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# *Drosophila* glutathione *S*-transferase 1-1 shares a region of sequence homology with the maize glutathione *S*-transferase III

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**ABSTRACT** **We have characterized a *Drosophila* glutathione *S*-transferase (RX:glutathione R-transferase, EC 2.5.1.18) cDNA encoding a protein of 209 amino acids. The cDNA was expressed in *Escherichia coli* harboring the expression plasmid construct pGTDml-KK. The active enzyme, designated as *Drosophila* glutathione *S*-transferase 1-1, had a specific activity toward 1-chloro-2,4-dinitrobenzene comparable to that for the mammalian glutathione *S*-transferases but did not have as broad a substrate specificity pattern. There is a region of 44 amino acids in this enzyme that shares 66% identity with an analogous region of maize glutathione *S*-transferase III. *Drosophila* glutathione *S*-transferase 1-1 had no obvious homology to any mammalian or parasitic glutathione *S*-transferases. The gene was found to be a member of a multigene family.**

The glutathione *S*-transferases (RX:glutathione R-transferases EC 2.5.1.18; GSTs) are a family of multifunctional proteins (for recent reviews, see refs. 1 and 2). High multiplicity of GSTs with overlapping substrate specificities may be essential to their multiple roles in xenobiotic metabolism, drug biotransformation, and protection against peroxidative damage. This isozyme is ubiquitous among eukaryotes. The rat and human GSTs are products of their respective gene superfamilies (3–6). In plants, GSTs are involved in the detoxification of certain herbicides. Different specificities of plant GSTs are thought to be the basis of selective actions of some herbicides (7–10). It has also been proposed that resistance to certain selected pesticides in insects may be related to changes in their GST expression (11, 12). The parasitic helminths of the genus *Schistosoma* have surface antigens that are glutathione *S*-transferases. Acquired immunity in mice, rats, hamsters, and monkeys against this antigen from *Schistosoma japonicum* or *Schistosoma mansoni* has provided protection against schistosomiasis, a chronic debilitating disease in several parts of the world (13–15). The major squid lens crystallins may be themselves GSTs or evolutionarily related to GSTs (16, 17). In this communication, we report the molecular characterization of a *Drosophila* GST gene[§] and its heterospecific expression in *Escherichia coli*.

## MATERIALS AND METHODS

**Materials.** Chemicals, *S*-hexylglutathione (GSH)-linked agarose and antibiotics were purchased from Sigma and/or Merck. Radioactive nucleotides were products of Amersham or DuPont/NEN. $^{125}$I-labeled protein A was purchased from ICN. Restriction endonucleases and T4 DNA ligase were products from New England Biolabs. A nick-translation kit was purchased from BRL. $Kc_0$ cells were grown in spinner flasks to a density of $\approx 5 \times 10^6$ cells per ml, according to a published procedure (18). Twelve- to 16-hour-old *Drosophila* embryos (Oregon R) were collected and washed before use. A *Drosophila* head cDNA library in the λgtll vector was provided by P. Salvaterra of the Beckman Research Institute (Duarte, CA) (19). A genomic library in the λEMBL4 vector was constructed from Oregon R genomic DNA as described (20).

**Purification of GSTs from $Kc_0$ Cells and *Drosophila* Embryos.** One unit of GST activity catalyzes the formation of 1-chloro-2,4-dinitrobenzene (CDNB)–GSH conjugate at a rate of 1 μmol/min. $Kc_0$ cells ($\approx 1 \times 10^{11}$) were lysed with Nonidet P-40 and cycles of freezing-and-thawing. After removal of nuclei by centrifugation at 5000 × *g* (6000 rpm, SS34 rotor, Sorvall centrifuge), the supernatant fractions were brought to 70% saturation by adding solid ammonium sulfate. The precipitated proteins, which contained nearly all the GST activities determined by CDNB conjugation, were recovered by centrifugation (8000 × *g* for 20 min). The precipitate was dissolved in a minimum volume of 25 mM Tris·HCl (pH 8.0) and dialyzed against the same buffer (1 liter) overnight with one change. Traces of denatured proteins were removed from the dialyzed fraction by centrifugation before affinity chromatography on *S*-hexyl-GSH (3-ml bed volume, Econo-column from Bio-Rad). Approximately 35% of the CDNB-conjugation activities appeared in the flow-through fractions that did not bind to a second column of *S*-hexyl-GSH-linked agarose; these fractions were not processed further for the results reported here. The affinity column was washed with 25 mM Tris·HCl (pH 8.0) buffer that contained 0.2 M KCl, and GST activities were eluted according to published procedures (21, 22). The eluted fractions contained two major bands on SDS/PAGE: one band at 23.4 kDa and the other band at 28.5 kDa (data not shown). This enzyme sample was dialyzed against 50 mM 2-[*N*-morpholino]ethanesulfonic acid (MeS) (pH 6.1) and further purified by fast protein liquid chromatography on a Mono Q column. The elution was carried out with a gradient of 0–0.3 M KCl in MeS buffer (pH 6.1). The first activity peak (peak I), which appeared just before the beginning of the gradient, contained a single band (23.4 kDa) on SDS/PAGE. The rest of the GST activities were eluted between 60 mM and 140 mM KCl in two overlapping activity peaks, peak II and peak III. Fractions of peak III contained four bands between 23.4 kDa and 31 kDa on SDS/PAGE. Peak I and peak III GSTs were used separately to raise polyclonal antibodies in rabbits as described

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Abbreviations: GST, glutathione S-transferase; GSH, glutathione; CDNB, 1-chloro-2,4-dinitrobenzene.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. X14233).

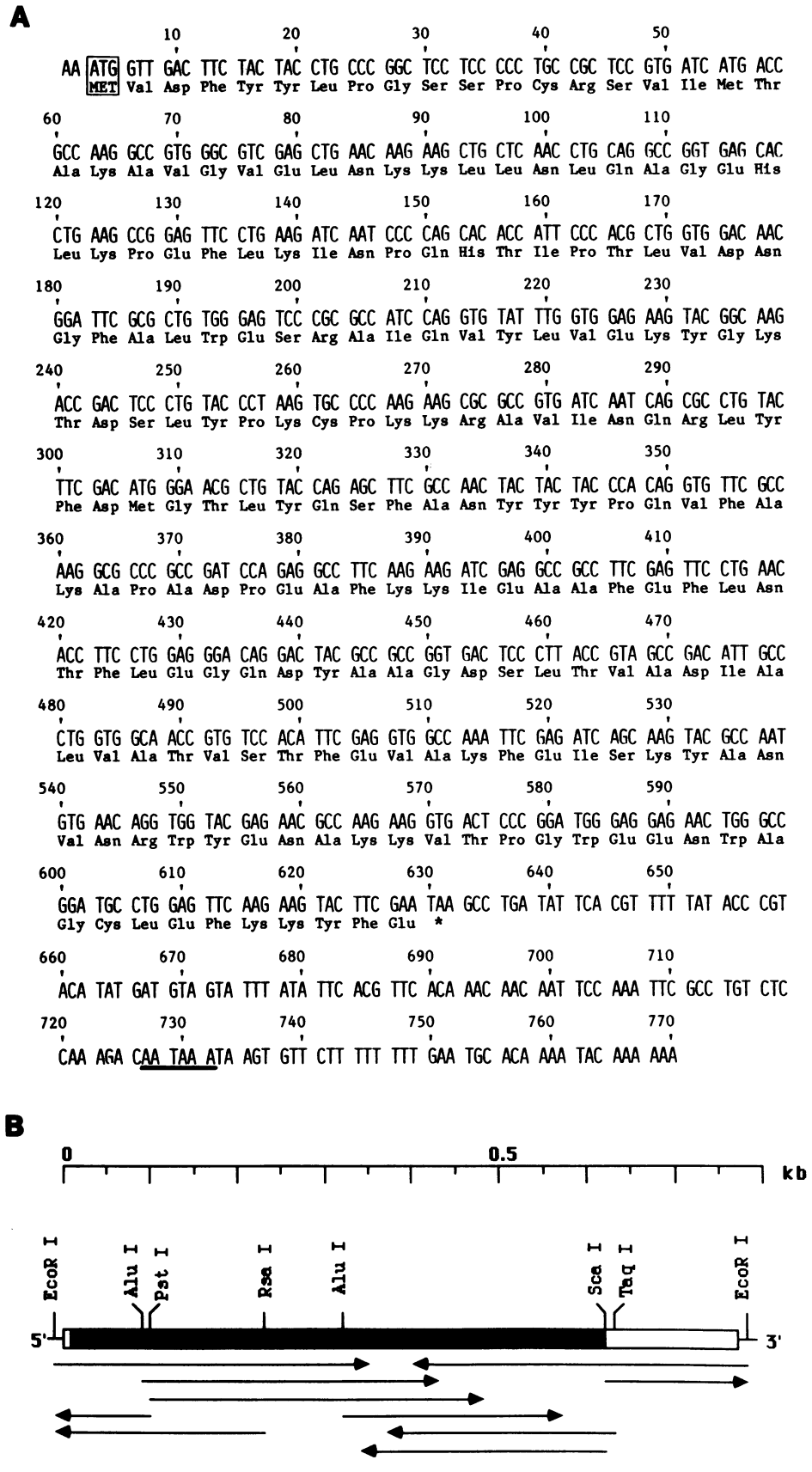


FIG. 1. Characterization of cDNA clone pGTDm1. (*A*) Nucleotide and deduced amino acid sequence. The ATG initiation codon is boxed, and the termination codon is indicated by an asterisk. The putative poly(A) addition signal is underlined. Arg-14, Pro-54, Asp-58, Ala-68, Ile-69, Gly-144, Asp-151, and Leu-160 are the eight residues identical to those conserved in mammalian GSTs (5, 6). (*B*) Partial restriction map and sequencing strategy. Only restriction sites pertinent to subcloning into M13mp18/19 phage are shown. Arrows and lines represent the direction and extent of the sequence determinations. The protein coding region is shown by the solid black bar.

(21, 23). Specificity of the two antisera was tested by Western (immunological) blotting experiments (24) using fast protein liquid chromatography-purified *Drosophila* GSTs.

GSTs were also purified from *Drosophila* embryos. Embryo cytoplasm (≈460 ml) was concentrated by $(NH_4)_2SO_4$ precipitation (70% saturation) and dialyzed against 25 mM Tris·HCl (pH 8.0) before affinity chromatography. The remaining procedures were similar to those in the purification of GSTs from $Kc_0$ cells. Peak I fractions, which were eluted from the Mono Q column just before the beginning of the gradient, were subjected to N-terminal sequencing for 20 cycles with an Applied Biosystems model 477A microsequenator.

**Screening of *Drosophila* λgt11 cDNA Library with Anti-GST Antisera.** The *Drosophila* cDNA library was screened as described (25, 26) with $^{125}I$-labeled protein A to label antibody-positive plaques on nitrocellulose filters. The antibody against the peak III GST did not produce any useful clones. Two positive signals were detected among 100,000 plaques with antibody against peak I GST. The two positive clones were designated λGTDm1 and λGTDm1-1; their subclones in pUC19 were designated as pGTDm1 and pGTDm1-1, respectively.

**Screening of λEMBL4 Genomic DNA Library.** The cDNA insert in pGTDm1 used as a hybridization probe was purified twice from a polyacrylamide gel (20). Three positive clones were then isolated from screening a total of 50,000 plaques. DNAs from two of these clones were identical in restriction patterns, and they were designated λGTDm101. The third clone was designated λGTDm102.

**Methods for Nucleic Acid Analysis.** Plasmid and phage DNA isolation procedures were those described (20), except that phage DNAs were further purified by KI gradient centrifugation (27). *Drosophila* genomic DNA was purified from Oregon R embryos by a published procedure (20). DNA fragments were subcloned into plasmid (pKK223-3 or pUC19) and M13 phage (mp 18, mp 19) vectors as described (20). Southern hybridization (genomic or cloned DNA) was accomplished on nitrocellulose filters with the full-length cDNA insert or a DNA fragment containing just the 3′-noncoding region (28). DNA sequence was analyzed from M13 subclones by the dideoxynucleotide chain-termination method with [α-$^{35}S$]dATP (29–31).

**Heterospecific Expression of a *Drosophila* GST cDNA in *E. coli*.** The cDNA insert of pGTDm1 was cloned into the *Eco*RI site of pKK223-3 in two orientations in *E. coli* JM105. The plasmid construct of correct orientation was designated as pGTDm1-KK, which expressed high levels of GST activity upon induction of the *tac* promoter by isopropyl β-D-thiogalactoside. The insert in the opposite orientation was designated as pGTDm1-KK′. To purify GST expressed from pGTDm1-KK, the procedures previously reported for the purification of *E. coli*-expressed $H_a$ subunit cDNAs and $Y_{b2}$ cDNA were followed up to the affinity chromatography step (32, 33). The proteins eluted from the *S*-hexyl-GSH affinity column were dialyzed thoroughly against 25 mM Tris·HCl (pH 8.0) and then separated by ion-exchange chromatography on a fast protein liquid chromatography Mono Q column. Enzyme activity was eluted in a single peak with a linear NaCl gradient (0–1 M) in 25 mM Tris·HCl (pH 8.0). The purified GST was dialyzed against 500 vol of 10 mM Tris·HCl (pH 8.0) and stored in aliquots at −20°C. Protein concentration was determined by the bincinchonic acid (BCA) protein assay (Pierce) with bovine serum albumin as standard.

## RESULTS

**Characterization of pGTDm1 and pGTDm1-1 Clones.** DNAs were isolated from λGTDm1 and λGTDm1-1 and analyzed after *Eco*RI digestion. Each clone contained an insert DNA of ≈750 base pairs (bp) in size. These clones were subcloned into M13mp18 and -mp19 vectors for sequence analysis either directly or after further digestion according to the strategy in Fig. 1. The two cDNAs were identical in sequence except that the pGTDm1-1 insert (data not shown) did not have six adenylate residues at the 3′ end (Fig. 1). The open reading frame of 209 amino acids had a calculated $M_r$ of 23,839. The deduced N-terminal sequence Met-Val-Asp-Phe-Tyr-Tyr-Leu-Pro-Gly-Ser-Ser-Pro-Cys-Arg-Ser-Val. . . was consistent with the protein sequence determined from peak I GST purified from $Kc_0$ cells or *Drosophila* embryos (Xaa-Xaa-Asp-Phe-(Ser)-Tyr-Leu-Pro-Gly-Ser-Xaa-Pro-(Trp)-Xaa-Ser-Xaa-Ile-Met-Thr-Ala-Xaa). The sequence match at the N-terminal region, the size of the open reading frame, and its positive identification by antibody against purified *Drosophila* GST make pGTDm1 a probable candidate for a *Drosophila* GST cDNA clone.

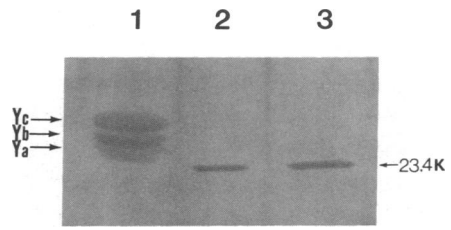


FIG. 2. Electrophoretic analysis of *Drosophila* GST 1-1. Lanes: 1, rat liver GSTs (≈2 μg) (Sigma); 2, *E. coli*-expressed *Drosophila* GST 1-1 (≈1 μg); 3, GST purified from *Drosophila* embryos (≈1 μg). Both *E. coli*-expressed and embryonic GSTs were purified by *S*-hexyl-GSH affinity chromatography and Mono Q column chromatography on fast protein liquid chromatography. The gel was silver-stained by using a Bio-Rad silver stain kit. The GST subunit 1 from *Drosophila* had an electrophoretic mobility at $M_r$ = 23,400. Rat liver GST $Y_a$, $Y_b$, and $Y_c$ subunits have $M_r$ values = 25,600, 27,000, and 28,000, respectively (1, 2).

**Heterospecific Expression of pGTDm1 cDNA in *E. coli*.** The two constructs in *E. coli* JM105 in opposing orientations, pGTDm1-KK and pGTDm1-KK′, were induced separately by isopropyl β-D-thiogalactoside (32, 33). The GST activity against CDNB could be detected only in sonicated extracts of cultures of pGTDm1-KK at a level of 0.09 unit per mg of protein. After purification of this activity from large-scale induced cultures (4-liter cultures) by affinity chromatography and fast protein liquid chromatography Mono Q column, we recovered the activity (17% yield) in a fraction containing a single band on SDS/PAGE. Its mobility (23.4 kDa) was identical to that of the purified embryo GST (Fig. 2). Thus, we have proven that the pGTDm1 cDNA insert encodes a *Drosophila* GST subunit, designated as subunit 1. When the fast protein liquid chromatography-purified GST (both *E. coli*-expressed and $Kc_0$ cell peak I enzyme) was chromatographed on a Sephadex G-100 column, CDNB-conjugation activities appeared at elution volumes of $M_r$ 48,500 relative to marker proteins (aprotinin, cytochrome *c*, carbonic anhydrase, bovine serum albumin). Therefore, GST subunit 1

Table 1. Substrate specificities of *E. coli*-expressed *Drosophila* GST 1-1

| Substrate | Specific activity, μmol/min per mg |
|---|---|
| 1-Chloro-2,4-dinitrobenzene | 24.08 ± 2.23* |
| Ethacrynic acid | 0.24 |
| 1,2-Dichloro-4-nitrobenzene | <0.01 |
| *p*-Nitrobenzyl chloride | <0.01 |
| 1,2-Epoxy-3-(*p*-nitrophenoxy)propane | <0.01 |
| Cumene hydroperoxide | <0.01 |

*Average of three determinations.

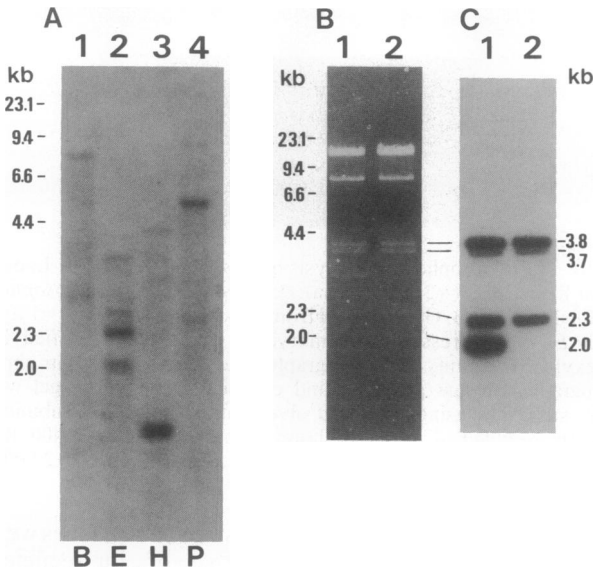


FIG. 3. Southern analysis of *Drosophila* genomic DNA and two *Drosophila* genomic clones, λGTDm101 and λGTDm102. (*A*) *Drosophila* genomic DNA (≈2.6 μg each) was digested overnight with 200 units each of *Bam*HI (B) (lane 1), *Eco*RI (E) (lane 2), *Hin*dIII (H) (lane 3), and *Pst* I (P) (lane 4) and separated by gel (0.8% agarose) electrophoresis. After transfer to a nitrocellulose membrane, the DNA was hybridized with the $^{32}$P-labeled *Eco*RI insert of pGTDm1. (*B*) *Eco*RI restriction pattern of two genomic clones, λGTDm101 (≈1 μg) (lane 1) and λGTDm102 (≈1 μg) (lane 2). (*C*) Autoradiogram of Southern analysis of λGTDm101 (lane 1) and λGTDm102 (lane 2) revealed by the pGTDm1 cDNA probe. Numbers at left of *A* and *B* indicate size markers (λ-*Hin*dIII) in kb. Numbers at right of *C* indicate sizes of positive DNA fragments. Lines between *B* and *C* indicate corresponding *Eco*RI fragments. Hybridizations were done in 5× SSC (1× SSC is 0.15 M sodium chloride/0.015 M sodium citrate, pH 7)/5× Denhardt's solution (1× Denhardt's solution is 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin)/50 mM sodium pyrophosphate/0.1% NaDodSO$_4$/denatured salmon testis DNA at 100 μg/ml, 40% formamide (vol/vol) at 40°C for 40 hr. The cDNA probe had a specific activity of 2 × 10$^8$ dpm/μg of DNA. The filters were washed at room temperature three times in 2× SSC/0.1% NaDodSO$_4$ for 90 min and then washed in 0.1× SSC/0.1% NaDodSO$_4$ at 55°C for 2 hr.

most probably exists as active dimers, designated as *Drosophila* GST 1-1. The substrate specificity of GST 1-1 is described in Table 1. CDNB was the most active substrate, but this protein (at 19.6–392 nM enzyme concentration) did not have any detectable GSH peroxidase activity against cumene hydroperoxide relative to a similar concentration of human GST 1-1 (33).

**Genomic Complexity Detected by pGTDm1 cDNA.** Genomic Southern blot analysis with pGTDm1 cDNA insert as a probe revealed multiple bands upon various restriction endonuclease digestions (Fig. 3). The 2.0-kilobase (kb) fragment in λGTDm101 was also strongly positive toward the 3′-noncoding probe of pGTDm1 (data not shown). In the *Eco*RI-digested sample, the 2.8-kb band has not been cloned in the two genomic clones λGTDm101 and λGTDm102. Other restriction digestions revealed multiple bands >4 kb in size (Fig. 3*A*).

## DISCUSSION

We have isolated and characterized a cDNA encoding the *Drosophila* GST subunit 1 of $M_r$ = 23,839, which contains 209 amino acids. The CDNB conjugation activity (24 μmol/min per mg of protein) of *Drosophila* GST 1-1 is comparable to that for mammalian GSTs and is approximately twice the reactivity of heterodimeric GST of *Drosophila* larvae and adults, as characterized by Cochrane *et al.* (34). The *Drosophila* GST did not have as broad a substrate specificity pattern as most mammalian GSTs relative to the substrates tested in Table 1. The antibody against peak I GST does not cross-react with other *Drosophila* GSTs. It is clear that *Drosophila* GST is an isozyme family composed of at least three classes of subunits of 23.4 kDa, 28.5 kDa, and 35 kDa (ref. 34 and this study).

The size of *Drosophila* GST subunit 1 (209 amino acids) makes it most analogous to the rat and human GST P or π subunits (210 amino acids). These proteins, however, do not share much amino acid sequence homology. Indeed, *Drosophila* GST subunit 1 does not share any extended amino acid sequence homology to the mammalian or parasitic GST sequences in the literature, except for 8 (Arg-14, Pro-54, Asp-58, Ala-68, Ile-69, Gly-144, Asp-151, and Leu-160 in Fig. 1) of the 12 amino acid residues conserved in all GST sequences known to date (2–6). Interestingly, subunit 1 does have significant homology to the maize GST III sequence, as shown in Fig. 4 (35). In a region of 44 amino acids, 22 amino acids were identical between the two GSTs at homologous positions (50% identity). In addition, there are 7 amino acids with similar side-chain groups and 6 more with similar polarity for a maximum of 79% sequence homology. This conservation is striking considering the evolutionary difference between maize and *Drosophila melanogaster*. It is therefore tempting to propose functional significance for this stretch of 44 amino acids.

The major difference in chromatographic behavior between maize GST III and *Drosophila* GST 1-1 is that maize GST III can bind to a GSH–agarose affinity column but not to a *S*-hexyl-GSH–agarose affinity column (ref. 36; K. P. Timmerman and C-P.D.T., unpublished results). Neither enzyme has GSH peroxidase activity. Therefore, these 44 conserved residues may be important in binding xenobiotic substrates. On the other hand, the difference in chromatographic behavior may reflect a subtle difference between the two GSTs in binding the common substrate GSH. The evolutionary significance of this sequence conservation remains to be elucidated.

The pGTDm1 cDNA has apparently detected a multigene family on genomic Southern blots. We have also isolated two different, but potentially overlapping, genomic clones (Fig. 3). Partial sequence analysis of the genomic clone λGTDm102 has revealed two sequences homologous to the N-terminal region of pGTDm1 cDNA sequence (C-P.D.T., N. M. Simkovich, Y-P.S.T., and L. Tu, unpublished results). The lack of immunological cross-reactivity between antibody against peak I GST and other potential GST subunits suggests that the 28.5-kDa band, if indeed a GST subunit, may not be closely related to GST subunit 1. Therefore, *Drosophila* GSTs may be encoded by different gene families, which likely

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                    40          50          60          70
GSTIII --- LTTGAHKQPDFLALNPFGQIPALVDGDEVLFESRAINRYIASKY ---
pGTDm1 --- LQAGEHLKPEFLKINPQHTIPTLVDNGFALWESRAIQVYLVEKY ---
                    40          50          60          70
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FIG. 4. Partial amino acid sequence comparison between maize GST III and *Drosophila* GST 1–1. The two sequences (in one-letter code) are aligned for maximal homology. Identical amino acids are represented by boldfaced letters. Those amino acid residues that are in the same group of side-chain type (i.e., small polar, S, G, D, and N; large polar, E, Q, K, and R; intermediate polarity, Y, H, and W; large nonpolar, F, M, L, I, and V; and small nonpolar, C, P, A, and T) are labeled with asterisks.

constitute a gene superfamily, as is the case for mammalian systems. GSH *S*-transferase(s) has been suggested to impart pesticide resistance against dichlorodiphenyltrichloroethane (DDT) and organophosphorus compounds in houseflies (*Musca domestica* L.). Molecular analysis of *Drosophila* GSTs should provide further understanding of a family of important xenobiotic metabolizing enzymes and their relationship to pesticide resistance.

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