Cloning and expression of a novel Mu class murine glutathione transferase isoenzyme

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The present study describes the cDNA cloning, expression and characterization of a novel Mu class murine glutathione transferase (GST) isoenzyme. Screening of a cDNA library from the small intestine of a female A/J mouse using consensus probes derived from Mu class murine GST genes (*mGSTM1*–*mGSTM5*) resulted in the isolation of a full-length cDNA clone of a previously unknown Mu class GST gene (designated as *mGSTM7*). The choice of tissue was based on our previous identification in female A/J mouse small intestine of a potentially novel Mu class GST isoenzyme. The deduced amino acid sequence of mGSTM7, which comprises of 218 amino acid residues, exhibited about 67–78% identity with other Mu class murine GSTs. Recombinant mGSTM7-7 cross-reacted with anti-(GST Mu) antibodies, but not with anti-(GST Alpha) or anti-(GST Pi) antibodies. The pI and the reverse-phase-HPLC elution profile of recombinant mGSTM7-7 were different from those of other Mu class murine GSTs. The substrate specificity of mGSTM7-7 was also different compared with other Mu class murine GSTs. Interestingly, mGSTM7 had a higher identity with the human Mu class isoenzyme hGSTM4 (87% identity and 94% similarity in the amino acid sequence) than with any of the known mouse Mu class GSTs. Specific activities of recombinant mGSTM7-7 and human GSTM4-4 were comparable towards several substrates. For example, similar to hGSTM4-4, recombinant mGSTM7-7 was poorly active in catalysing the GSH conjugation of 1-chloro-2,4-dinitrobenzene and ethacrynic acid, and lacked activity towards 1,2-dichloro-4-nitrobenzene and 1,2-epoxy-3-(*p*-nitrophenoxy)propane. These results suggested that hGSTM4-4 might be the human counterpart of mouse GSTM7-7. Reverse transcription-PCR analysis using mGSTM7-specific primers revealed that *mGSTM7* is widely expressed in tissues of female A/J mice, including liver, forestomach, lung, kidney, colon and spleen.

Key words: detoxification, glutathione conjugation, mouse, small intestine, xenobiotics.

INTRODUCTION

Glutathione transferases (GSTs) can detoxify a wide variety of electrophilic xenobiotic compounds, chiefly by catalysing their conjugation with GSH [1,2]. Toxicologically relevant substrates for GSTs include activated metabolites of the polycyclic aromatic hydrocarbon family of environmental and dietary carcinogens (e.g. benzo[*a*]pyrene 7,8-diol-9,10-epoxide), pesticides (e.g. alachlor), antibiotics (e.g. fosfomycin) and products of oxidative damage (e.g. acrolein, fatty acid hydroperoxides and 4-hydroxynonenal) (reviewed in [2]). GST-catalysed GSH conjugation reactions are not always advantageous. For example, GST-catalysed GSH conjugation of certain clinically useful anticancer agents or their metabolites, including melphalan, chlorambucil, thiotepa and cyclophosphamide, is an important mechanism by which tumour cells acquire resistance to these agents [3]. GSTs may also participate in the metabolism of certain endogenous compounds, such as leukotriene A_4 and Δ^5-androstene-3,17-dione (Δ^5-AD) [4,5]. Furthermore, GSTs can non-catalytically bind a number of chemicals, such as steroid and thyroid hormones, bile acids and bilirubin, and presumably facilitate their transport (reviewed in [2]). Recent studies have also suggested that certain GST isoenzymes may be involved in the regulation of stress-activated cell signalling pathways through direct physical association with c-Jun N-terminal kinase and apoptosis signal-regulating kinase-1 [6,7].

The cytosolic GST activity in mammalian tissues is often due to multiple isoenzymes that are dimers of either identical or structurally different subunits [1,2]. Mammalian cytosolic GSTs have been grouped into several distinct classes, namely Alpha, Mu, Pi [8], Theta [9], Sigma [10], Kappa [11], Zeta [12] and Omega [13], based on their structural and functional properties. Moreover, each class comprises one or more distinct gene products [1,2]. For example, at least five distinct Mu class murine GST genes (*mGSTM1*–*mGSTM5*) have been characterized [14–18]. Recent studies from our laboratory suggested that a previously uncharacterized Mu class GST isoform with a pI of 5.5 might be expressed in the small intestine of female A/J mice [19]. Here we report cDNA cloning of a novel Mu class GST isoenzyme gene (designated as *mGSTM7* according to the

Abbreviations used: Δ^5-AD, Δ^5-androstene-3,17-dione; (±)-*anti*-BPDE, racemic *anti*-benzo[*a*]pyrene-7,8-diol-9,10-epoxide; BSP, bromosulphophthalein; CDNB, 1-chloro-2,4-dinitrobenzene; CUOH, cumene hydroperoxide; DCNB, 1,2-dichloro-4-nitrobenzene; EA, ethacrynic acid; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; EST, expressed sequence tag.; GST, glutathione transferase; IPTG, isopropyl β-thiogalactopyranoside; LB, Luria–Bertani; 4-NBC, 4-nitrobenzyl chloride; ORF, open reading frame; RT, reverse transcription.

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The nucleotide sequence data reported in the present paper have been submitted to the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AF464943.

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nomenclature recommended for GSTs). Our studies also indicate that recombinant mGSTM7-7 is catalytically distinct from other Mu class murine GST isoenzymes.

EXPERIMENTAL

Materials

A custom-prepared cDNA library from the small intestine of a female A/J mouse in Lambda ZAP II was obtained from Stratagene (La Jolla, CA, U.S.A.). Female A/J mice were purchased from the National Cancer Institute (Frederick, MD, U.S.A.). The Institutional Animal Care and Use Committee approved the use of mice for the present studies. Restriction endonucleases and isopropyl β-thiogalactopyranoside (IPTG) were from Promega (Madison, WI, U.S.A.); T4 DNA ligase, BL21(DE3) competent cells and pET11a vector were from Novagen (Madison, WI, U.S.A.). Advantage cDNA polymerase was obtained from Clontech (Palo Alto, CA, U.S.A.). Megaprime DNA labelling system was from Amersham Biosciences (Piscataway, NJ, U.S.A.), and epoxy-activated Sepharose 6B, GSH, 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), ethacrynic acid (EA), 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP), bromosulphophthalein (BSP), 4-nitrobenzyl chloride (4-NBC) and cumene hydroperoxide (CUOH) were from Sigma (St. Louis, MO, U.S.A.). Racemic *anti*-benzo[*a*]pyrene 7,8-diol-9,10-epoxide [($\pm$)-*anti*-BPDE] was from Chemsyn Science Laboratories (Lenexa, KS, U.S.A.), 4-hydroxynonenal was from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.), and Δ^5-AD was from Steraloids, Inc. (Newport, RI, U.S.A.). Antibodies against Alpha, Mu and Pi classes of human GSTs were purchased from Oxford Biomedical Research (Oxford, MI, U.S.A.).

Cloning strategy

By alignment of known Mu class murine GST gene sequences (*mGSTM1*–*mGSTM5*) [14–18], two highly conserved exons were identified. These consensus exons were amplified by PCR and used as probes for low-stringency screening of a small-intestine cDNA library. The choice of tissue was based on the previous identification of a potentially novel Mu class GST isoenzyme (GST 5.5) in the A/J mouse small intestine that did not correspond to any of the known Mu class murine GSTs [19]. The resulting cDNA clones were sequenced, and those identical with known Mu class sequences were discarded. A clone highly similar to, but not identical with, known *mGSTM* sequences was identified in the screen and was named *mGSTM7*.

DNA probes for *mGSTM7* cloning

Two fragments were amplified by PCR using mouse genomic DNA isolated from the blood of female A/J mice as the template, and the following primers: sense primer A (5′-ATGTACCACT-ACTCTCAC-3′), antisense primer B (5′-TGTGACCAGAAGA-AT-3′), sense primer C (5′-CCTTCCCTGCTTGTCCATCC-3′), and antisense primer D (5′-CCCTGCAGGCAGCCCCAC-3′). While the primers were derived from intronic sequences of the *mGSTM5* gene [18], they amplify exons that are highly conserved in all known *mGSTM* genes [14–18]. Primers A and B are within introns that flank exon 2, and primers C and D are within introns that flank exon 4 of *mGSTM5*. PCR was performed for 40 cycles of denaturation (93 °C, 10 s), annealing (50 °C, 30 s), primer extension (72 °C, 1 min) followed by 10 min at 72 °C. The products were labelled by the random priming method using [α-^{32}P]dCTP and the Megaprime DNA labelling system according to the manufacturer's protocol.

Screening of female A/J mouse small-intestine cDNA library and clone isolation

The cDNA library was plated on *Escherichia coli* XL1-Blue MRF′, and 6×10^5 plaques were screened by filter hybridization with a mixture of the two probes described above. The positive phages were plaque-purified, the pBluescriptSK(–) phagemids were excised from the lambda ZAP II vector, and the inserts were released by digestion with *Eco*RI. Five independent clones, named m1a, m2a, m2d, m5a and m8c, were obtained, and yielded inserts of 1.2, 0.4 and 0.7, 1.4, 1.0, and 1.8 kb respectively. Partial sequences of the five clones were compared with published sequences deposited in the GenBank® database. Partial sequences of clones m1a, m2a, m5a and m8c matched with *mGSTM1*, *mGSTM3*, *mGSTM5* and *mGSTM3* respectively, and these clones were not pursued further. On the other hand, clone m2d exhibited 70–80 % identity with the known murine Mu class GSTs, and this clone (designated *mGSTM7*) was selected for further investigations.

Isolation of the 5′ end of mGSTM7 cDNA

Sequencing of the m2d insert and its alignment with other Mu class murine GSTs indicated that m2d was lacking approx. 170 bp of the open reading frame (ORF) at the 5′ end. To isolate the missing 5′ fragment, pBluescriptSK(−) phagemids were excised from the small-intestine cDNA library in lambda ZAP II, and were used as the template for PCR with the internal antisense primer 5′-TGGCATTGCTCTGCGTGATCT-3′ (located approx. 50 bp downstream of the 5′ end of the available m2d clone) and sense primer 5′-CGGGATATCACTCAGCATAATG-3′ (homologous with the T7 promoter of the pBluescript vector). The PCR yielded a 320 bp product, the sequence of which partially overlapped with the 5′ end of the m2d clone. In addition, the presence in the library of clone(s) containing the uninterrupted ORF for *mGSTM7* was demonstrated by PCR using the phagemid templates described above in conjunction with primers 5′-CCAGCATCATGCCTATGAC-3′ (sense; spanning the translational start site) and 5′-TGGATGAAAGAAAAGAGG-3′ (antisense; located approx. 130 bp downstream of the stop codon). The PCR yielded the expected product of approx. 780 bp, the identity of which was confirmed by sequencing.

Construction of *mGSTM7* expression vector

The phagemid template described in the previous section was used for PCR with the upstream primer 5′-GGAATTC-<u>CATATG</u>CCTATGACACTGGGTTA-3′, and the downstream primer 5′-ACGC<u>GGATCC</u>AGCACTAGGGCTACA-3′. This introduced an *Nde*I site (underlined) spanning the start codon, and a *Bam*HI site (underlined) approx. 70 bp downstream of the stop codon, respectively. The PCR product was digested with *Nde*I and *Bam*HI, and subcloned into the expression plasmid pET11a previously cut with the same restriction enzymes, yielding plasmid pET11a/mGSTM7. The identity of the insert was verified by sequencing. The identity of the sequence with that of the 780 bp fragment obtained in a separate amplification (see previous subsection) indicated the absence of PCR-related mutations.

Expression and purification of mGSTM7-7

E. coli BL21(DE3) was transformed with plasmid pET11a/mGSTM7. Bacteria were grown overnight in Luria–Bertani (LB) medium containing 100 μg/ml carbenicillin at 30 °C. The

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caggcaccag catcatgcct atgacactgg gttactggga catccgtggg ctagctcacg   60
ctattcggct gctcctagaa tacacaggct caagctatga agagaagaga tacaccatgg  120
gagacgctcc tgactatgac cgaagccagt ggctgagtga gaagttcaaa ttgggcctgg  180
actttcccaa tttgccttac ttgattgatg ggtcacacaa gatcacgcag agcaatgcca  240
tcctgcgcta cattgcccgc aagcacaacc tgtgtgggga gacagaggaa gagaagattc  300
gcgtggacat tttggagaac caggctatgg atgtctccaa tcagctggct cgagtctgtt  360
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agctcttctc acagttcctg ggccagcgga catggtttgt tggtgaaaag attacttttg  480
tagatttcct ggcttacgat atcctggacc tgcaccttat attcgaaccc acgtgcctgg  540
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ggatcctgtc tctgatgtct tcagagtctc ccgtattggt caccagggat gggggccatc 1440
ttggttaatc cctcctcttt gtgacccccc gtgaaataaa ttcttcatgc tttcgtaaaa 1500
aaaaaaaaaa aaaaa 1515
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Figure 1 Nucleotide sequence of mGSTM7 cDNA, including the 3′-untranslated region

Start and stop codons are identified by underlining with continous and dotted lines respectively.

overnight culture was diluted 1:50 with LB medium and further incubated at 30 °C for 2 h. IPTG was added to the culture at a final concentration of 2 mM. After further incubation for 6 h, bacteria were harvested by centrifugation at 8000 ***g*** for 5 min and stored at −80 °C. Recombinant mGSTM7-7 was purified by affinity chromatography over a column of GSH linked to epoxy-activated Sepharose 6B by the method of Simons and Vander Jagt [20] as described previously [21]. The purity of the mGSTM7-7 preparation was ascertained by SDS/PAGE [22].

Characterization of recombinant mGSTM7-7

The pI and the reverse-phase-HPLC elution profile of recombinant mGSTM7-7 were determined as described previously [23,24]. Western-blot analysis was performed by the method of Towbin et al. [25] as described previously [26]. The molecular mass of recombinant mGSTM7-7 was determined by gel filtration on a column (1.5 cm × 100 cm) of Sephadex G-100 (Pharmacia, Piscataway, NJ, U.S.A.) equilibrated and eluted with 50 mM Tris/HCl buffer containing 0.1 M KCl. The affinity-purified preparation of recombinant mGSTM7-7 (about 0.5 mg of protein) was co-chromatographed (at a column flow rate of 5 ml/h) with gel-filtration standards (Bio-Rad Laboratories, Hercules, CA, U.S.A.) consisting of bovine thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa) and vitamin B_{12} (1350 Da). Fractions were monitored for absorbance at 280 nm and GST activity towards CDNB [27].

Substrate specificity of recombinant mGSTM7-7

Specific activities of recombinant mGSTM7-7 towards CDNB, DCNB, EA, EPNP, BSP, and 4-NBC were determined as described by Habig et al. [27]. Activities towards CUOH and 4-hydroxynonenal were determined by the methods described by Awasthi et al. [28] and Alin et al. [29] respectively. Activity towards Δ^5-AD was determined as described by Johansson and Mannervik [5]. Specific activity of recombinant mGSTM7-7

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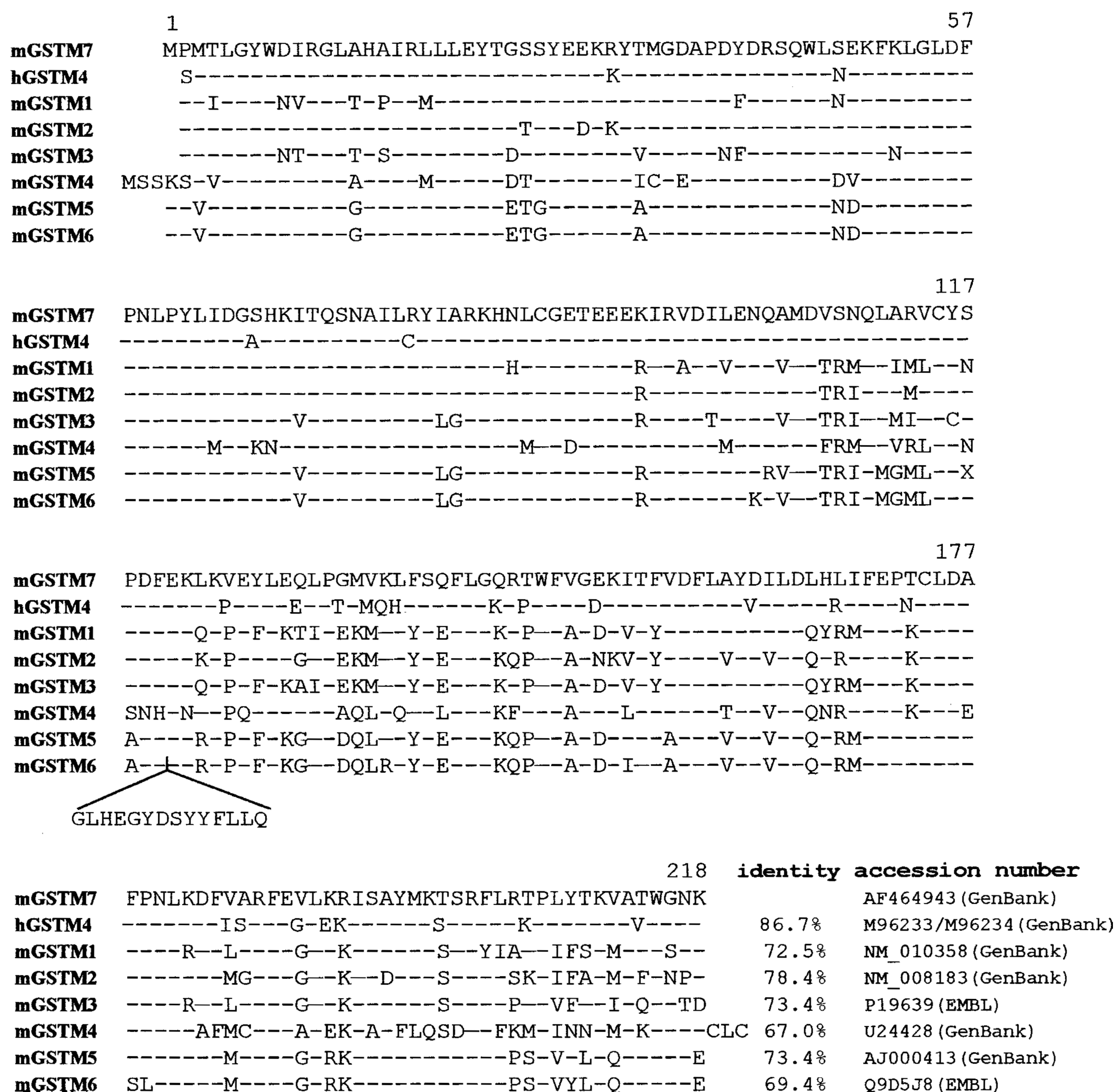

Figure 2 Alignment of the deduced amino acid sequence of mGSTM7 with those of hGSTM4 and all currently known murine Mu class GSTs

Residues identical with those of mGSTM7 are indicated by dashes. Amino acid sequences for hGSTM4, mGSTM1, mGSTM2, mGSTM3, mGSTM4, and mGSTM5 were taken from Comstock et al. [36], Pearson et al. [14], Townsend et al. [15], Reinhart and Pearson [16], Fulcher et al. [17] and de Bruin et al. [18] respectively. The numbering used in the alignment reflects the mGSTM7 sequence. The deduced amino acid sequence for mGSTM6 contains an insert of 14 amino acids that is absent from other Mu class murine GSTs, including mGSTM7.

towards (±)-*anti*-BPDE was determined as described previously [23]. Protein content was determined by the Bradford method [30].

Reverse transcription (RT)-PCR analysis for expression of *mGSTM7* transcript

Tissue distribution of the *mGSTM7* transcript was determined by RT-PCR. Briefly, total RNA from the desired tissue was prepared using TRIZOL reagent (Invitrogen, Carlsbad, CA, U.S.A.). RT-PCR was carried out using the Access RT-PCR system (Promega) with the specific antisense primer 5′-TAAGCAGAGATCCTCTTCAGTA-3′ and the sense primer 5′-GAGAACCAGGCTATGGATG-3′. Both primers were designed to match *mGSTM7* but to diverge in both the ultimate and penultimate nucleotide at their 3′ ends from the remaining six isoforms of murine Mu GSTs (mGSTM1–mGSTM6). In addition, the two primers were designed to have a similar melting

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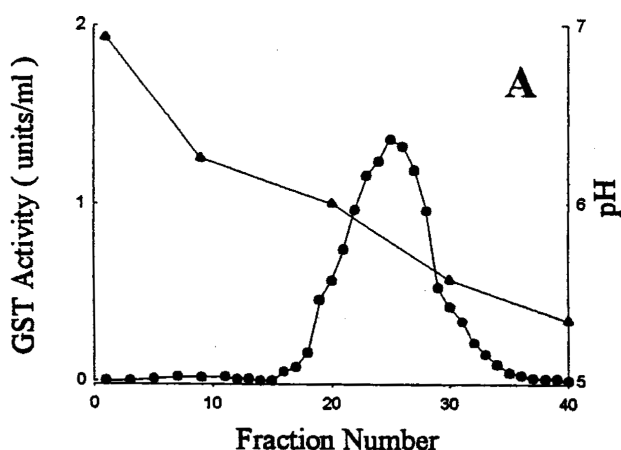
Table 1 Substrate specificity of recombinant mGSTM7-7

ND, activity could not be detected in the present study; –, activity not reported.

	Specific activity (μmol/min per mg of protein)			
Substrate	mGSTM7-7*	mGSTM1-1†	mGSTM3-3†	hGSTM4-4†
CDNB	3.0 ± 0.4	148	22.2	1.25
DCNB	ND	4.4	0.08	ND
EA	0.14 ± 0.006	0.12	0.012	0.04
EPNP	ND	0.48	–	ND
BSP	ND	0.58	0.009	–
4-NBC	ND	–	0.50	–
CUOH	0.13 ± 0.08	0.11	0.02	ND
4-HNE	0.3 ± 0.03	6.0	–	–
(±)-*anti*-BPDE	0.032 ± 0.001	–	–	–
Δ^5-AD	0.10 ± 0.03	0.043	–	–

* Results from present study; values are means ± S.D. of three determinations; enzyme activities towards 4-hydroxynonenal (4-HNE) and Δ^5-AD were measured at 30 °C, whereas the activity towards (±)-*anti*-BPDE was determined at 37 °C; activities towards other substrates were determined at 25 °C.

† Data for mGSTM1-1, mGSTM3-3 and hGSTM4-4 are taken from Warholm et al. [33], Benson et al. [34] and Ross and Board [35], respectively.



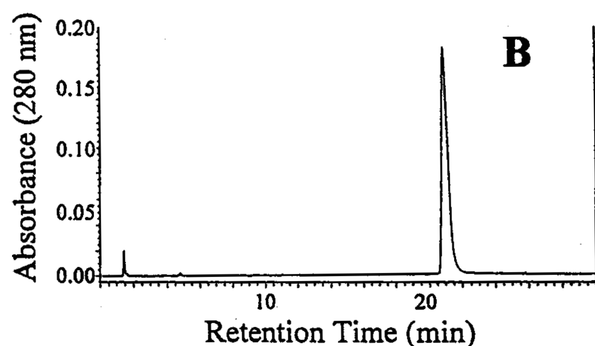


Figure 3 Structural characterization of recombinant mGSTM7-7

(**A**) Chromatofocusing of GSH-affinity-purified preparation of recombinant mGSTM7-7 in the pH range 7–4. Chromatofocusing was performed according to the manufacturer's instructions using reagents from Pharmacia. Fractions (1 ml each) were collected and monitored for pH (▲), and GST activity towards CDNB (●). (**B**) Reverse-phase-HPLC elution profile for recombinant mGSTM7-7. Approx. 5 μg of purified recombinant mGSTM7-7 was injected into the column.

temperature (53.6 °C and 53.0 °C respectively). A product of 290 bp was expected. The selectivity of the primers for *mGSTM7* versus other Mu sequences under the RT-PCR conditions used

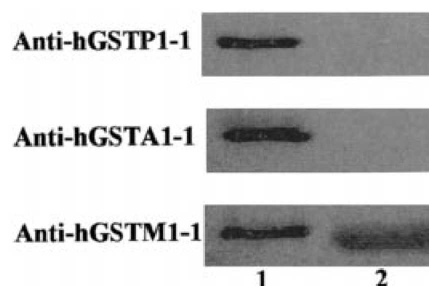


Figure 4 Western-blot analysis of recombinant mGSTM7-7

Blots were probed using antibodies against recombinant hGSTP1-1 (top panel), hGSTA1-1 (middle panel) and hGSTM1-1 (bottom panel). Lane 1 in all three panels contained 1–5 μg of appropriate positive control, and lane 2 contained 5 μg of purified recombinant mGSTM7-7.

in the present work was established experimentally: the primers yielded the expected product when approx. 10 ng of plasmid containing *mGSTM7* was used as the template, but no product was obtained from the same amount of plasmids with *mGSTM1*, *mGSTM3*, or *mGSTM5* inserts. Furthermore, the specificity of amplification from total tissue RNA was verified by sequencing some of the RT-PCR products. Additional control reactions without template RNA or without reverse transcriptase were run in parallel for some tissues (small intestine and liver) to rule out reagent contamination and amplification from genomic DNA respectively.

RESULTS AND DISCUSSION

Previous studies from our laboratory suggested that a novel Mu class GST isoenzyme (pI 5.5) might be expressed in the small intestine of female A/J mice [19]. This isoenzyme, which cross-reacted with anti-(GST Mu) antibodies, was consistently detected during chromatofocusing of the GSH-affinity purified GST preparation from the female A/J mouse small intestine [19]. Low-stringency screening of a cDNA library from the small intestine of female A/J mice using consensus probes spanning two highly conserved exons in Mu class murine GSTs [14–18]

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resulted in a clone (m2d) that was similar to, but not identical with, known Mu class GSTs. Sequencing of the m2d insert and its alignment with *mGSTM1–mGSTM5* revealed that m2d lacked about 170 bp of the ORF at the 5′ end. A cDNA clone containing the entire ORF was subsequently isolated by PCR. Searches of the GenBank® database indicated that the nucleotide sequence of this clone was about 70–80% similar to those of previously characterized Mu class murine GSTs. The nucleotide sequence of this clone, including the 3′-untranslated region, is shown in Figure 1. According to the recommended nomenclature for GSTs [2,31] we have assigned the name mGSTM7-7 to the isoenzyme described in the present study. The GenBank® database contains an entry for *mGSTM6* under accession number NM_008184. However, the nucleotide sequence of NM_008184 is identical with that of *mGSTM5* (accession numbers AJ000412 and AJ000413). An expressed-sequence-tag (EST) clone, AK015265, from adult C57BL/6J mouse testis [32] has been identified as encoding a novel murine Mu class GST that was named mGSTM6-6 (EMBL accession number Q9D5J8). We have assigned the consecutive name, mGSTM7-7, to the isoenzyme described in the present study.

The deduced amino acid sequence of mGSTM7 (the present study) and the amino acid sequences of other known Mu class murine GSTs are shown in Figure 2. Including initiator methionine, the deduced amino acid sequence for mGSTM7 comprises 218 amino acid residues. The amino acid sequence identity between mGSTM7 and mGSTM1, mGSTM2, mGSTM3, mGSTM4, mGSTM5, and mGSTM6 was 67–78%. Interestingly, mGSTM7 had a higher identity with human hGSTM4 (87% identity and 94% similarity in the amino acid sequence) than with any of the known mouse Mu class GSTs (Figure 2).

mGSTM7 was expressed in *E. coli*, and the recombinant protein was purified to determine its relationship with other Mu class murine GSTs. As shown in Figure 3(A), the pI value for recombinant mGSTM7-7 (5.65) was different from those of other Mu class murine GSTs [14–18,23,24]. Similarly, the reverse-phase HPLC elution profile for recombinant mGSTM7-7 (Figure 3B; 21.5 min) was different from those of mGSTM1-1 and mGSTM2-2 (13.3 and 14.0 min respectively) [23]. Gel-filtration studies under non-dissociating conditions gave a molecular mass of approx. 50 kDa for the affinity-purified recombinant mGSTM7-7 (results not shown). A single protein band with apparent molecular mass of about 26 kDa was observed during SDS/PAGE, indicating that mGSTM7-7 is a dimer. As shown in Figure 4, recombinant mGSTM7-7 cross-reacted with anti-(GST Mu) antibodies (Figure 4, bottom panel), but not with the antibodies against Alpha or Pi class GSTs. These results clearly indicated that mGSTM7-7 belongs to class Mu.

The substrate specificity of recombinant mGSTM7-7 towards several commonly used electrophilic substrates is summarized in Table 1. Specific activities for mGSTM1-1 and mGSTM3-3 [33,34] are included for comparison. The specific activity of recombinant mGSTM7-7 towards model substrate CDNB was significantly lower as compared with those of mGSTM1-1 and mGSTM3-3. The CDNB-conjugating activity of recombinant mGSTM7-7 (the present study) was, however, comparable with that of tissue-isolated GST5.5 from female A/J mouse small intestine (J. Guo and S. V. Singh, unpublished work). The specific activity of mGSTM7-7 towards EA was comparable with that of mGSTM1-1. Unlike mGSTM1-1, however, recombinant mGSTM7-7 lacked detectable activity towards DCNB, EPNP or BSP (Table 1). However, there are similarities in the substrate specificities of mGSTM7-7 and the human enzyme hGSTM4-4 [35]. For example, both mGSTM7-7 and hGSTM4-4 are poor catalysts for the GSH-conjugation of model substrate CDNB,

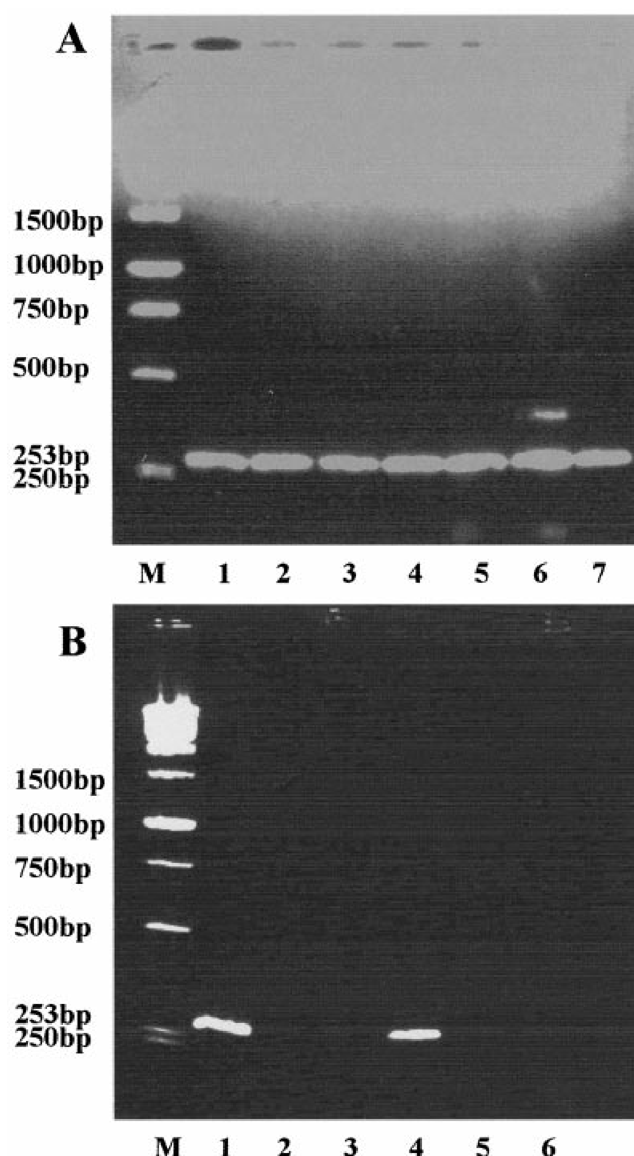

Figure 5 Expression of *mGSTM7* transcript

(**A**) RT-PCR analysis of the tissue distribution of the *mGSTM7* transcript in female A/J mice. Lane 1, small intestine; lane 2, forestomach; lane 3, lung; lane 4, liver; lane 5, kidney; lane 6, colon; lane 7, spleen. Lane M contained a DNA ladder. (**B**) Controls for RT-PCR analysis. Lanes 1 and 4 contained reverse transcriptase and template RNA for small intestine and liver respectively. In lanes 2 and 5, the template RNA was omitted. Lanes 3 and 6 contained template RNA for small intestine and liver respectively, but the reverse transcriptase was omitted.

and lack activity towards DCNB and EPNP. These results suggested that hGSTM4-4 might be the human counterpart of mouse GSTM7-7.

RT-PCR using *mGSTM7*-specific primers was performed to determine the tissue distribution of the *mGSTM7* transcript in the female A/J mouse. As shown in Figure 5(A), a 290 bp product corresponding to mGSTM7 was detected in every tissue examined, i.e. small intestine, forestomach, colon, liver, lung, kidney and spleen. Additional controls were included in RT-PCR reactions to establish the specificity of the amplification, since the transcript was detected in every tissue. As in Figure 5(A), a 290 bp PCR product was observed in the small intestine

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(Figure 5B, lane 1) and liver (Figure 5B, lane 4) in reaction mixtures containing RNA and the reverse transcriptase (positive control). Product was not detected in reactions where template RNA was omitted (Figure 5B, lanes 2 and 5 for small intestine and liver respectively). This technical control establishes the lack of sample and/or reagent contamination. More importantly, a product of 290 bp was not observed in reaction mixtures which contained template RNA but no reverse transcriptase (Figure 5B, lanes 3 and 6 for small intestine and liver respectively). This establishes that the template is in fact RNA, rather than contaminating genomic DNA.

It is noteworthy that, in spite of the ubiquitous expression of mGSTM7 mRNA which encodes a protein with pI 5.65 (Figure 3A), in our previous studies on the purification of GSTs we could detect a Mu class GST with a pI close to 5.5 only in the small intestine, but not in colon, liver, forestomach, or lung of female A/J mice [19,23,24]. Low abundance of mGSTM7-7 protein may have prevented its detection in other mouse tissues. Even in small intestine, mGSTM7-7 accounts for less than 1 % of total GST protein [19].

An EST clone (accession number AK003418) similar to *mGSTM7* has been recently deposited in the GenBank® database. The clone, derived from a cDNA library from an 18-day mouse embryo (strain C57BL/6J), was missing the initial 36 nucleotides of the coding sequence, and differed from *mGSTM7* in only three positions within the ORF (causing $Glu^{92} \rightarrow$ Ala and $Glu^{93} \rightarrow$ Gln substitutions), and in an additional eight nucleotides in the 3′ untranslated region. While the AK003418 clone was not annotated as a member of the GST superfamily and was not further characterized, it confirms the cloning results reported in the present paper. In addition, it indicates that mGSTM7-7 expression is not limited to the A/J strain used in the present study. The minor sequence differences between *mGSTM7* and AK003418 could be due to strain differences.

Detailed characterization of tissue-isolated GST5.5 has not been possible, mainly due to difficulty in obtaining sufficient amounts of purified protein. Even though the recombinant mGSTM7-7 and tissue-isolated GST5.5 resemble each other with respect to pI value and CDNB-conjugating activity, further studies are needed to firmly establish their identity. Studies are also needed to establish the physiological function of mGSTM7-7.

Note added in proof (received 25 July 2002)

During the review of this manuscript, Hayes and co-workers [37] published a paper describing the cloning of a murine GST isoform designated Gstm7. The deduced amino acid sequence of Gstm4 is identical with that of mGSTM7 described in the present study.

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