Cloning, expression and biochemical characterization of one Epsilon-class (GST-3) and ten Delta-class (GST-1) glutathione S-transferases from *Drosophila melanogaster*, and identification of additional nine members of the Epsilon class

Rafał SAWICKI*, Sharda P. SINGH*, Ashis K. MONDAL*, Helen BENEŠ† and Piotr ZIMNIAK*‡§[1]

*Department of Medicine, University of Arkansas for Medical Sciences, Little Rock, AR 72205, U.S.A., †Department of Anatomy and Neurobiology, University of Arkansas for Medical Sciences, Little Rock, AR 72205, U.S.A., ‡Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Little Rock, AR 72205, U.S.A., and §Central Arkansas Veterans Healthcare System, Medical Research (151/LR), 4300 West 7th Street, Little Rock, AR 72205, U.S.A.

From the fruitfly, *Drosophila melanogaster*, ten members of the cluster of Delta-class glutathione S-transferases (GSTs; formerly denoted as Class I GSTs) and one member of the Epsilon-class cluster (formerly GST-3) have been cloned, expressed in *Escherichia coli*, and their catalytic properties have been determined. In addition, nine more members of the Epsilon cluster have been identified through bioinformatic analysis but not further characterized. Of the 11 expressed enzymes, seven accepted the lipid peroxidation product 4-hydroxynonenal as substrate, and nine were active in glutathione conjugation of 1-chloro-2,4-dinitrobenzene. Since the enzymically active proteins included the gene products of *DmGSTD3* and *DmGSTD7* which were previously deemed to be pseudogenes, we investigated them further and determined that both genes are transcribed in *Drosophila*. Thus our present results indicate that *DmGSTD3* and *DmGSTD7* are probably functional genes. The existence and multiplicity of insect GSTs capable of conjugating 4-hydroxynonenal, in some cases with catalytic efficiencies approaching those of mammalian GSTs highly specialized for this function, indicates that metabolism of products of lipid peroxidation is a highly conserved biochemical pathway with probable detoxification as well as regulatory functions.

Key words: detoxification, Diptera, electrophile, glutathione conjugation, lipid peroxidation.

INTRODUCTION

Partially reduced forms of oxygen, including the superoxide radical anion, hydrogen peroxide and the hydroxyl radical, are collectively known as reactive oxygen species (ROS). The formation of ROS is an inevitable consequence of aerobic metabolism. The majority of ROS is generated by mitochondria, although other enzyme systems also contribute; transition metal ions catalyse interconversions of individual ROS, especially the formation of the highly reactive hydroxyl radical. The latter can react, among other targets, with polyunsaturated fatty acids and thus initiate the lipid peroxidation chain reaction [1], which produces multiple molecules of lipid hydroperoxides per initiating event. By either spontaneous or enzyme-catalysed reactions [2], lipid hydroperoxides give rise to α,β-unsaturated aldehydes such as 4-hydroxynonenal (4-HNE) [3]. These aldehydes are highly reactive but considerably more selective than the parent ROS. Specific modification of proteins by 4-HNE and similar aldehydes often affects protein function. This is probably the basis of the signalling role of 4-HNE at physiological concentrations [4,5] and the toxicity elicited by this compound at high concentrations [3].

The generation of 4-HNE, primarily from arachidonic acid [3], and the formation of 4-HNE–protein adducts [6] are well-established phenomena in mammalian tissues. Although the fruitfly, *Drosophila melanogaster*, lacks arachidonic acid [7,8], 4-HNE can be formed from any ω-6 polyunsaturated fatty acid [9]. In addition to arachidonic acid, this also includes linoleic acid, which is present in large amounts in larval and adult stages of *Drosophila* [8,10]. Although the ability of *Drosophila* to desaturate fatty acids is limited, dietary polyunsaturated fatty acids are readily incorporated into phospholipids [8]. Therefore 4-HNE and/or similar 4-hydroxyalkenals are expected to be generated in *Drosophila*. We have previously confirmed the presence of 4-HNE–protein adducts in *Drosophila* by immunoblotting [11], and Yan and Sohal [12] have demonstrated the formation of such adducts in the housefly.

In mammals, the major route of 4-HNE metabolism is via glutathione conjugation [13], catalysed by a specialized subclass of Alpha-class glutathione S-transferases (GSTs) [14,15], although a reductive pathway may be significant in some tissues. Because of the apparently universal and obligatory formation of 4-hydroxyalkenals in aerobes, we examined which of the *Drosophila* GSTs has the ability to conjugate 4-HNE. In a previous study [11], we investigated DmGSTS1-1 (see the Materials and methods section for an explanation of the nomenclature), which was previously assumed to be a structural muscle protein or perhaps a stretch sensor devoid of enzymic properties [16]. However, DmGSTS1-1 has moderate glutathione-conjugating activity for 4-HNE, and due to its high level of expression, it accounts for most of this activity in adult *Drosophila* [11]. In the course of that study, we also found a less abundant GST (or mixture of GSTs), probably belonging to the Delta class, which had a higher specific activity for 4-HNE conjugation than DmGSTS1-1 [11]. We have now characterized a number of *Drosophila* GSTs, both of the Delta and the novel Epsilon class, and have shown that some, but not all, of them participate in 4-HNE metabolism.

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; EST, expressed sequence tag; GST, glutathione S-transferase; 4-HNE, 4-hydroxynonenal; ORF, open reading frame; poly(A)$^+$, polyadenylated; ROS, reactive oxygen species; RT, reverse transcriptase.

[1] To whom correspondence should be addressed (e-mail zimniakpiotr@uams.edu).

MATERIALS AND METHODS

Nomenclature of *Drosophila* GSTs

In the present study, we adhere to a recently proposed unified nomenclature that includes insect as well as mammalian GSTs [17]. Accordingly, we use the term 'Delta class' for *Drosophila* GST-1 and its homologues [18], refer to the protein originally described as GST-2 [19] as DmGSTS1-1 and designate *Drosophila* GST-3 [20] as DmGSTE1-1, a prototypical member of the Epsilon class of GSTs (Professor Philip G. Board, personal communication). This nomenclature has been recently adopted by Flybase (www.flybase.org).

Cloning of *Drosophila* Delta- and Epsilon-class GSTs

PCR primers specific for the ten Delta-class genes and for *DmGSTE1* were used to amplify the sequences from *Drosophila* genomic DNA. The primers are listed in Table 1. All upstream primers were designed to span the translation initiation codon and to introduce an *Nde*I restriction site to facilitate subsequent subcloning. For the same reason, the downstream primers introduced a unique restriction site, as specified in Table 1. One hundred male adult flies were homogenized, and total genomic DNA was isolated using the DNeasy Tissue Kit (Qiagen). Each PCR reaction contained, in a total volume of 25 μl, 50 ng of *Drosophila* genomic DNA as the template and 25 ng each of the respective primers. Reactions were initiated by the addition of *Taq* polymerase using the hot start technique and were continued for 32 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 48 °C for *DmGSTD6*, 50 °C for *DmGSTD10*, ramped from 52.2 to 49 °C over 32 cycles (touch-down PCR) for *DmGSTD7* and from 56.4 to 50 °C for *DmGSTD1–5*, *DmGSTD8*, *DmGSTD9* and *DmGSTE1*, followed by extension for 1 min at 72 °C/cycle. The specificity of the amplification was verified by sequencing of all PCR products.

Bacterial expression and purification of *Drosophila* GSTs

The PCR-amplified fragments encoding the *Drosophila* GSTs were digested with *Nde*I and a restriction enzyme appropriate for the antisense primer (see Table 1) and subcloned between the corresponding sites of pET-30a(+) (Novagen). *Escherichia coli* BL21(DE3)pLysS cells were used for expression of all proteins except for DmGSTD3-3, DmGSTD7-7 and DmGSTE1-1, which were expressed in the BL21 Star(DE3)pLysS strain, which carries a truncated RNAse E, leading to increased mRNA stability. Expression was induced with 1 mM isopropyl β-D-thiogalactoside at absorbance A_{600} 0.4–0.6 and it was continued for 8 h at 37 °C. The bacteria were collected by centrifugation and the pellets were frozen at −20 °C until use. Bacterial cells were lysed and the GSTs, except for DmGSTD3-3 and DmGSTE1-1, were purified by glutathione affinity chromatography using a 1 ml prepacked glutathione Sepharose 4 Fast Flow column (Amersham) according to the manufacturer's instructions. DmGSTD3-3 and DmGSTE1-1 did not bind to the glutathione column. The bacterial lysate (from 100 ml culture) containing DmGSTD3-3 was fractionated by ammonium sulphate precipitation. The 25–50 % fraction was dialysed against 10 mM potassium phosphate (pH 7.4) and further fractionated on a Sephadex G-200 column (1 cm × 50 cm). Fractions containing the GST were pooled and loaded on a hydroxyapatite column (1 cm × 8.5 cm). The column was eluted with a gradient of 10–400 mM potassium phosphate (pH 6.5). Fractions containing DmGSTD3-3 eluted at 300 mM KCl and were identified by SDS/PAGE. DmGSTE1-1 was purified by ion-exchange chromatography. Frozen bacterial cells from 500 ml of culture were lysed by sonication in 20 mM Tris/HCl (pH 7.5), 50 mM KCl, centrifuged for 30 min at 30000 ***g*** and the supernatant was passed over a DEAE column (Macro-Prep DEAE Support; Bio-Rad Laboratories), which did not retain DmGSTE1-1. The flow-through was then applied to a Macro-Prep High Q Support column (0.5 cm × 3 cm; Bio-Rad Laboratories), which was washed with 20 mM Tris/HCl (pH 7.5), 50 mM KCl and eluted with a step gradient of KCl in 20 mM Tris/HCl (pH 7.5). The 4-HNE-conjugating activity eluted at 0.1 M KCl. The fraction was dialysed against 50 mM Tris/HCl (pH 7.5) and 1.3 mM 2-mercaptoethanol.

Determination of enzyme activity

GST activities were measured spectrophotometrically in a microtitre plate reader (SpectraMax Plus; Molecular Devices, Sunnyvale, CA, U.S.A.). Conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) was measured at 25 °C. Enzyme activity with 4-HNE was calculated from the rate of consumption of this substrate at 30 °C [13]. To determine kinetic constants of 4-HNE conjugation, the GST activities of the enzymes were measured by varying the concentrations of 4-HNE at fixed concentrations of reduced glutathione. The results were analysed by least-squares non-linear fitting of a Michaelis–Menten hyperbola or the Hill

Table 1 Primers used to amplify *D. melanogaster* GST genes

Forward primers were designed to introduce an *Nde*I restriction site (underline) which includes the initiation codon ATG. Reverse primers spanned all or part of the stop codon (double underline) and were designed to introduce a unique restriction site (lower-case boldface; *Eco*RI for *DmGSTD1*, *DmGSTD3*, *DmGSTD5*, *DmGSTD7–9* and *DmGSTE1*; *Bam*HI for *DmGSTD2* and *DmGSTD10*; *Kpn*I for *DmGSTD4*; *Sac*I for *DmGSTD6*).

Clone	Forward primer	Reverse primer
GSTD1	5′-CTTCTACAGTCATATGGTTGACTTCTACTAC	5′-AAAACGT**gaattc**CAGGCTTATTC
GSTD2	5′-GCAATATCCCCCATATGGACTTTTACTAC	5′-ATAGTTG**ggatcc**CGATCACTTAGC
GSTD3	5′-CGCTCCGTTCATATGGTGGGCAAG	5′-TC**gaattc**AATGAATTATTTAGCAGCATTCTG
GSTD4	5′-CGCCCCAGCCATATGGATTTCTACT	5′-GTTT**ggtacc**ACTGTAGTTACTTTAATGAG
GSTD5	5′-ACACTCAAATAACCCATATGGATTTCTATT	5′-TT**gaattc**TTATTGCTTCGCCGCC
GSTD6	5′-GCCCTTCTGCATATGGATCTCTATA	5′-CG**gagctc**TCTATAATATTTAATAACTTTTC
GSTD7	5′-GTGAGTTCTACATATGACAAACATATTC	5′-GT**gaattc**AAAACTATTCTACGGTG
GSTD8	5′-GGACCTTTCCATATGGACTTTTACTACC	5′-CATTTACTGCAG**gaattc**TCATCAAATC
GSTD9	5′-CAGCGAGTACATATGTTGGACTTCTAC	5′-GAT**gaattc**TTACAGACCCGTCATTG
GSTD10	5′-GAGATAGCGTCATATGGATTTATACTATAG	5′-TCG**ggatcc**TATATTCAGTTATCGAAG
GSTE1	5′-ACAACACATCATATGTCGAGCTCTGG	5′-TT**gaattc**ACTTGTCGCCCAGC

equation. Glutathione peroxidase activity with cumene hydroperoxide as substrate was determined as described in [21].

Reverse transcriptase (RT)–PCR

The presence of *DmGSTD3* transcripts was determined in total RNA isolated from wandering third-instar *Drosophila* larvae using the RNeasy Mini Kit (Qiagen). The RNA was treated with RNase-free DNase I (Roche Applied Science, Indianapolis, IN, U.S.A.; 1 unit of DNase I/μg of RNA in 25 mM Tris/HCl, 5 mM $MgCl_2$, 0.1 mM EDTA, at 37 °C for 30 min, followed by heat inactivation of DNase at 75 °C for 15 min). RNA (200 ng) was used for each RT–PCR reaction (Titan One Tube RT–PCR System; Roche Applied Science). The sense primer was 5′-CGCTCCGTTCTGATGGTGGGCAAG, and the antisense primer was 5′-TCGTTATAAATGAATTATTTAGCAGCATTCTG. The reverse transcription step was performed at 50 °C for 30 min, followed by inactivation of the RT at 94 °C for 2 min. The conditions for the subsequent PCR step were as follows: 10 cycles of 94 °C, 10 s; 58 °C, 30 s; 68 °C, 45 s and then 25 cycles of 94 °C, 10 s; 58 °C, 30 s; 68 °C, initially 45 s with cycle elongation by 5 s for each cycle; 1 cycle at 94 °C, 10 s; 58 °C, 30 s; 68 °C for 7 min. In control reactions, the mixture of RT and thermostable DNA polymerase supplied in the Titan One Tube RT–PCR System was substituted with *Taq* polymerase (Biolase; Bioline USA, Canton, MA, U.S.A.). For detection of the *DmGSTD7* transcript, polyadenylated [poly(A)$^+$] RNA was isolated from 25 μg of total larval RNA using the Oligotex Direct mRNA Mini Kit (Qiagen), and one-tenth of each of the recovered poly(A)$^+$ RNA was used for every RT–PCR or control reaction under conditions described above for *DmGSTD3*, with the sense primer 5′-GTGAGTTCTAACAATGACAAACATATTC and the antisense primer 5′-GTCAAACGAAAACCATTCTACGGTG.

RESULTS

Identification of gene clusters encoding Delta- and Epsilon-class GSTs in *Drosophila* genome

The genomic sequence of *D. melanogaster* was examined, using the BLAST software, for the presence of open reading frames (ORFs) homologous with previously described Delta [18] and Epsilon [20] GSTs. The Delta cluster at chromosomal position 87B11–87B13 spanned approx. 18 kb and contained the eight genes previously designated as *D1*, *D21*, *D22*, *D23*, *D24*, *D25*, *D26* and *D27* [18,22,23]. In keeping with a recent nomenclature proposal [17], we refer to these genes as *DmGSTD1* to *DmGSTD8* respectively. In addition, the Delta cluster contained two recently reported [17] but not further characterized genes *DmGSTD9* and *DmGSTD10*. Three genes (*DmGSTD1*, *DmGSTD9* and *DmGSTD10*) were located on the same DNA strand, whereas the remaining Delta-class genes were on the opposite strand. The orientation and relative positions of the Delta ORFs are depicted in Figure 1(A).

A novel Theta class-related GST gene *Gst-3* has been described recently [20]. According to a suggestion from Professor Philip G. Board (personal communication), we refer to this gene as *DmGSTE1*, a member of the Epsilon class of GSTs. By BLAST searches of the *Drosophila* genomic sequence, we identified nine additional genes (*DmGSTE2–DmGSTE10*) with high homology to *DmGSTE1*. The Epsilon genes form a tight cluster spanning approx. 13 kb at chromosomal position 55C9. Within that cluster (Figure 1B), one gene (*DmGSTE10*) has an antiparallel orientation relative to the remaining genes.

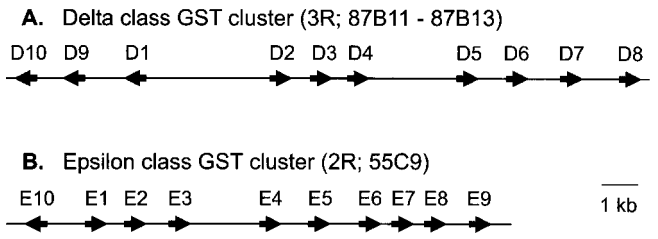

Figure 1 Clusters of Delta- and Epsilon-class glutathione transferase genes in *D. melanogaster*

The map is drawn to scale and is based on the *Drosophila* genomic sequence. The arrows denote the positions and 5′ → 3′ direction of the coding sequences of the genes; none of the Delta and Epsilon genes contains introns within the ORF. The chromosomal positions of the clusters are indicated.

Table 2 Context of the initiation codon of *D. melanogaster* Delta and Epsilon GST genes

Gene	−4	−3	−2	−1	Start	+4
DmGSTD1	T	A	A	A	ATG	G
DmGSTD2	C	A	A	C	ATG	G
DmGSTD3	T	C	T	G	ATG	G
DmGSTD4	C	A	A	C	ATG	G
DmGSTD5	C	G	A	A	ATG	G
DmGSTD6	G	A	C	G	ATG	G
DmGSTD7	A	A	C	A	ATG	A
DmGSTD8	C	A	T	C	ATG	G
DmGSTD9	A	A	T	C	ATG	T
DmGSTD10	T	A	A	G	ATG	G
DmGSTE1	T	A	T	C	ATG	T
DmGSTE2	C	A	T	C	ATG	T
DmGSTE3	A	G	A	C	ATG	G
DmGSTE4	G	A	G	A	ATG	G
DmGSTE5	A	A	A	C	ATG	G
DmGSTE6	C	A	A	G	ATG	G
DmGSTE7	C	A	A	G	ATG	C
DmGSTE8	C	G	G	C	ATG	T
DmGSTE9	A	G	C	G	ATG	G
DmGSTE10	C	A	C	A	ATG	G
Drosophila Consensus [31]	3% G 32% A 8% T 57% C	13% G 82% A 1% T 3% C	9% G 56% A 10% T 25% C	18% G 38% A 8% T 36% C	ATG ATG ATG ATG	26% G 37% A 22% T 15% C

Sequence similarities within the Delta and Epsilon clusters of GSTs

It has been previously reported that the coding sequences of the Delta-class genes [18,24,25] as well as of the *DmGSTE1* gene [20] contain no introns. We have now extended this observation to *DmGSTD9*, *DmGSTD10* and the nine novel *DmGSTE* genes. All of the Delta and Epsilon genes can be conceptually translated. Their initiation codons are in a context appropriate for *Drosophila* [26] (Table 2) and they are followed by an ORF of a size expected for glutathione transferases (see the Discussion section for additional details). The alignment of the resulting protein sequences is shown in Figure 2. The Delta-class sequences formed a tight cluster, with the exception of DmGSTD7-7, which carried a 47-amino-acid N-terminal extension and was truncated at the C-terminus by approx. 50 amino acids, and of DmGSTD3-3, which was shorter by approx. 15 residues at the N-terminus, as compared with the remaining Delta-class proteins. The

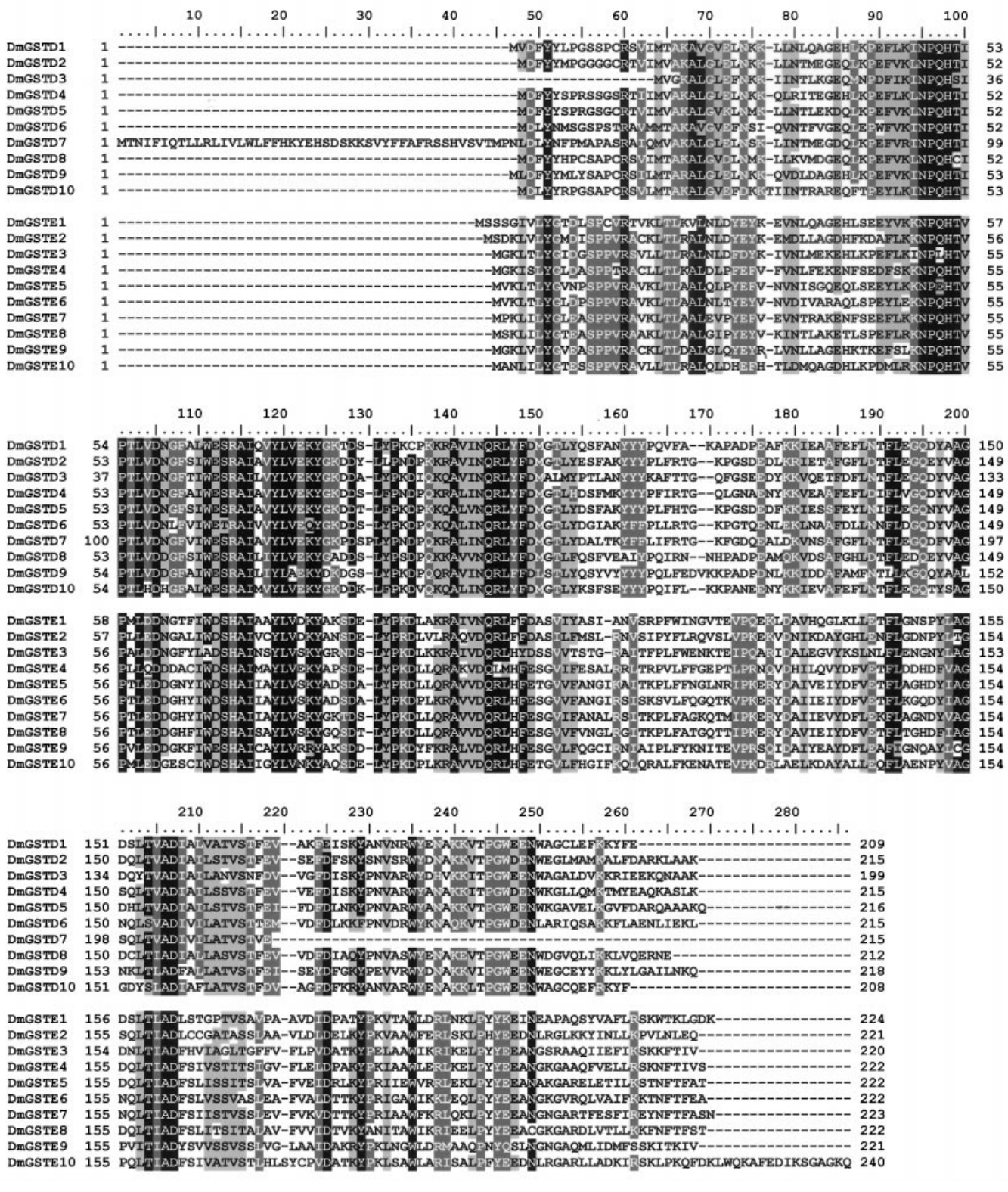

Figure 2 Alignment of the predicted protein sequences of *D. melanogaster* Delta- and Epsilon-class glutathione transferases

Residues identical in at least 18 of the 20 sequences are shaded in black, and residues similar (as defined by the BLOSUM62 matrix) in at least 18 of the 20 sequences are shown as black letters shaded in light grey. Residues that are, by the above criteria, neither identical nor similar in the entire set of 20 sequences but are similar (or identical) in at least 9 out of 10 sequences in one of the two GST clusters are shown as white letters shaded in dark grey. Thus amino acid positions denoted as white letters shaded in dark grey are conserved within the Delta or the Epsilon cluster, but not between the two clusters.

	D1	D2	D3	D4	D5	D6	D7	D8	D9
D2	67/79								
D3	56/68	60/75							
D4	65/78	75/86	60/73						
D5	65/78	79/89	59/77	72/89					
D6	56/71	60/75	53/68	59/74	59/74				
D7	40/49	46/53	38/45	44/51	42/51	45/54			
D8	65/79	64/79	50/67	61/79	62/76	53/72	38/49		
D9	63/79	59/74	46/61	56/74	53/71	51/62	35/44	58/74	
D10	71/85	60/76	55/69	59/72	61/76	55/71	36/47	59/73	56/74

Figure 3 Percentage of identity/similarity respectively for pairwise alignments of *Drosophila* Delta-class protein sequences

The PAM250 matrix was used to calculate similarity scores.

similarities and identities of all pairs of the Delta-class sequences are presented in Figure 3. Similarly, all Epsilon-class proteins could be brought into close alignment, with DmGSTE10-10 being the most divergent sequence because of a C-terminal extension of approx. 15 amino acids (Figure 2). The similarity between the Delta and Epsilon clusters was less pronounced. For example, the similarity and identity between the DmGSTD1-1 and DmGSTE1-1 proteins was only 36 and 49% respectively. The relationship between the two GST classes can be visualized as a hypothetical phylogenetic tree, which shows that the Delta and Epsilon nucleotide sequences form distinct clusters, with both clusters only distantly related to the remaining characterized *Drosophila* GST gene, *DmGSTS1* (Figure 4). The sequence homology within each of the two clusters, together with the physical proximity of all Delta genes on chromosome 3 and all Epsilon genes on chromosome 2 (Figure 1), suggests that each cluster was probably formed by repeated duplication events of a Delta and an Epsilon ancestral gene respectively. Interestingly, in the Epsilon cluster, similarities in sequence and thus probable evolutionary relationships of individual genes are reflected in their physical proximity. For example, the *DmGSTE5* and *DmGSTE6* genes, which have probably diverged most recently on an evolutionary time scale (Figure 4), are located next to each other on the genomic DNA (Figure 1). This generalization holds for all members of the Epsilon group of genes, but not for the Delta cluster. This indicates that, in the Epsilon cluster, individual genes (rather than blocks of genes) underwent duplication, with no subsequent rearrangement.

Cloning and bacterial expression of Delta- and Epsilon-class GSTs

The ten Delta-class genes (*DmGSTD1–DmGSTD10*) as well as *DmGSTE1* were amplified by PCR using genomic DNA as the template, as described in Materials and methods section. Their intron-free coding sequences were used directly for expression of the proteins in *E. coli*. All proteins could be expressed, albeit with different yields. Although the expression level of DmGSTD5-5 and DmGSTD7-7 was low (results not shown), it was sufficient to determine the enzymic activity of the proteins in bacterial lysates. Enzymes selected because of their ability to conjugate 4-HNE (see below) were further purified (Figure 5).

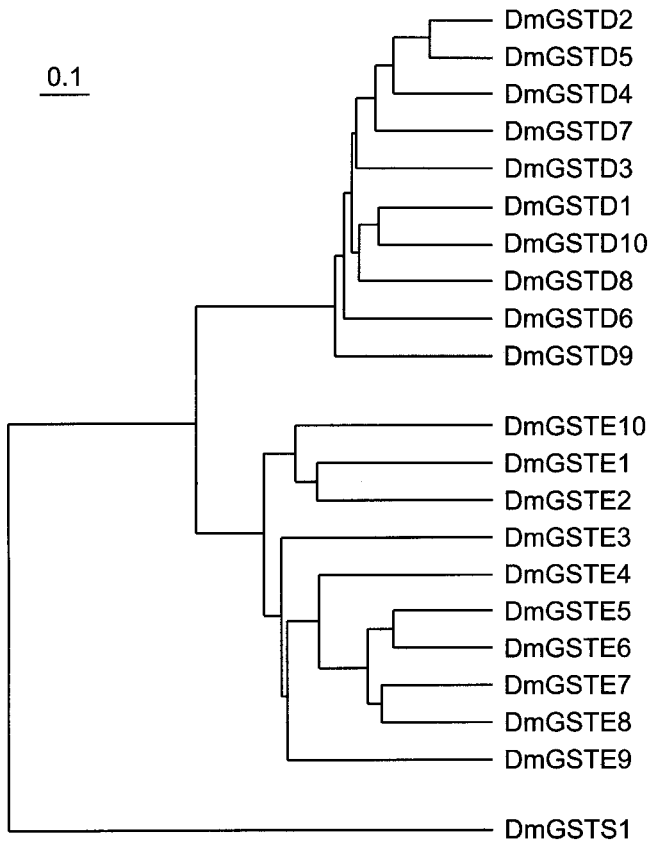

Figure 4 Phylogenetic tree of *D. melanogaster* GSTs

The tree representing similarities among the full-length protein-encoding nucleotide sequences was calculated using the KITSCH program [49], as implemented in the BioEdit program suite (www.mbio.ncsu.edu/BioEdit). The program assumes a constant evolutionary rate; the bar indicates the time in which 0.1 nucleotide substitution occurs at any given site.

Catalytic activity of *Drosophila* GSTs

Except for DmGSTD3-3, all Delta-class GSTs conferred CDNB-conjugating activity on lysates of bacterial cells in which they were expressed. In contrast, DmGSTD3-3 and DmGSTE1-1 had no activity with CDNB but were able to conjugate 4-HNE in crude bacterial lysates. This pattern of activities resembles that of the quantitatively predominant *Drosophila* GST, DmGSTS1-1 ([11] and Table 3). Six of the 10 Delta-class GSTs had 4-HNE-conjugating activity. Thus each of the 11 enzymes under study was active with either 4-HNE or CDNB. Enzymes able to catalyse the conjugation of 4-HNE were of special interest in the context of the present study and were selected for purification and further characterization. The kinetic parameters of the latter GSTs are shown in Table 3.In this group, DmGSTD1-1 had the highest catalytic efficiency for 4-HNE conjugation, only 4-fold lower than the catalytic efficiency of the highly specialized murine enzyme mGSTA4-4 [21]. DmGSTD7-7 had an appreciable catalytic efficiency for 4-HNE which exceeded that of DmGSTS1-1. The remaining four Delta-class GSTs had lower but clearly measurable activities for 4-HNE. As observed by us previously [11], DmGSTS1-1 contributes most of 4-HNE-conjugating activity of adult *Drosophila* because of the high abundance of the protein. Our present results indicate that DmGSTD1-1 and several additional GSTs have higher or equal catalytic-centre activities and/or catalytic efficiencies compared with DmGSTS1-

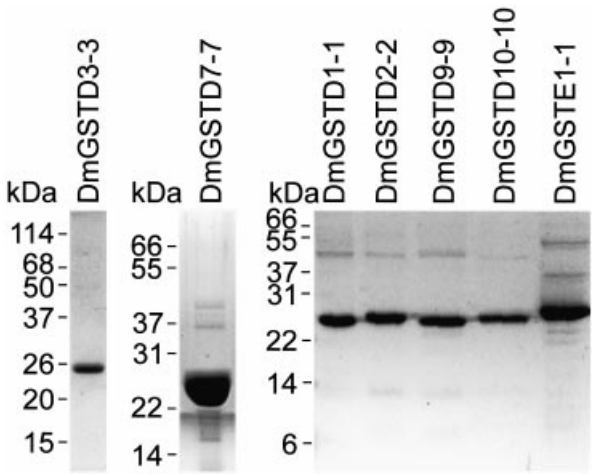

Figure 5 Purification of bacterially expressed *Drosophila* GSTs

See the Materials and methods section for the purification procedures. The purified proteins were separated by SDS/PAGE, and the gels were stained with Coomassie Blue.

1. Thus the Delta-class GSTs, and perhaps also Epsilon-class GSTs, may be relevant in the metabolism of lipid peroxidation products in specific tissues in which they are expressed.

Most of the enzymes listed in Table 3 adhered to Michaelis–Menten kinetics. In several cases, the Hill equation gave a marginally better fit than a Michaelis–Menten hyperbola, but the Hill coefficient was close to 1. There were, however, several notable deviations from Michaelis–Menten kinetics. DmGSTD2-2 and DmGSTE1-1 showed positive cooperativity with 4-HNE, as did DmGSTD9-9 with CDNB. In these three cases, the Hill coefficient was approx. 2 (Table 3). This finding contrasts with the behaviour of the murine mGSTA4-4, which shows negative co-operativity with 4-HNE due to a functional linkage of the active sites in the two subunits via a chain of arginine residues which favours a half-site reactivity of the enzyme [27]. The mechanistic basis and possible physiological significance of the positive co-operativity in some of the *Drosophila* GSTs is of considerable interest.

Mammalian Alpha-class GSTs which act on 4-HNE also have glutathione peroxidase activity for lipid hydroperoxides [21] and can thus metabolize the precursor of 4-HNE as well as 4-HNE itself. We assayed the *Drosophila* GSTs for glutathione peroxidase activity using the model substrate cumene hydroperoxide. In the present study, only DmGSTD1-1, but not the remaining Delta-class enzymes or DmGSTE1-1, had measurable glutathione peroxidase activity in bacterial lysates of *E. coli* expressing the proteins. The specific activity of purified DmGSTD1-1 with cumene hydroperoxide was 0.38 ± 0.02 μmol $\cdot$ min^{-1} $\cdot$ mg^{-1}, similar to that found previously by others [22] and consistent with the postulated antioxidant role of *Drosophila* GSTs [22].

Expression of the *DmGSTD3* and *DmGSTD7* genes in *Drosophila*

It has been hypothesized previously that *DmGSTD3* and *DmGSTD7* may be pseudogenes [18]. Since both genes have uninterrupted ORFs and yield enzymically active proteins upon bacterial expression, we tested whether these genes are normally expressed in *Drosophila*. RT–PCR on RNA isolated from *Drosophila* larvae using primers specific for the two genes yielded products of the expected size (marked by arrows in Figure 6), in addition to several extraneous bands. Partial sequencing of the bands marked by arrows confirmed that they were *DmGSTD3* and *DmGSTD7* respectively. Control reactions from which RT was omitted yielded no product, indicating that the template for amplification was cDNA rather than contaminating genomic DNA. Although RT–PCR with primers specific for *DmGSTD3* yielded a strong band when total RNA was used as the starting material, the band with *DmGSTD7*-specific primers was weak

Table 3 Kinetic parameters of *D. melanogaster* GSTs

In initial experiments, *Drosophila* GSTs were expressed in *E. coli* as described in the text and 4-HNE-conjugating activity was measured in bacterial lysates. Active enzymes were purified and their kinetic parameters were determined by non-linear fitting of both the Michaelis–Menten and the Hill equations to the data. The Hill coefficient is listed for enzymes which gave a better fit to the Hill equation. Enzymes which gave a better fit to the Michaelis–Menten equation are denoted by '(1)' (i.e. implied Hill coefficient = 1). Catalytic efficiency was calculated by direct division of k_{cat} (obtained using the molecular mass of each protein dimer derived from its amino acid composition) by K_M. Catalytic efficiencies calculated from initial reaction rates at low substrate concentration ([S] $\ll K_M$) were similar to those obtained by dividing k_{cat} by K_M (results not shown). For comparison, kinetic parameters of *Drosophila* DmGSTS1-1 [11] and the murine mGSTA4-4 [47] are also listed. Values are means $\pm$ S.D.; the S.D. of the catalytic efficiency was estimated from the means and variances of k_{cat} and K_M according to [48, p. 91]. Negligible, below assay sensitivity; nd, not determined.

	4-HNE					CDNB				
GST	Activity in lysate	V_{max} (μmol/mg $\cdot$ min)	K_M (μM)	Hill coefficient	k_{cat}/K_M (s^{-1} $\cdot$ mM^{-1})	Activity in lysate	V_{max} (μmol/mg $\cdot$ min)	K_M (μM)	Hill coefficient	k_{cat}/K_M (s^{-1} $\cdot$ mM^{-1})
D1-1	+	31.5 ± 1.6	63 ± 9	(1)	399 ± 59	+	24.3 ± 1.1	543 ± 77	(1)	36 ± 1
D2-2	+	1.3 ± 0.1	22 ± 3	1.9 ± 0.4	46 ± 6	+	0.21 ± 0.04	851 ± 249	1.4 ± 0.3	0.2 ± 0.1
D3-3	+	1.1 ± 0.1	50 ± 5	(1)	17 ± 4	−	Negligible	nd	nd	nd
D4-4	−	nd	nd	nd	nd	+	nd	nd	nd	nd
D5-5	−	nd	nd	nd	nd	+	nd	nd	nd	nd
D6-6	−	nd	nd	nd	nd	+	nd	nd	nd	nd
D7-7	+	40.0 ± 3.6	169 ± 27	(1)	193 ± 35	+	nd	nd	nd	nd
D8-8	−	nd	nd	nd	nd	+	nd	nd	nd	nd
D9-9	+	1.0 ± 0.2	107 ± 30	(1)	8 ± 3	+	7.0 ± 0.6	240 ± 24	2.1 ± 0.5	25 ± 3
D10-10	+	8.8 ± 3.8	190 ± 113	1.3 ± 0.2	38 ± 28	+	2.4 ± 0.3	326 ± 65	1.4 ± 0.2	6 ± 1
E1-1	+	0.6 ± 0.1	63 ± 7	2.3 ± 0.4	8 ± 1	−	Negligible	nd	nd	nd
S1-1		8.4 ± 0.4	123 ± 12	(1)	63		Negligible	nd	nd	nd
A4-4 (mouse)		120	72		1520		38	5300	(1)	6

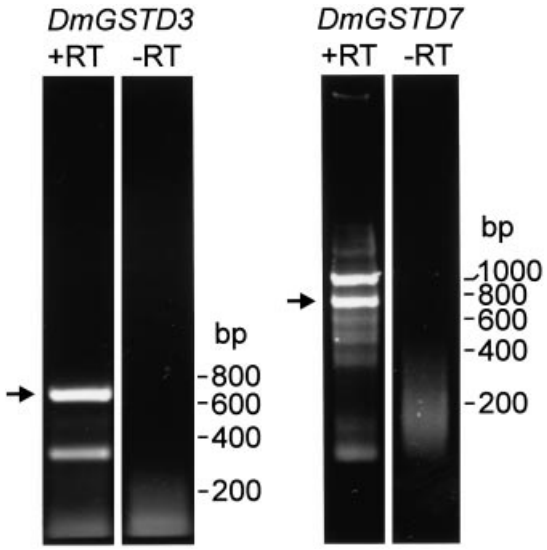

Figure 6 Identification of *DmGSTD3* and *DmGSTD7* transcripts in *Drosophila* larvae by RT–PCR

Total RNA was used as the starting material for amplification of *DmGSTD3*, and poly(A)$^+$ RNA was used to amplify *DmGSTD7*. The identity of the amplification products (denoted by arrows) was confirmed by partial sequencing; the additional bands were probably the result of mispriming and were not further analysed. Control reactions lacking RT (lanes labelled —RT) did not yield amplification products.

(results not shown). Therefore the poly(A)$^+$ RNA fraction was purified from total RNA for amplification of the latter sequence. The results of RT–PCR demonstrate that the *DmGSTD3* and *DmGSTD7* genes are expressed in *Drosophila* larvae, although the level of the *DmGSTD7* mRNA may be lower than that of *DmGSTD3*.

DISCUSSION

The cluster of *D. melanogaster* Delta-class GSTs has been originally described, and several of the enzymes have been biochemically characterized in a series of elegant papers by C.-P. D. Tu et al. [18,22–24,28,29]. Two additional members of the cluster have been identified recently by bioinformatics approaches [17] but have not been biochemically characterized. Similarly, the *DmGSTE1* (*Gst-3*) gene has been reported [20], but the functional properties of the protein remained unknown. We have now cloned, expressed in *E. coli* and characterized all ten Delta-class GSTs and DmGSTE1-1. Furthermore, we identified, but have not further characterized, nine additional members of the Epsilon cluster. Thus both the Delta- and the Epsilon-class GSTs form families of at least ten members each, probably as the result of repeated gene duplications.

DmGSTE1-1 and all ten Delta-class GSTs could be expressed in *E. coli* as enzymically active proteins. This result appears to contradict the earlier conclusion that *DmGSTD3* and *DmGSTD7* are likely to be pseudogenes [18]. *DmGSTD3* was presumed to be a pseudogene on the basis of a sub-optimal Kozak context of the translation-initiation codon, which may prevent its synthesis, and a 15-amino-acid N-terminal truncation, which may render the protein inactive even if synthesized [18]. Our demonstration of bacterial expression of DmGSTD3-3 does not address the question of whether *mGSTD3* mRNA can be efficiently translated in eukaryotic, and specifically *Drosophila*, cells. However, the database of all known and predicted *Drosophila* transcripts ([30]; file available as 'na_gadfly.dros.RELEASE2' on www.fruitfly.org) contains, in addition to *DmGSTD3*, at least two other sequences that have an identical context of the initiator ATG (TCTG **ATG** G) and encode identifiable proteins: a Zn-dependent exopeptidase (Flybase symbol CG10073) and a cell adhesion protein (CG5550). This is consistent with the fact that the Kozak consensus is not an absolute requirement for translation, but rather one of several interdependent factors that co-determine initiation efficiency. Moreover, *Drosophila* appears to tolerate more deviation from the 'ideal' context of the initiator codon than mammalian cells [26,31]. In fact, suboptimal determinants of protein synthesis initiation may render the process more amenable to regulation [32], an aspect that may be relevant for some of the GSTs (see below). An expressed sequence tag (EST) clone (LP11313, GenBank® accession nos. AI297103 and AY118350) has been isolated that fully matches the genomic *DmGSTD3* sequence, except for the presence of a poly(A)$^+$ tail at the 3′-end of the EST clone. Finally, we identified a *DmGSTD3* transcript by RT–PCR. This demonstrates that the *DmGSTD3* gene is expressed, at least up to the stage of transcription and transcript processing. This argues against the possibility that *DmGSTD3* is a pseudogene.

As noted previously [18], DmGSTD3-3 lacks the N-terminal sequence (cf. Figure 2) that includes the highly conserved tyrosine residue involved in the catalytic cycle of GSTs. Strikingly, DmGSTD3-3 is active towards 4-HNE, with kinetic parameters similar to those of other Delta-class GSTs (Table 3). This indicates that other residues may take the place of the active-site tyrosine.

DmGSTD7 was deemed to be a pseudogene because of a C-terminal truncation which may affect the electrophile-binding pocket of the active site and/or protein stability [18]. We have demonstrated by RT–PCR the presence of *DmGSTD7* mRNA in *Drosophila* larvae. Furthermore, our results show that the DmGSTD7-7 protein is active towards both 4-HNE and CDNB (Table 3). In fact, its catalytic efficiency for 4-HNE was second highest among the enzymes tested in the present study. The examples of DmGSTD3-3 and DmGSTD7-7 show that GSTs can tolerate significant deletions, probably through compensatory changes in the folding of the polypeptide.

It is noteworthy that six out of the ten Delta-class GSTs, as well as DmGSTE1-1, had significant activity for 4-HNE conjugation. Together with the previously characterized DmGSTS1-1 [11], there are at least seven distinct GSTs in *Drosophila* with this activity. The catalytic efficiency of one of the Delta-class enzymes, DmGSTD1-1, was lower by only a factor of 4 when compared with that of mammalian Alpha-class GSTs highly specialized for 4-HNE conjugation, exemplified by mGSTA4-4 [21]. At least three of the enzymes, namely DmGSTD3-3, DmGSTE1-1 (Table 3) and DmGSTS1-1 [11], are active towards 4-HNE but not towards the near-universal GST model substrate CDNB. Finally, 4-HNE-conjugating activity probably arose on multiple independent occasions during evolution, since it is associated with some, but not all, of the Theta-related enzymes in *Drosophila* [DmGSTS1-1 [11] and certain Delta- and Epsilon-class GSTs (this work)], with some, but again not all, Alpha-class mammalian GSTs [15,33] and with at least one Pi-class GST in *Caenorhabditis elegans* [34]. Collectively, these findings suggest that conjugation of 4-HNE evolved, and is maintained, by selective pressure, rather than being an adventitious activity of some GSTs.

In addition to toxicity at supraphysiological levels, 4-HNE has signalling functions affecting, among others, cell proliferation, differentiation and apoptosis (see e.g. [35–39]). It is tempting to speculate that the multiplicity of GSTs involved in 4-HNE

metabolism is necessary to modulate and terminate these functions of 4-HNE in a variety of physiological situations, perhaps including embryogenesis and insect metamorphosis. The lack of *DmGSTD3*, *DmGSTD5*, *DmGSTD7* and *DmGSTD8* transcripts detectable by Northern blotting in adult *Drosophila* [18] may indicate that their expression is spatially and temporally restricted, perhaps to pre-adult stages. For example, the EST representing *DmGSTD3* (LP11313; Berkeley Drosophila Genome Project, direct submission to GenBank®) has been isolated from a larval/early pupal stage of *Drosophila*, and we have demonstrated the presence of *DmGSTD3* and *DmGSTD7* transcripts in third instar larvae. In addition to transcriptional regulation, the suboptimal Kozak context of the translational start site of some of the above GSTs is consistent with translational regulation. Low and tightly controlled expression is characteristic of regulatory proteins, but would be unusual for an enzyme whose primary function is general detoxification.

Previously, insect GSTs have been studied primarily because of their possible involvement in the metabolic inactivation of insecticides [40]. Our work indicates that several *Drosophila* GSTs participate in defence mechanisms against oxidative stress, and in the metabolism of endogenously formed lipid peroxidation products. In this, they functionally resemble mammalian Alpha-class GSTs [41,42]. A possible role of lipid peroxidation products, and thus GSTs that metabolize them, in the modulation of embryogenesis and development has been already discussed. Another process in which oxidative stress plays a key role is organismal aging [43,44]. In this context, it is intriguing that lifespan in *Drosophila* correlates with the expression level of DmGSTS1-1 [45] and DmGSTD1-1 [46], two enzymes identified by us as capable of metabolizing 4-HNE.

The above examples demonstrate that the physiological roles of GSTs transcend simple detoxification and affect a number of fundamental biological processes. *Drosophila* is well suited to the study of these processes because of its known genetics and availability of methods for molecular interventions. Since basic biological processes are often conserved between invertebrates such as *Drosophila* and mammals, including humans, we expect that the elucidation of the physiological functions of *Drosophila* GSTs will have broad biological implications.

We thank Mr Venu Varma for computational help with the identification, in the database of genes potentially expressed in *Drosophila*, of transcripts with a suboptimal Kozak context which corresponds to that of *DmGSTD3*. This investigation was supported in part by the National Institute of Aging (USPHS grant no. AG018845) and the National Institute of Environmental Health Sciences (grant no. ES07804).

REFERENCES

1 Halliwell, B. and Gutteridge, J. M. C. (1999) Free Radicals in Biology and Medicine, Oxford University Press, Oxford
2 Schneider, C., Tallman, K. A., Porter, N. A. and Brash, A. R. (2001) Two distinct pathways of formation of 4-hydroxynonenal: mechanisms of nonenzymatic transformation of the 9- and 13-hydroperoxides of linoleic acid to 4-hydroxyalkenals. J. Biol. Chem. **276**, 20831–20838
3 Esterbauer, H. (1993) Cytotoxicity and genotoxicity of lipid-oxidation products. Am. J. Clin. Nutr. **57**, 779S–786S
4 Dianzani, M. U. (1998) 4-Hydroxynonenal and cell signalling. Free Radic. Res. **28**, 553–560
5 Uchida, K., Shiraishi, M., Naito, Y., Torii, Y., Nakamura, Y. and Osawa, T. (1999) Activation of stress signaling pathways by the end product of lipid peroxidation. 4-Hydroxy-2-nonenal is a potential inducer of intracellular peroxide production. J. Biol. Chem. **274**, 2234–2242
6 Uchida, K. and Stadtman, E. R. (1992) Modification of histidine residues in proteins by reaction with 4-hydroxynonenal. Proc. Natl. Acad. Sci. U.S.A. **89**, 4544–4548
7 Stark, W. S., Lin, T. N., Brackhahn, D., Christianson, J. S. and Sun, G. Y. (1993) Fatty acids in the lipids of *Drosophila* heads: effects of visual mutants, carotenoid deprivation and dietary fatty acids. Lipids **28**, 345–350
8 Draper, H. H., Philbrick, D. P., Agarval, S., Meidiger, R. and Phillips, J. P. (2000) Avid uptake of linoleic acid and vitamin E by *Drosophila melanogaster*. Nutr. Res. **20**, 113–120
9 Pryor, W. A. and Porter, N. A. (1990) Suggested mechanisms for the production of 4-hydroxy-2-nonenal from the autoxidation of polyunsaturated fatty acids. Free Radic. Biol. Med. **8**, 541–543
10 Jones, H. E., Harwood, J. L., Bowen, I. D. and Griffiths, G. (1992) Lipid composition of subcellular membranes from larvae and prepupae of *Drosophila melanogaster*. Lipids **27**, 984–987
11 Singh, S. P., Coronella, J. A., Beneš, H., Cochrane, B. J. and Zimniak, P. (2001) Catalytic function of *Drosophila melanogaster* glutathione S-transferase DmGSTS1-1 (GST-2) in conjugation of lipid peroxidation end products. Eur. J. Biochem. **268**, 2912–2923
12 Yan, L. J. and Sohal, R. S. (1998) Mitochondrial adenine nucleotide translocase is modified oxidatively during aging. Proc. Natl. Acad. Sci. U.S.A. **95**, 12896–12901
13 Alin, P., Danielson, U. H. and Mannervik, B. (1985) 4-Hydroxyalk-2-enals are substrates for glutathione transferase. FEBS Lett. **179**, 267–270
14 Awasthi, Y. C., Zimniak, P., Awasthi, S., Singhal, S. S., Srivastava, S. K., Piper, J. T., Chaubey, M., Petersen, D. R., He, N.-G., Sharma, R. et al. (1996) A new group of glutathione S-transferases with protective role against lipid peroxidation. In Glutathione S-transferases: Structure, Function and Clinical Implications (Vermeulen, N. P. E., Mulder, G. J., Nieuwenhuyse, H., Peters, W. H. M. and van Bladeren, P. J., eds.), pp. 111–124, Taylor & Francis, London
15 Hubatsch, I., Ridderstrom, M. and Mannervik, B. (1998) Human glutathione transferase A4-4: an Alpha class enzyme with high catalytic efficiency in the conjugation of 4-hydroxynonenal and other genotoxic products of lipid peroxidation. Biochem. J. **330**, 175–179
16 Clayton, J. D., Cripps, R. M., Sparrow, J. C. and Bullard, B. (1998) Interaction of troponin-H and glutathione S-transferase-2 in the indirect flight muscles of *Drosophila melanogaster*. J. Muscle Res. Cell Motil. **19**, 117–127
17 Chelvanayagam, G., Parker, M. W. and Board, P. G. (2001) Fly fishing for GSTs: a unified nomenclature for mammalian and insect glutathione transferases. Chem. Biol. Interact. **133**, 256–260
18 Toung, Y.-P. S., Hsieh, T. and Tu, C.-P. D. (1993) The glutathione *S*-transferase *D* genes. A divergently organized, intronless gene family in *Drosophila melanogaster*. J. Biol. Chem. **268**, 9737–9746
19 Beall, C., Fyrberg, C., Song, S. and Fyrberg, E. (1992) Isolation of a *Drosophila* gene encoding glutathione S-transferase. Biochem. Genet. **30**, 515–527
20 Singh, M., Silva, E., Schulze, S., Sinclair, D. A. R., Fitzpatrick, K. A. and Honda, B. M. (2000) Cloning and characterization of a new theta-class glutathione S-transferase (GST) gene, *gst-3*, from *Drosophila melanogaster*. Gene **247**, 167–173
21 Zimniak, P., Singhal, S. S., Srivastava, S. K., Awasthi, S., Sharma, R., Hayden, J. B. and Awasthi, Y. C. (1994) Estimation of genomic complexity, heterologous expression, and enzymatic characterization of mouse glutathione S-transferase mGSTA4-4 (GST 5.7). J. Biol. Chem. **269**, 992–1000
22 Tang, A. H. and Tu, C.-P. D. (1994) Biochemical characterization of *Drosophila* glutathione S-transferases D1 and D21. J. Biol. Chem. **269**, 27876–27884
23 Lee, H. C. and Tu, C.-P. D. (1995) *Drosophila* glutathione S-transferase D27: functional analysis of two consecutive tyrosines near the N-terminus. Biochem. Biophys. Res. Commun. **209**, 327–334
24 Toung, Y. P., Hsieh, T. S. and Tu, C.-P. D. (1991) The *Drosophila* glutathione S-transferase 1-1 is encoded by an intronless gene at 87B. Biochem. Biophys. Res. Commun. **178**, 1205–1211
25 Lougarre, A., Bride, J. M. and Fournier, D. (1999) Is the insect glutathione S-transferase I gene family intronless? Insect Mol. Biol. **8**, 141–143
26 Feng, Y., Gunter, L. E., Organ, E. L. and Cavener, D. R. (1991) Translation initiation in *Drosophila melanogaster* is reduced by mutations upstream of the AUG initiator codon. Mol. Cell. Biol. **11**, 2149–2153
27 Xiao, B., Singh, S. P., Nanduri, B., Awasthi, Y. C., Zimniak, P. and Ji, X. (1999) Crystal structure of a murine glutathione S-transferase in complex with a glutathione conjugate of 4-hydroxynon-2-enal in one subunit and glutathione in the other: evidence of signaling across the dimer interface. Biochemistry **38**, 11887–11894
28 Toung, Y. P. and Tu, C.-P. D. (1992) *Drosophila* glutathione S-transferases have sequence homology to the stringent starvation protein of *Escherichia coli*. Biochem. Biophys. Res. Commun. **182**, 355–360
29 Tang, A. H. and Tu, C.-P. D. (1995) Pentobarbital-induced changes in *Drosophila* glutathione S-transferase D21 mRNA stability. J. Biol. Chem. **270**, 13819–13825
30 Rubin, G. M., Yandell, M. D., Wortman, J. R., Gabor Miklos, G. L., Nelson, C. R., Hariharan, I. K., Fortini, M. E., Li, P. W., Apweiler, R., Fleischmann, W. et al. (2000) Comparative genomics of the eukaryotes. Science **287**, 2204–2215
31 Cavener, D. R. (1987) Comparison of the consensus sequence flanking translational start sites in *Drosophila* and vertebrates. Nucleic Acids Res. **15**, 1353–1361
32 Kozak, M. (1999) Initiation of translation in prokaryotes and eukaryotes. Gene **234**, 187–208

33 Cheng, J. Z., Yang, Y., Singh, S. P., Singhal, S. S., Awasthi, S., Pan, S. S., Singh, S. V., Zimniak, P. and Awasthi, Y. C. (2001) Two distinct 4-hydroxynonenal metabolizing glutathione S-transferase isozymes are differentially expressed in human tissues. Biochem. Biophys. Res. Commun. **282**, 1268–1274
34 Engle, M. R., Singh, S. P., Nanduri, B., Ji, X. and Zimniak, P. (2001) Invertebrate glutathione transferases conjugating 4-hydroxynonenal: CeGST 5.4 from *Caenorhabditis elegans*. Chem. Biol. Interact. **133**, 244–248
35 Barrera, G., Pizzimenti, S., Muraca, R., Barbiero, G., Bonelli, G., Baccino, F. M., Fazio, V. M. and Dianzani, M. U. (1996) Effect of 4-hydroxynonenal on cell cycle progression and expression of differentiation-associated antigens in HL-60 cells. Free Radicals Biol. Med. **20**, 455–462
36 Liu, W., Akhand, A. A., Kato, M., Yokoyama, I., Miyata, T., Kurokawa, K., Uchida, K. and Nakashima, I. (1999) 4-Hydroxynonenal triggers an epidermal growth factor receptor-linked signal pathway for growth inhibition. J. Cell Sci. **112**, 2409–2417
37 Pizzimenti, S., Barrera, G., Dianzani, M. U. and Brusselbach, S. (1999) Inhibition of D1, D2, and A-cyclin expression in HL-60 cells by the lipid peroxidation product 4-hydroxynonenal. Free Radicals Biol. Med. **26**, 1578–1586
38 Cheng, J.-Z., Singhal, S. S., Saini, M., Singhal, J., Piper, J. T., Van Kuijk, F. J. G. M., Zimniak, P., Awasthi, Y. C. and Awasthi, S. (1999) Effects of mGST A4 transfection on 4-hydroxynonenal-mediated apoptosis and differentiation of K562 human erythroleukemia cells. Arch. Biochem. Biophys. **372**, 29–36
39 Rinaldi, M., Barrera, G., Spinsanti, P., Pizzimenti, S., Ciafre, S. A., Parella, P., Farace, M. G., Signori, E., Dianzani, M. U. and Fazio, V. M. (2001) Growth inhibition and differentiation induction in murine erythroleukemia cells by 4-hydroxynonenal. Free Radical Res. **34**, 629–637
40 Ranson, H., Rossiter, L., Ortelli, F., Jensen, B., Wang, X. L., Roth, C. W., Collins, F. H. and Hemingway, J. (2001) Identification of a novel class of insect glutathione S-transferases involved in resistance to DDT in the malaria vector *Anopheles gambiae*. Biochem. J. **359**, 295–304
41 Cheng, J.-Z., Sharma, R., Yang, Y., Singhal, S. S., Sharma, A., Saini, M. K., Singh, S. V., Zimniak, P., Awasthi, S. and Awasthi, Y. C. (2001) Accelerated metabolism and exclusion of 4-hydroxynonenal through induction of RLIP76 and hGST5.8 is an early adaptive response of cells to heat and oxidative stress. J. Biol. Chem. **276**, 41213–41223
42 Yang, Y., Cheng, J. Z., Singhal, S. S., Saini, M., Pandya, U., Awasthi, S. and Awasthi, Y. C. (2001) Role of glutathione S-transferases in protection against lipid peroxidation. Overexpression of hGSTA2-2 in K562 cells protects against hydrogen peroxide-induced apoptosis and inhibits JNK and caspase 3 activation. J. Biol. Chem. **276**, 19220–19230
43 Sohal, R. S. and Orr, W. C. (1998) Oxidative stress may be a causal factor in senescence. Age **21**, 81–82
44 Parkes, T. L., Elia, A. J., Dickinson, D., Hilliker, A. J., Phillips, J. P. and Boulianne, G. L. (1998) Extension of *Drosophila* lifespan by overexpression of human SOD1 in motorneurons. Nat. Genet. **19**, 171–174
45 Seong, K.-H., Ogashiwa, T., Matsuo, T., Fuyama, Y. and Aigaki, T. (2001) Application of the gene search system to screen for longevity genes in *Drosophila*. Biogerontology **2**, 209–217
46 Kang, H. L., Benzer, S. and Min, K. T. (2002) Life extension in *Drosophila* by feeding a drug. Proc. Natl. Acad. Sci. U.S.A. **99**, 838–843
47 Nanduri, B., Hayden, J. B., Awasthi, Y. C. and Zimniak, P. (1996) Amino acid residue 104 in an alpha-class glutathione S-transferase is essential for the high selectivity and specificity of the enzyme for 4-hydroxynonenal. Arch. Biochem. Biophys. **335**, 305–310
48 Armitage, P. and Berry, G. (1987) Statistical Methods in Medical Research. Blackwell Scientific Publications, Oxford
49 Felsenstein, J. (1993) PHYLIP (Phylogeny Inference Package), version 3.5c, Department of Genetics, University of Washington, Seattle, WA

Received 14 August 2002/8 November 2002; accepted 20 November 2002
Published as BJ Immediate Publication 20 November 2002, DOI 10.1042/BJ20021287