA box (−68 to −63 bp) was assessed by comparing the T/−68 and C/−68 constructs with the otherwise identical −174/+1859 construct (fig. 1). Substitution with either T or C increased activity by two- to fourfold in a subset of adult activity measures: 1- and 5-day-old adult females and male and female hemolymph, about an 8-fold effect in unidentified male tissue(s), and about 3-fold effects in unidentified female tissue(s) and the male reproductive tract (fig. 4).

Finally, we note that the 45 pairwise correlations between the various activity measures across constructs are all positive (data not shown). Forty-three of the 45 correlation coefficients are between 0.4 and 0.9 and the other 2 are 0.2 and 0.3. The latter are from comparisons of male reproductive tract activities with those of whole larvae. The results of the correlation analysis support the evidence from the comparisons of construct effects cited above that the majority of DNA regions defined here affect multiple activity measures.

Sequences Regulating EST6 Activity in Hemolymph and Ejaculatory Duct/Bulk Complex

The construct comparisons above show that six DNA regions have some effect on at least one of the three hemolymph activities and each of these activities is modulated by five DNA regions (figs. 3 and 4). Three of these regions have positive regulatory effects at all developmental stages: one region lies 3’ of the coding region (+1860 to +2731 bp) with the other two in the 5’ DNA (−613 to −284 bp and −171 to −25 bp). The remaining three regions all exert negative regulatory effects and all are located 5’ of the coding region. One of these (−1174 to −845 bp) influences activity at all stages and appears to be largely specific for hemolymph activity. −844 to −614 bp influences male hemolymph activity and −283 to −172 bp influences larval and female hemolymph activities.

There are substantial differences in the sizes of the effects conferred on hemolymph activity by these DNA regions. Activity in all three stages is increased approximately 20-fold by the sequences between −171 and −25 bp. All the other regions that influence hemolymph activity confer small effects (two- to threefold) and, given the combination of positive and negative effects, their net effect is essentially zero. This suggests that only 171 bp of 5’ DNA is required for wild-type hemolymph activity. These results were confirmed from comparisons of EST6 activities in hemolymph of lines carrying construct −171/+1859 and the wild-type strain Dm145, which revealed no activity differences in hemolymph from larvae, males, or females (Dunnett t18 = 0.4, 0.5, and 1.3, respectively; P > 0.05).

Wild-type EST6 activity in the ejaculatory duct/bulb complex requires four DNA regions that all have positively regulating effects: one is located 3’ of the coding region and the other three are in the 1,174 bp of 5’ flanking DNA (figs. 3 and 4). Sequences from +1860 to +2731 bp, −613 to −284 bp, and −283 to −172 bp each confer two- to threefold effects on activity while the effect of sequences at −844 to −614 bp is four- to fivefold. The effects of −844 to −614 bp and −283 to −172 bp appear to be specific for reproductive tract activity while the other two regions exert similar effects on both reproductive tract and other tissues. The distribution and size of these effects are such that the relatively long construct −613/+1859 expresses less than 10% of the wild-type activity. Together these data suggest that the two more distal regions (+1860 to +2731 bp and −844 to −614 bp) are primarily responsible for activity in the ejaculatory duct/bulb complex.

Histochemical Analyses of EST6 Activities in the Ejaculatory Duct and Bulb

Histochemical analysis of dissected male reproductive tracts from the wild-type strain Dm145 showed only trace amounts of EST6 activity in the outer epithelial layer of the accessory glands and the most posterior region of the testes (table 2). Most activity is found in epithelial cells of the ejaculatory duct, predominantly in the anterior portion of the duct, with smaller amounts in the ejaculatory bulb, confirming the results of Sheehan, Richmond, and Cochrane (1979) and Ludwig, Tamarin, and Richmond (1993).

Similar analyses of lines carrying modified Est6 genes (table 2) revealed that two of the four DNA
Table 2
EST6 Activities in the Ejaculatory Duct and Bulb

<table>
<thead>
<tr>
<th>Construct</th>
<th>Ejaculatory Duct</th>
<th>Ejaculatory Bulb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>++++++</td>
<td>+</td>
</tr>
<tr>
<td>−1174/+4174</td>
<td>++++++</td>
<td>+</td>
</tr>
<tr>
<td>−1174/+2731</td>
<td>++++++</td>
<td>+</td>
</tr>
<tr>
<td>−1174/+1859</td>
<td>++++++</td>
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<td>++++++</td>
<td>+</td>
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<tr>
<td>T/−68</td>
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<td>+</td>
</tr>
<tr>
<td>C/−68</td>
<td>++++++</td>
<td>+</td>
</tr>
</tbody>
</table>

**NOTE.**—EST6 activity was visualized histochemically as described in Materials and Methods. ++++++ indicates the highest level of activity, + the lowest, and − not detectable.

regions that influence activity in the ejaculatory duct/bulb complex, +1860 to +2731 bp and −613 to −284 bp, have similar effects on both the duct and bulb. However, sequences between −844 and −614 bp are essential for ejaculatory duct, but not bulb, activity; EST6 activity was present in the ejaculatory duct of lines with the −844/+1859 construct, but not in lines with the −613/+1859 construct, while activity was present in the ejaculatory bulbs of both sets of lines. In contrast, sequences essential for ejaculatory bulb, but not duct activity, are located between −283 and −172 bp: lines carrying the −283/+1859 construct have EST6 activity in the ejaculatory bulb while lines with the −171/+1859 construct have essentially no bulb activity. Neither of these lines have detectable activity in the ejaculatory duct. These results indicate that qualitative aspects of EST6 activities in the ejaculatory duct and bulb are independently regulated by the −844- to −614-bp and −283- to −172-bp regions, respectively.

### Specific Sequence Motifs within Est6 Regulatory Regions

The sequences flanking Est6 contain nine copies of the A/TGATAA/G element, which is the binding site for the dGATA transcription factor that appears to regulate Adh expression in the larval fat body (Abel, Michelson, and Maniatis 1993; table 3). Each of the five regions which our deletion analyses above have shown to modulate EST6 activity in whole larvae contains at least one of these elements and the elements are not found outside these regions. The A/TGATAA/G element is also present in the regulatory sequences of six other genes whose proteins are present in the larval fat body and its presence in the Est6 larval regulatory sequences is consistent with the larval fat body being a site of EST6 activity (Healy, Dumancic, and Oakeshott 1991).

Male EST6 activity is inducible by ecdysone (Richmond and Tepper 1983) and the sequences flanking Est6 contain seven copies of the consensus ecdysone response elements identified by Luo, Amin, and Voellmy (1991) and Antoniewski, Laval, and Lepesant (1993; table 3). Six of these elements are in two of the five regions that influence activity in male hemolymph and/or ejaculatory duct/bulb complex. Seven copies of the
ecdysone half sites (Pongs 1988) are also present, five copies within four of the six regions that influence male-specific activity measures. Thus, most copies of the putative ecdysone response element lie in regions affecting male-specific activity measures but there are also some such regions which lack these elements.

Three elements have been proposed to regulate activity of another enzyme primarily expressed in the ejaculatory duct of D. melanogaster, glucose dehydrogenase (GLD; Cavener 1992; Quine et al. 1993). One of these, the palindromic Gpal element (C/GAGCTTA/GCTTGAA), is not present in the Est6 flanking sequences. The other two elements are closely related (TTAGAAT/GCTT), sharing the first five of seven nucleotides. No copies of TTAGAAT are present in the Est6 flanking DNA while one copy of TTAGAAT lies in the 3' region that contributes to EST6 activity in the ejaculatory duct/bulb complex. The region carrying sequences essential for ejaculatory duct/bulb activity in particular tissues (table 3). Seven copies of the core TTAGA element are present, with three in the +1860- to +2731-bp region that modulates ejaculatory duct/bulb activity but none in the other three regions influencing activity in this tissue. Thus the locations of three elements proposed to control ejaculatory duct/bulb activity of GLD do not correlate well with regions regulating the ejaculatory duct/bulb activity of EST6 and it is notable that none of these elements falls within the -844- to -641-bp and -283- to -172-bp regions defined here as being important for qualitative aspects of EST6 activity in these tissues.

We also screened for motifs in the Est6 flanking DNA that recur within or between regions regulating activity in particular tissues (table 3). Within the -844- to -641-bp region essential for ejaculatory duct activity, the motif TGCAAG is repeated three times in the space of 35 bp (-643 to -638 bp, -660 to -655 bp, and -666 to -661 bp) and the copy at -666 to -661 bp overlaps an ecdysone half site. This motif also occurs twice in the 3' regulatory regions (+2365 to +2370 bp and +2386 to +2381 bp), with the element at +2365 to +2370 bp falling within a region which contributes substantially to EST6 activity in the ejaculatory duct/bulb complex. The region carrying sequences essential for ejaculatory bulb activity (-283 to -172 bp) contains a CAATACT motif (-257 to -251 bp), which is repeated at -518 to -512 bp, close to the 16-bp palindrome (-538 to -523 bp) which our earlier work indicates is associated with male-specific activity (Game and Oakeshott 1990; Odgers, Healy, and Oakeshott 1995).

Two sequence motifs recur within the -171- to -25-bp region essential for hemolymph activity (table 3). Of these, the ACTGGTT motif is tandemly duplicated (-120 to -114 bp and -112 to -106 bp) and it is also imperfectly repeated at -208 to -202 bp and +2632 to +2638 bp. The other, TGTTCA, occurs twice adjacent to the ACTGGTT motif (-199/-194 bp and -106/-101 bp), three times elsewhere in the 5' flanking DNA (-1061 to -1056 bp, -930 to -925 bp, and -551 to -546 bp), and once in the 3' DNA (+3504 to +3509 bp).

**Discussion**

The Organization of Est6 Regulatory Sequences

Our results demonstrate that essentially the wild-type pattern and level of EST6 activity in both third-instar larvae and adults are conferred by sequences that include the Est6 coding region and intron and flanking sequences lying up to +1,174 bp 5' and 1,046 bp 3'. The finding that the 1,174 bp of 5' DNA is sufficient for at least some aspects of wild-type expression agrees with the findings of Ludwig, Tamarina, and Richmond (1993) for D. melanogaster and Karotam and Oakeshott (1993) for D. simulans. However, the possibility that additional elements contributing to quantitative aspects of expression lie upstream of this region cannot be excluded given previous evidence that insertions between -1174 and -1600 bp in three wild-caught lines of D. melanogaster are associated with reductions in larval EST6 activity (Oakeshott et al. 1994).

An unexpected feature of the organization of Est6 regulatory sequences is that 3' flanking sequences modulate several EST6 activity measures. While the 5' regulatory sequences lie relatively distant from the adjacent transcription unit (at least 400 bp further 5'; Collet et al. 1990), the 3' sequences lie immediately upstream of, or within, the adjacent EstP transcription unit. Specifically, they fall within the region that starts 20 bp 5' of the EstP initiation codon (42 bp downstream of its putative TATA box) and extends 853 bp into the EstP coding DNA. Such an overlap of regulatory and transcribed sequences has been described in other duplicated genes that are transcribed in the same (e.g., Sgs-3/7; Gianandrè, Mettling, and Richards 1987) or opposite directions (e.g., yp1/y2p; Logan, Garabedian, and Wensink 1989).

Each of the 10 activity measures we have monitored is modulated quantitatively, or in some cases qualitatively, by at least three regulatory regions. While this might be expected for whole-organism measures, activities in individual tissues are also modulated by multiple regulatory regions, raising the possibility of functionally redundant or combinatorially acting regulatory elements as proposed by Dover (1992) and Dickinson (1988). Our data also indicate that several of the DNA regions individually exert different quantitative effects on several activity measures (e.g., -283 to -172 bp and -171 to -25 bp). This is particularly apparent in the -171- to -25-bp region, which sequence data suggest contains the transcriptional initiation signals common to all activity measures, but which also exerts differential quantitative effects. While this may imply that individual regions contain multiple regulatory elements, it could also reflect differences in the interactions of specific elements with transcription factors. Further dissections of the regions are required to distinguish between these possibilities.

The level of complexity in the organization of Est6 regulatory sequences evident in our quantitative analysis is substantially greater than the relatively simple organization of sequences conferring qualitative aspects of tissue-specific expression identified by Ludwig, Tamar-
ina, and Richmond (1993). However, the complexity now evident in the Est6 regulatory sequences is comparable to that in other promoters that have been subjected to detailed analyses (e.g., Adh; Abel, Bhatt, and Maniatis 1992; Abel, Michelson, and Maniatis 1993; Ayer et al. 1993, and Ddc; Lundell and Hirsh 1992; Mastick and Scholnick 1992).

Mutating the single nucleotide at position −68 changes the stage- and tissue-specific EST6 activity. This nucleotide is the 1st in the element AATAAA which is proposed to function as the Est6 TATA box. While we have no empirical evidence that it functions as a TATA box, two lines of evidence suggest that it does. First, the element is located 31 bp upstream of the most 5′ of the 3 major transcription initiation sites at −38 bp relative to the coding region, as determined by primer extension (Collet et al. 1990). Second, only the first nucleotide does not conform to the consensus sequence for eukaryotic promoters, and 20% of these promoters deviate from consensus at this position (Bucher 1990).

Substitution of the first nucleotide in the putative Est6 TATA box with either the TATA box consensus nucleotide T or another nonconsensus nucleotide C results in two- to threefold higher levels of EST6 activity in whole adult females and adult hemolymph but not in the other measures of activity monitored. In many genes deletion or mutation of the TATA box reduces the level of transcription and/or disrupts the position of initiation without otherwise affecting the overall pattern of expression (e.g., Maniatis, Goodbourn, and Fischer 1987). However, recent studies have revealed that modification of the TATA box may also result in tissue specific changes in transcription (e.g., Ravid et al. 1991). This may occur because of competition between the TATA binding protein and other transcription factors whose binding sites overlap the TATA box (e.g., Ravid et al. 1991; Fong and Emerson 1992). We propose that similar interactions occur at the Est6 TATA box to repress EST6 activities in adult females and adult hemolymph.

Sequences Regulating Activity in Ancestral Activity Sites

Many of the conserved sites of EST6 activity are regulated by the 450 bp immediately 5′ of the Est6 translation start site. The major conserved site of activity, the hemolymph, shows some EST6 activity in all 25 species tested to date, spanning nine subgroups and two subgenera (Oakeshott, Healy, and Game 1990). Here we have demonstrated that wild-type hemolymph activities in third-instar larvae and adult males and females can be achieved with positively regulating sequences that lie adjacent to the translation start site (−171 to −25 bp). Hemolymph activity in each of the three life stages is further modulated by two positively and two negatively regulating sequences that are located more distally, but each of these has a relatively small effect and their net effect is essentially zero. Some of the complexity in the regulation of hemolymph activity may be a consequence of contributions of EST6 activity from different cell types. At least some of the activity derives from the hemocytes (MH, MD, AC, and JO, unpublished data) but it is possible that some of the activity is secreted into the hemolymph from tissues such as the fat body in which EST6 is also active (Healy, Dumancic, and Oakeshott 1991).

Conserved minor activity sites, including the mouthparts, antennae, gut, thorax, and ejaculatory bulb, have been demonstrated in at least some representative species from the repleta and virilis groups in the subgenus Drosophila and from the obscura and melanogaster groups in the subgenus Sophophora (Oakeshott, Healy, and Game 1990). Ludwig, Tamarina, and Richmond (1993) have shown that activities in mouthparts, antennae, and respiratory system within the thorax are regulated qualitatively by sequences between −371 and −276 bp, while salivary gland activity is quantitatively controlled by the −445- to −372-bp region. These minor activity sites may explain some of the whole-adult effects identified here that are not attributable to hemolymph or whole-male reproductive tract activity, particularly in the −613- to −284-bp region.

We and Ludwig, Tamarina, and Richmond (1993) have demonstrated that the sequences regulating qualitative aspects of ejaculatory bulb activity also fall within the proximal 450 bp. The combined results of the two studies refine the location of these sequences to −273 to −172 bp. We have also identified two more distally located regions (−613 to −284 bp and +1860 to +2731 bp) that may further modulate activity in the ejaculatory duct/bulb complex, although it is not clear if these act in both the duct and the bulb.

Therefore, our data suggest that the qualitative aspects of hemolymph and ejaculatory bulb activities are regulated by the proximal 450 bp, while more distal sequences modulate quantitative aspects of their expression. The complex regulation of these activities may be a consequence of the selective constraint to maintain these activity sites despite the acquisition of other sites of expression such as the ejaculatory duct.

Sequences Regulating Activity in the Acquired Ejaculatory Duct Site

We have found that distal sequences located between −844 and −614 bp regulate qualitative aspects of expression in the acquired ejaculatory duct site. This is consistent with, but substantially refines, the findings of Ludwig, Tamarina, and Richmond (1993), who attributed qualitative control of duct activity to the −1174- to −553-bp region. We also identified two additional regions, discussed above (−613 to −284 bp and +1860 to +2731 bp), that may modulate duct activity quantitatively. We propose that the ejaculatory duct activity evolved through changes to the distal sequences that did not disrupt the sequences conferring the ancestral sites of activity and thus their highly constrained functions.

Our findings of quantitative effects on the male reproductive tract activity by the −613- to −284-bp region are consistent with RFLP and allelic sequencing analyses of the region. These analyses revealed a block of three nucleotide polymorphisms in a 20-bp interval.
a pseudoobscura sequences are interpreted by the D. melanogaster regulatory proteins to give the D. pseudoobscura expression pattern (Brady and Richmond 1990). It is therefore of great interest to determine if the putative regulatory elements identified in Est6 in our study correspond with the few conserved sequences in Est5B.

The four species for which sequence information is available have moderate levels of EST6 activity in the hemolymph. Of the two internally repeated motifs within regions that regulate hemolymph activity, the TGTTCA motif is not present in the 5' flanking DNA of Est5B from D. pseudoobscura. However, the other motif, ACTGGTT at −120 to −114 bp, is found in Est5B as well as Est6 of D. simulans and D. mauritiana and on this basis is proposed as a candidate hemolymph control element. Two additional sequences within the regions defined here as essential for hemolymph activity are also conserved in Est6 from the four species. One of these (−52 to −32 bp) is likely to contain the primary transcription initiation sites (Collet et al. 1990). The other (−171 to −137 bp) shows no obvious similarities with other flanking DNA but its location suggests a role in the regulation of hemolymph activity.

The high level of similarity in the expression profiles of D. melanogaster, D. simulans, and D. mauritana suggest that EST6 is likely to be present in the ejaculatory bulb, although this has not been empirically tested in the latter two species to our knowledge. Both copies of the CAAACCT motif proposed to regulate ejaculatory bulb activity are present in these species. However, EST5B from D. pseudoobscura is not present in the ejaculatory bulb and the distal copy of the CAAACCT motif is not conserved in Est5B, although a single imperfect copy of the proximal motif is retained at −233 to −227 bp, just 8 bp 3' of the 16-bp palindrome. Thus, the position of this CAAACCT motif relative to the palindrome is conserved in all four species. An imperfect copy of the motif is also present in the D. virilis gene encoding an inactive esterase found in the ejaculatory bulb (at −337 bp; Sergeev et al. 1993) and within the region that confers temporal and spatial spec

(−544 to −522 bp) which contains a 16-bp palindrome.

A haplotype defined by these three polymorphisms in combination with others in the −613- to −284-bp region is associated with 15% less whole-organism activity in adult males, but not in adult females or preadults (Oakeshott et al. 1994; Odgers, Healy, and Oakeshott 1995).

Interspecific Conservation of Regulatory Sequences

While the EST6 activity levels in the hemolymph are conserved across four Drosophila species in the melanogaster and obscura groups, activity levels in the ejaculatory bulb and ejaculatory duct are variable. Comparisons of EST6 activities at these three sites and conserved sequences across the species reveal a good correlation between conservation of the activity site and conservation of the motifs that we have found associated with activity at the sites (table 4).

The sequence and position of three sets of motifs proposed to control EST6 activity in hemolymph, ejaculatory bulb, and ejaculatory duct are strongly conserved in the homologous genes of the closely related species D. simulans and D. mauritana (Karotam, Delves, and Oakeshott 1993). This is consistent with the overall high level of sequence conservation among D. melanogaster, D. simulans, and D. mauritana, which is greater than 90% in the distal 720 bp of 5' DNA studied here and greater than 97% in the proximal 450 bp (Karotam, Delves, and Oakeshott 1993). In addition, D. simulans and D. mauritana are very similar to D. melanogaster in their EST6 activity levels across a variety of life stages and tissues (Karotam and Oakeshott 1993).

However, comparisons of the flanking sequences in the more distantly related D. melanogaster Est6 and D. pseudoobscura Est5B revealed little sequence similarity. Less than 100 bp of the 570 bp immediately 5' of the coding regions can be aligned and these form four short regions of similarity (Brady, Richmond, and Oakeshott 1990). Despite this the two species share many ancestral sites of expression (Brady, Richmond, and Oakeshott 1990; Healy, Dumancic, and Oakeshott 1991), and transformation experiments demonstrated that the D. melanogaster and obscura EST5B can be aligned and these form four short regions of similarity (Brady, Richmond, and Oakeshott 1990).
ificity on this gene (Sergeev et al. 1995). Therefore, the functions and evolution of the CAAACCT elements remain to be clarified.

In contrast, the presence of the putative ejaculatory duct element (TGCAAG) is correlated with activity in this tissue. All three 5’ copies of the element are conserved in Est6 from D. simulans and D. mauritiana, which have ejaculatory duct expression, but not in D. pseudoobscura EstSB or the gene encoding the inactive esterase from D. virilis, neither of which have ejaculatory duct expression (Sergeev et al. 1993).

Thus, the comparisons of homologous genes across species support our identification of regulatory elements controlling EST6 expression in at least the hemolymph and ejaculatory duct. In addition, the data suggest that the hemolymph expression site conserved between D. melanogaster and D. pseudoobscura may be directed, at least in part, by these conserved sequences despite otherwise minimal sequence conservation. However, it is not clear if other ancestral sites of activity are regulated by the short regions of alignable sequences.

In contrast, comparison of the sequences regulating Est6 and the nonhomologous Gld gene suggest that their shared expression sites can be achieved with different sequences. Of the three motifs proposed to contribute to regulation of GLD activity in the ejaculatory duct and ejaculatory bulb (Cavener 1992; Quine et al. 1993; Gunaratne et al. 1994), only a single copy of one of these motifs lies within DNA that contributes to the regulation of EST6 activity in the ejaculatory duct/bulb complex and neither of the other two motifs are present anywhere in the Est6 flanking DNA. In addition, neither of the motifs that we propose to regulate EST6 activity in these tissues is present in the Gld 5′ flanking DNA. Thus, even though there is a strong correlation between GLD and EST6 activities in the ejaculatory duct/bulb complex across the Drosophila phylogeny (Schiff et al. 1992; Ross, Fong, and Cavener 1994), the regulatory sequences mediating their activities are not closely related.

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