Molecular cloning and heterologous expression of an alternatively spliced human Mu class glutathione S-transferase transcript

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Two cDNA clones encoding a new Mu class glutathione S-transferase (GST) have been isolated from a human testis cDNA library. Both clones are incomplete and appear to result from alternative splicing. One clone is missing the sequence encoding exon 4 and the other is missing exon 8. The complete sequence of the previously undescribed isoenzyme can be deduced from the two cDNA clones. This is the first report of alternative splicing in a GST transcript and may represent either a novel form of regulation in this mutigene family or illegitimate transcription and experimental alternative splicing as part of the evolutionary process. By combining components from each clone a complete cDNA has been constructed and the encoded protein expressed in *Escherichia coli*. In general, the recombinant enzyme has relatively low activity when compared with all the previously described human Mu class GST isoenzymes.

INTRODUCTION

The glutathione S-transferases (GSTs) are a large family of multifunctional enzymes. Multiple GST isoenzymes have been identified in all mammalian species investigated so far and they appear to be expressed in a developmentally regulated and tissue-specific manner (Mannervik and Danielson, 1988; Board et al., 1990). Mammalian GSTs have at least two distinct functions. They have several catalytic activities, the most prominent being the conjugation of glutathione to an extensive range of electrophilic compounds. In addition, they can serve as intracellular binding and transport proteins for compounds such as bilirubin and haem (for review see Board et al., 1990). The documented substrates for many GSTs are mainly xenobiotics, including many carcinogens and several therapeutic compounds. GSTs have been shown to conjugate some cytotoxic drugs and have been implicated in the development of drug resistance in cancer chemotherapy (Wolf et al., 1990; Tew et al., 1990; Tsuchida and Sato, 1992). Endogenous substrates are less well known; however, some hydroperoxides and some of the end-products of lipid peroxidation such as alkene-dienals are metabolized by GSTs, and it has been postulated that the by-products of oxygen metabolism may be the primary endogenous substrates (Mannervik and Danielson, 1988).

GSTs from many species have now been characterized and mammalian cytosolic GSTs have been divided into four evolutionary classes, Alpha, Mu, Pi and Theta, on the basis of their biochemical characteristics, immunological relationships and N-terminal sequences (Mannervik et al., 1985, 1992). Studies of the human Mu class isoenzymes in a number of tissues have suggested that the class is quite extensive (Suzuki et al., 1987, 1991; Board et al., 1988; Campbell et al., 1990; Tsuchida et al., 1990; Hussey et al., 1991; Singhal et al., 1991). So far, at least three functional Mu class genes have been identified by the isolation of cDNA clones (Seidegård et al 1988; De Jong et al., 1988; Campbell et al., 1990; Vorachek et al., 1991).

RNA splicing is common to all eukaryotes, and regulated alternative splicing either may lead to the production of multiple protein isoforms from a single pre-mRNA or can function in a regulatory manner such as an on–off switch in development. Given the number of genes now known to be regulated by alternative splicing, this is clearly a significant option in addition to the regulation of promoter activity. The diversity of protein isoforms thus generated often show either developmental or tissue specificity (for reviews see Andreadis et al., 1987; Smith et al., 1989; McKown 1992). Although many of the processes regulating mRNA splicing are now understood, there is still much to be learnt about the recognition and regulation involved in alternative splicing and its role in gene regulation. The varying patterns of GST expression suggest that their regulatory mechanisms may be complex. Although the mechanisms described so far are largely pre-transcriptional (Daniel et al., 1989; Okuda et al., 1989; Rushmore and Pickett, 1990) there is some evidence that expression of the Pi class enzyme GSTP1-1 may be regulated by a post-transcriptional mechanism (Morrow et al., 1992).

As part of a programme to gain a greater understanding of the genetic diversity of the human GSTs we have investigated the GSTs expressed in human testis and have now identified cDNA clones that encode a new human Mu class GST isoenzyme that shows evidence of alternative splicing. Heterologous expression of cDNA in *Escherichia coli* has permitted the characterization of the new isoenzyme and its comparison with the previously described human Mu class GSTs.

MATERIALS AND METHODS

Materials

Reagents used in the bacterial culture media and buffers were supplied by Difco Laboratories (Detroit, MI, U.S.A.), Sigma Chemical Co. (St. Louis, MO, U.S.A.) and Ajax Chemical Co, (Sydney, NSW, Australia). A λgt11 human testes cDNA library was purchased from Clontech Laboratories (Palo Alto, CA, U.S.A.). The restriction endonucleases and buffers were purchased from Boehringer Mannheim Australia, Pharmacia Australia or Progen Industries (Darra, Queensland, Australia). The nitrocellulose filters and the T7 sequencing kit were from

Abbreviations used: GST, glutathione S-transferase; IPTG, isopropyl β-D-thio-galactoside; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; Npep, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; *t*-PBO, *trans*-4-phenyl-3-buten-2-one; pNPA, *p*-nitrophenyl acetate.

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The nucleotide sequence data reported here have been submitted to GenBank and have the accession numbers M99421 and M99422.

Amersham (U.K.). The [γ-^{32}P]dATP (4000 Ci/mmol) was purchased from Bresatec (Adelaide, Australia). The expression plasmid pKK261 was kindly provided by Dr. Rohan Baker of JCSMR, Australian National University. Pentylglutathione–agarose was prepared according to the method of Mannervik and Guthenberg (1981) from reduced glutathione supplied by Sigma Chemical Co. The enzyme substrates were purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) with the exception of the tritiated *trans*-stilbene oxide, which was supplied by Chemsyn Science Laboratories (Lenexa, KS, U.S.A.). Oligonucleotides were prepared on an Applied Biosystems 380B DNA synthesizer (Applied Biosystems, Foster City, CA, U.S.A.).

Isolation of cDNA clones encoding a new human GST Mu class subunit

A human testis λgt11 library was screened using a synthetic oligonucleotide probe (GT1 2B:5′ AGGAGACTGCTATCATGCCCATGATACTG 3′) based on the known sequence of GSTM1b (Seidegård et al., 1988). *E. coli* (Y1090) was infected with the human testis cDNA λgt11 library and plated at a density of 20000 plaques per 150 mm NZCYM plate by procedures described by Sambrook et al. (1989). The plates were incubated for 16 h and the resulting plaques were screened by the filter hybridization method of Benton and Davis (1977), with the oligonucleotide probe labelled with [γ-^{32}P]dATP by polynucleotide kinase. Plaque purification was achieved by replating and successive hybridizations with the original oligonucleotide probe. The hybridizations were conducted at 65 °C for 16 h in 5 × Denhardts solution and filters were washed, with several changes, in 2 × SSC containing 0.1 % (w/v) SDS at 65 °C as described by Sambrook et al. (1989).

DNA sequence analysis

The cDNA inserts in the cross-hybridizing λ phage were excised by *Eco*RI digestion and, after isolation by agarose gel electrophoresis and purification with Geneclean II (Bio 101, La Jolla, CA, U.S.A.), the fragments were subcloned into pUC18 and subsequently into M13 phage mp18 or mp19. Single-stranded DNA for sequencing was prepared by the miniprep method of Sanger et al. (1980) and the sequencing reactions were resolved on 6 % polyacrylamide gels according to the methods described by Sambrook et al. (1989). All sequences were obtained on both strands and all restriction sites used in subcloning were overlapped.

Construction of a full-length GSTM4 cDNA transcript

To determine whether the GSTM4 protein was functional, a plasmid containing a combined cDNA was constructed from the λGST-T and λT7.3 sequences and placed downstream of a *tac* promoter by the strategy shown in Figure 1. The plasmid subclones pGST-T and pT7.3 were digested with *Nco*I, which cuts once within the cDNA, and *Hin*dIII, which cuts only in the polylinker. The vector containing the 5′ portion of insert from pT7.3 and the *Nco*I to *Hin*dIII insert fragment (consisting of the 3′ sequence) from pGST-T were recovered by agarose gel electrophoresis. These fragments were ligated together and transformed into *E. coli* (TG1). The structure of the resulting clone (pGSTM4) was verified by restriction analysis with *Bam*HI and *Bgl*II. To facilitate cloning and expression an *Eco*RI site and a ribosome binding site were added to the GSTM4 cDNA by PCR amplification with the 5′ forward oligonucleotide primer (GTT ExA). The 3′ reverse oligonucleotide primer (GT1 ExB) incorporated a *Sal*I restriction site (primers are detailed in Table 1). PCR was carried out with Taq polymerase (Promega, Madison, WI, U.S.A.), with an initial five cycles with the denaturing step at 93 °C for 1 min, annealing at 40 °C for 1 min and extension at 70 °C for 1 min. This was followed by 25 cycles with the denaturing step at 93 °C for 1 min, and annealing and extension at 70 °C for 2 min. The PCR product was digested with *Eco*RI and *Sal*I and isolated by agarose gel electrophoresis. The fragment was purified using Geneclean II, ligated with the expression vector pKK261 and transformed into *E. coli* (TG1). This vector has been modified from pKK223-3 by removing a *Bam*HI–*Sal*I fragment from the backbone of the vector. Screening of the clones was carried out by the growth of isopropyl β-D-thiogalactoside (IPTG)-induced 5 ml cultures, sonication and a spectrophotometric activity assay with 1-chloro-2,4-dinitrobenzene (CDNB). A selection of clones showing activity were recloned into M13 and sequenced for verification. The clone used

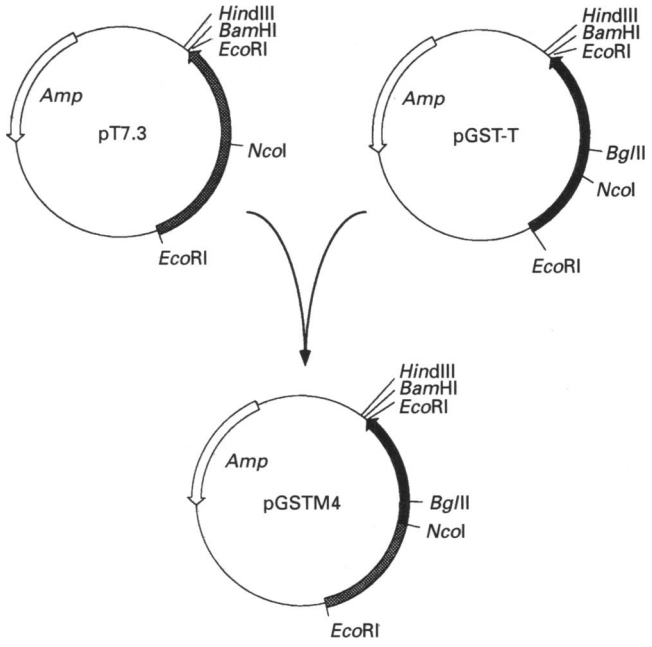

Figure 1 Schematic diagram showing the construction of pGSTM4

The *Nco*I to *Hin*dIII fragment was removed from pT7.3 and replaced by the equivalent fragment from pGST-T. The backbone plasmid is pUC18.

Table 1 Oligonucleotide primers used for the amplification and addition of restriction sites to human Mu class cDNAs

The names of the forward primers end in A and the names of the reverse primers end in B. The sequences were obtained from the following sources: GSTM2, Vorachek et al. (1991); GSTM3, Campbell et al. (1990).

cDNA	Oligonucleotide	Sequence
GSTM2	GT4 ExA	5′ TCGAATTCAGGAGACTGCTATCATGCCCATGACACTG 3′
	GT1 ExB	5′ AAGTCGACCCTACTTGTTGCCCCCAGAC 3′
GSTM3	GTM3 ExA	5′ TCGAATTCAGGAGACTGCTATCATGTCGTGCGAGTG 3′
	GTM3 ExB	5′ AACAAGCTTGCAAGTCTGCCTCCTGC 3′
GSTM4	GTT ExA	5′ TCGAATTCAGGAGACTGCTATCATGTCCATGACACTG 3′
	GT1 ExB	5′ AAGTCGACCCTACTTGTTGCCCCAGAC 3′

in the following studies for the expression of GSTM4-4 was termed pKKGSTM4.

Generation of other Mu class GST sequences for expression in *E. coli*

To compare the properties of all the known human Mu class GSTs we expressed and purified the enzymes GSTM1b-1b (clone pKKGST1; generously provided by Dr. Gordon S. Rule, Department of Biochemistry, University of Virginia, Charlottsville, VA, U.S.A.), GSTM2-2 (pKKGSTM2) and GSTM3-3 (pKKGSTM3). The latter two were generated by PCR amplification from the testis cDNA library. The primers were derived from the published sequences and are detailed in Table 1. Procedures used for the PCR amplification and cloning of GSTM2-2 and GSTM3-3 were similar to those described for the production of pKKGSTM4, except that the 3′ reverse primer for GSTM3 has a *Hin*dIII site instead of a *Sal*I site.

Clones expressing GSTM2-2 and GSTM3-3 were sequenced for confirmation. In comparison with the published nucleotide sequence (Vorachek et al., 1991) the GSTM2 sequence had a single T to C substitution at base 223 (numbered from the initiating ATG). This is a silent change and does not alter the amino acid sequence. In the GSTM3 sequence we identified a T to G substitution at base 440. This results in a Trp-147 to Gly substitution which may be a natural polymorphism. A PCR error can be discounted because the same substitution has been identified independently (M. Widersten and B. Mannervik, personal communication). This substitution is unlikely to have a critical effect on function as this residue is Gly in GSTM1, GSTM2 and GSTM4.

Purification of GST enzymes from *E. coli*

E. coli (TG1) containing the required expression plasmid was grown in two 500 ml cultures of RS both (2% tryptone, 1.5% yeast extract, 0.5% NaCl) to a D_{555} of 0.4 and then induced with 0.2 mM IPTG and grown overnight. Bacteria were collected by centrifugation, resuspended in an equal volume of 50 mM Tris/HCl, pH 7.2, 50 mM EDTA, 15% glucose and 1 mg/ml lysozyme, and incubated on ice for 1 h. After addition of 170 μM phenylmethanesulphonyl fluoride the cells were sonicated (Branson Sonifer 250) for 3 × 20 s at setting 4 and the soluble fraction was collected after centrifugation at 25000 ***g*** for 20 min. The extraction procedure was repeated on the pellet and the soluble fractions were pooled.

All enzymes were purified by affinity chromatography on pentylglutathione–agarose according to a modification of the method described by Mannervik and Guthenberg (1981) using 50 mM Tris/HCl, pH 7.2. Purification was monitored by an activity assay using CDNB (Habig and Jakoby, 1981). The GSTs were eluted from the column in 50 mM Tris, pH 7.2, containing 5 mM pentylglutathione. The fractions exhibiting activity were dialysed against 50 mM Tris/HCl, pH 7.2, 1 mM EDTA and 0.2 mM dithiothreitol. The enzyme was then concentrated in a Diaflo PM10 ultrafilter (Amicon Corp., Lexington, MA, U.S.A.) and immediately assayed for activity with various substrates. All operations in the purification procedure were performed at 4 °C. The purified enzymes were examined by SDS/PAGE by the method of Laemmli (1970). To determine the isoelectric point, the recombinant proteins were subjected to isoelectric focusing on polyacrylamide gels with standard proteins of known pI (Pharmacia). Protein assays were carried out using the method of Peterson (1977).

Table 2. Sizes of native recombinant Mu class GSTs determined by gel filtration

Values for the native protein are means of two determinations.

	Molecular mass (kDa)	
GST	Deduced subunit	Native protein
GSTM1b-1b	25.7	40.6
GSTM2-2	25.7	41.7
GSTM3-3	26.7	49.0
GSTM4-4	25.6	41.9

Enzyme assays

Specific activities were determined with a range of substrates in spectrophotometric assays (see Table 3) according to the procedures of Habig and Jakoby (1981) and Brophy et al. (1989); gluthathione peroxidase activity was determined by the method of Beutler (1975). All assays were conducted at 30 °C. Activity with tritiated *trans*-stilbene oxide was determined by a procedure based on the method of Seidegård et al. (1984) and Seidegård and Pero (1985). The activity was measured in 250 mM Tris/HCl, pH 7.2, containing 5 mM reduced glutathione and 4.75 μM *trans*-stilbene oxide. Initially the reaction mixture was incubated at 37 °C and incubations were terminated after 5, 10 or 15 min. Over the longer time span it was found that the reaction rate was non-linear; therefore all subsequent reactions were determined over 5 min. All reactions were terminated by extraction with 2 vol. of hexanol. Vortexing and centrifugation produced phase separation. The amount of tritiated product was determined in a 20 μl aliquot of the aqueous phase by liquid scintillation counting.

Amino acid sequencing

The four recombinant Mu class enzymes studied were subjected to N-terminal amino acid sequencing for at least six cycles on an Applied Biosystems automated sequencer, model 477A.

Immunological characterization

Purified proteins were separated on SDS/12%-PAGE and electroblotted on to nitrocellulose filters as described by Towbin et al. (1979). The filters were then probed with antiserum directed against either denatured GSTM1b-1b or native GSTM2-2 (Board et al., 1988).

Native size determination

Purified recombinant GSTs were passed through an f.p.l.c. Superose-12 column (Phamacia). Elution volumes were determined in a buffer consisting of 150 mM NaCl and 10 mM $NaPO_4$, pH 7.2, in comparison with protein standards of known size (BSA, 66 kDa; ovalbumin, 45 kDa, trypsinogen, 24 kDa).

RESULTS

Characterization of the new GST cDNA clones

The sequencing of cross-hybridizing cDNA clones from the testis library has identified four clones with a high level of similarity to the GSTM1 sequence. Two clones encoded GSTM2 (formerly GST4), which has been described by Board et al. (1988) and cloned by Vorachek et al. (1991). Two other clones were clearly

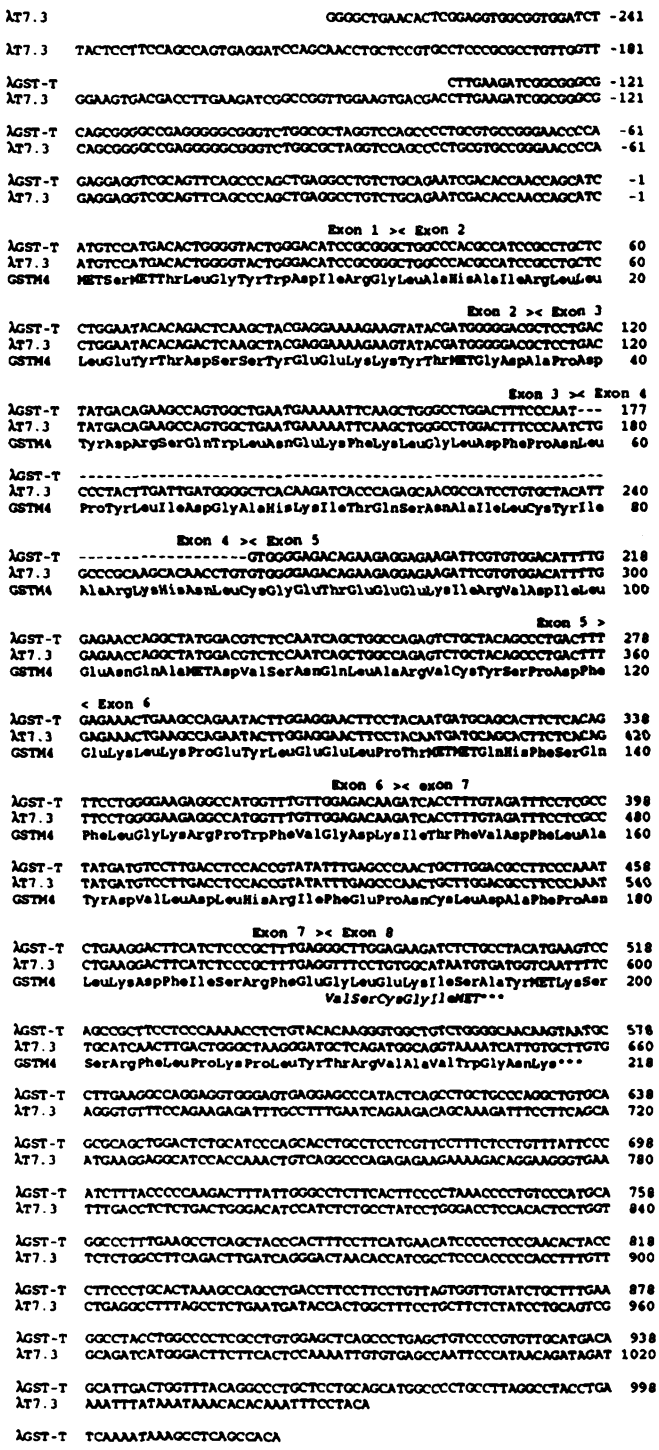

Figure 2 Alignment of the nucleotide sequences of two cDNA clones encoding the GSTM4 subunit

The predicted splice sites are shown above the sequence. The six substituted residues at the new truncated C-terminal of the protein encoded by λT7.3 are shown below the GSTM4 sequence at the start of exon 8.

related and encoded transcript of a new GST gene. The first clone sequenced (designated λGST-T) is 1158 nucleotides long with a 95-nucleotide poly(A) tail extension (Figure 2). This clone contains a 5′ non-coding region of 147 nucleotides and a 3′ non-coding region of 462 nucleotides. It was found to have an 82-nucleotide deletion which corresponds to exon 4 in the other known Mu class genes. The deletion is excised exactly at the splice junctions expected from comparison with rat and human Mu class genes (Lai et al., 1988; Taylor et al., 1991). The deletion of exon 4 causes a shift in the reading frame resulting in 36 missense amino acids before a stop codon.

The other clone (λT7.3) isolated from the same testis library is 1323 nucleotides long and also finishes with a poly(A) tail (Figure 2). It has a 5′ non-coding region of 270 nucleotides and was found to contain the exon 4 sequence deleted from λGST-T. However, the similarity of the coding region stops at the 3′ boundary of exon 7 and continues with unrecognizable sequence that may be intronic in origin (Figure 2). The junction between the coding and non-recognizable regions is again precisely at the predicted splice site, and has resulted in the omission of exon 8 which, based on λGST-T, contains sequence for 23 amino acids of the C-terminal region as well as the 3′ untranslated region before the poly(A) tail. The presence of putative intronic sequence results in the substitution of six alternative amino acids before an in-frame stop codon.

Apart from the deleted region encoding exon 4 and the substituted exon 8, the complete coding sequences of λGST-T and λT7.3 are identical and it appears likely that each clone represents a separate transcript of the same Mu class gene. The λT7.3 clone has a relatively long 5′ non-coding region that extends to −271 nucleotides. Although we cannot be absolutely certain that this is not a cloning artefact, the presence of the identical sequence to the 5′ limit of λGST-T confirms that it is a true copy of the transcript to at least −147 nucleotides.

Features of the encoded GST subunit

The complete amino acid sequence of the isoenzyme subunit can be deduced by combining sequences from λGST-T and λT7.3, and is compared in Figure 3 with other previously described human Mu class sequences. After discussion with other members of the GST nomenclature group (Mannervik et al., 1992) it is appropriate that this new enzyme be termed GSTM4 in agreement with the new nomenclature system.

GSTM4 consists of a peptide of 218 amino acids and is identical in length to GSTM1 and GSTM2. The deduced sequence of GSTM4 indicates that it has a molecular mass of 25561 Da. This is not substantially different from the deduced molecular masses of GSTM1 (25697 Da) and GSTM2 (25744 Da), but is somewhat smaller than that of GSTM3 (26688 Da), which is 7 amino acids longer. The amino acid sequence of GSTM4 has 87% similarity with GSTM1, 83% similarity with GSTM2 and only 72% similarity with GSTM3, the only other human Mu class isoenzymes for which a complete sequence is known.

Characterization of the human Mu class GSTs expressed in *E. coli*

When pKKGSTM4 was grown in the presence of IPTG, significant quantities of recombinant GSTM4-4 were expressed and preliminary determinations demonstrated it was functional with CDNB as a substrate. Similar results were obtained with the plasmids pKKGST1, pKKGSTM2 and pKKGSTM3. Large-scale preparations showed that recombinant GSTM4-4 could be produced by pKKGSTM4 at the rate of 12 mg/l after purification by affinity chromatography. Recombinant GSTM1 was produced at the rate of 4 mg/l by pKKGST1, GSTM2-2 was produced by pKKGSTM2 at the rate of 14 mg/l and pKKGSTM3 produced GSTM3-3 at a rate of 6 mg/l.

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GSTM1    ----MPMILGYWDIRGLAHAIRLLLEYTDSSYEEKKYTMGDAPDYDRSQW   46
GSTM2    ----MPMTLGYWNIRGLAHSIRLLLEYTDSSYEEKKYTMGDAPDYDRSQW   46
GSTM3    MSCESSMVLGYWDIRGLAHAIRLLLEFTDTSYEEKRYTCGEAPDYDRSQW   50
GSTM4    ----MSMTLGYWDIRGLAHAIRLLLEYTDSSYEEKKYTMGDAPDYDRSQW   46
             * **** ****** ****** ** ***** ** * *********

GSTM1    LNEKFKLGLDFPNLPYLIDGAHKITQSNAILCYIARKHNLCGETEEEKIR   96
GSTM2    LNEKFKLGLDFPNLPYLIDGTHKITQSNAILRYIARKHNLCGESEKEQIR   96
GSTM3    LDVKFKLDLDFPNLPYLLDGKNKITQSNAILRYIARKHNMCGETEEEKIR  100
GSTM4    LNEKFKLGLDFPNLPYLIDGAHKITQSNAILCYIARKHNLCGETEEEKIR   96
         *  **** ********* **  ********* ******* *** * * **

GSTM1    VDILENQTMDNHMQLGMICYNPEFEKLKPKYLEELPEKLKLYSEFLGKRP  146
GSTM2    EDILENQFMDSRMQLAKLCYDPDFEKLKPEYLQALPEMLKLYSQFLGKQP  146
GSTM3    VDIIENQVMDFRTQLIRLCYSSDHEKLKPQYLEELPGQLKQFSMFLWKFS  150
GSTM4    VDILENQAMDVSNQLARVCYSPDFEKLKPEYLEELPTMMQHFSQFLGKRP  146
          ** *** **   **   **    ***** **  **       * ** *

GSTM1    WFAGNKITFVDFLVYDVLDLHRIFEPNCLDAFPNLKDFISRFEGLEKISA  196
GSTM2    WFLGDKITFVDFIAYDVLERNQVFEPSCLDAFPNLKDFISRFEGLEKISA  196
GSTM3    WFAGEKLTFVDFLTYDILDQNRIFDPKCLDEFPNLKAFMCRFEALEKIAA  200
GSTM4    WFVGDKITFVDFLAYDVLDLHRIFEPNCLDAFPNLKDFISRFEGLEKISA  196
         ** * * *****  ** *     * * *** ***** *  *** **** *

GSTM1    YMKSSRFLPRPVFSKMAVWGNK---   218
GSTM2    YMKSSRFLPRPVFTKMAVWGNK---   218
GSTM3    YLQSDQFCKMPINNKMAQWGNKPVC   225
GSTM4    YMKSSRFLPKPLYTRVAVWGNK---   218
         *  *  *   *     * ****
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Figure 3 Amino acid sequence of the known human Mu class GST isoenzymes aligned with the deduced sequence of GSTM4

Identical residues are identified (*). The sequences were obtained from the following sources: GSTM1, Seidegård et al. (1988); GSTM2, Vorachek et al. (1991); GSTM3, Campbell et al. (1990).

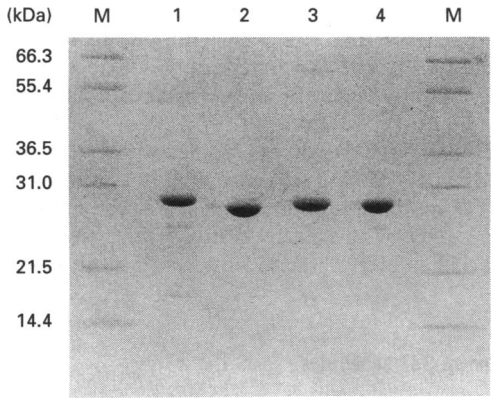

Figure 4 SDS/PAGE of recombinant human Mu class GSTs

Lane 1, GSTM1b; lane 2, GSTM2; lane 3, GSTM3; lane 4, GSTM4; lanes M, standard size markers.

Subunit and protein size, and immunological relationships

Samples of the purified GSTs were analysed by SDS/PAGE and are shown in Figure 4. The molecular masses of GSTM1, GSTM3 and GSTM4 are similar. In this experiment GSTM2 appears to be slightly smaller than the other subunits despite the fact that it has a deduced molecular mass similar to those of GSTM1 and GSTM4. In contrast, GSTM3 has a deduced molecular mass of approx. 1 kDa greater than that of the other subunits, but this difference could not be reliably detected in a number of experiments.

To confirm that the recombinant GSTs were assembling in *E. coli* as dimers, the purified proteins were subjected to gel filtration on an f.p.l.c. Superose-12 column in comparison with standard proteins of known size. The estimated sizes of the recombinant proteins are shown in Table 2 along with their deduced subunit molecular masses. Although they are slightly smaller than may be predicted from their deduced sequence, they are clearly in the range expected for dimers.

Western blots of SDS/PAGE gels were produced with antiserum raised against either denatured GSTM1-1 or native GSTM2-2 (results not shown). Antiserum directed against GSTM2-2 recognized GSTM1 and GSTM4; however, it failed to recognize GSTM3. In contrast, antiserum raised against denatured GSTM1-1 cross-reacted with all Mu class subunits. Possibly denaturation of the antigen reveals some epitopes that are conserved in all four isoenzymes.

Isoelectric focusing

The isoelectric points (pIs) of the recombinant GSTs were determined by isoelectric focusing in the range 4–6.5 in a polyacrylamide gel (Figure 5). The pI of GSTM1b-1b was 5.7, the pI of GSTM2-2 was 5.4 and the pI of GSTM4-4 was 5.2. A comparison of GSTM4-4 with GSTM1b-1b reveals six amino acid substitutions that result in charge differences. Although these charge changes are balanced, and there is no net change, the positions of the substitutions in the mature folded protein clearly influence its isoelectric point.

N-terminal sequencing

The N-terminal sequences for all four recombinant proteins were identical with those predicted from their respective cDNAs. Approx. 15% of the GSTM3 preparation retained the initiating

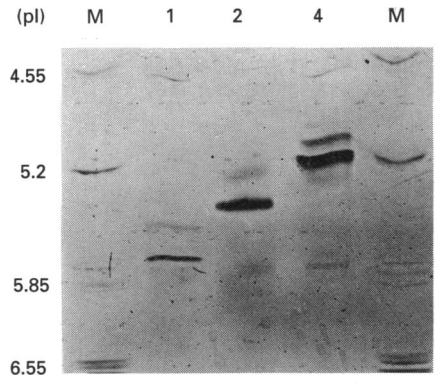

Figure 5 Isoelectric focusing of recombinant human Mu class enzymes

Lane 1, GSTM1b-1b; lane 2, GSTM2–2; lane 4, GSTM4–4; lanes M, standard proteins of known isoelectric point.

methionine, whereas this fraction was $\leqslant 5\%$ in the cases of GSTM1, GSTM2 and GSTM4. The N-terminal sequencing also detected a small ($\sim 2\%$) fraction of sequences where the first three residues were deleted, suggesting that in a small number of transcripts the translation of the protein is initiated from the second methionine in the sequence.

Substrate specificities

The compounds used for enzyme rate assays are known to be substrates for GST enzymes from different classes (Table 3). Recombinant GSTM4-4 has a lower level of activity with the model substrate CDNB compared with either GSTM1b-1b or GSTM2-2, but the value is similar to that of GSTM3-3. This is also true using *trans*-4-phenyl-3-buten-2-one (*t*-PBO) as the second substrate. All the Mu class enzymes showed a low but consistent activity with ethacrynic acid. GSTM4-4 has no discernible activity with either DCNB or 1,2-epoxy-3-(*p*-nitrophenoxy)propane (Npep), although all the other isoenzymes have some activity with the former and GSTM2 at least has activity with the latter. In this study GSTM1b-1b had no detectable activity with the second substrate *p*-nitrophenyl acetate (pNPA), and the activity exhibited by the remaining enzymes was low.

Mu class GST enzymes have been considered as efficient catalysts with some epoxide substrates (Seidegård and Pero, 1985; Mannervik and Danielson, 1988). However, in this comparison of Mu class recombinant enzymes GSTM1b-1b had at least a 1000-fold higher activity with *trans*-stilbene oxide than any of the other enzymes. The reaction rate of GSTM4-4 is marginally higher, though not significantly so, than those of GSTM2-2 and GSTM3-3. In the present study there was no detectable peroxidase activity in any of the recombinant enzymes; however, Campbell et al. (1990) reported a low level of activity against cumene hydroperoxide with GSTM-3 purified from human testis.

Although there was no detectable activity with the lipid hydroperoxide model substrates, all the Mu class enzymes showed conjugating activity with both classes of reactive carbonyl compounds, *trans*-alk-2-enals and *trans,trans*-alk-2,4-dienals. The activity of each enzyme increased with increasing carbonyl chain length in both series. GSTM1b-1b and GSTM2-2 are more reactive with the *trans,trans*-alk-2,4-dienals than either GSTM3-3 or GSTM4-4. However, GSTM3-3 has a higher activity with the *trans*-alk-2-enals than does GSTM2-2.

DISCUSSION

Evidence for a new Mu class locus

There is evidence that the human Mu class is the most extensive of all the classes in the human GST gene family. The isoenzymes GSTM1-1 and GSTM2-2 have been well characterized (Warholm et al., 1983; Vander Jagt et al., 1985; Soma et al., 1986; Suzuki et al., 1987; Board et al., 1988; Seidegård et al., 1988; De Jong et al., 1988; Vorachek et al., 1991). In addition, Campbell et al. (1990) have cloned and characterized a distinct Mu class isoenzyme (GSTM3-3) from brain and testis. Other studies by Tsuchida et al. (1990), Hussey et al. (1991), Singhal et al. (1991) and Suzuki et al. (1991) have provided limited N-terminal sequence and structural data suggesting that there are additional Mu class GSTs expressed in human tissues. In the present study four Mu class cDNA clones were identified; two were copies of GSTM2 which has been described by Board et al. (1988) and

Table 3 Comparison of specific activities of human Mu class isoenzymes with a range of known GST substrates

Values are expressed as μmol · min^{-1} · mg^{-1}, except those for *trans*-stilbene oxide activity, which are expressed as nmol · min^{-1} · mg^{-1}. All values are the means ± S.D. of triplicate determinations. n.d., not detectable.

Substrate	GSTM1	GSTM2	GSTM3	GSTM4
CDNB	107.4 ± 8.1	186.4 ± 7.1	6.0 ± 0.5	1.25 ± 0.02
DCNB	0.7 ± 0.30	3.4 ± 0.5	0.11 ± 0.01	n.d.
Npep	n.d.	7.0 ± 0.5	n.d.	n.d.
t-PBO	0.06 ± 0.01	0.07 ± 0.07	n.d.	n.d.
p-NPA	n.d.	1.7 ± 1.13	0.20 ± 0.02	0.03 ± 0.01
Ethacrynic acid	0.93 ± 0.23	3.22 ± 0.09	0.06 ± 0.01	0.04 ± 0.002
Cumene hydroperoxide	n.d.	n.d.	n.d.	n.d.
t-Butyl hydroperoxide	n.d.	n.d.	n.d.	n.d.
trans-Stilbene oxide	10477 ± 1059	0.29 ± 0.58	0.41 ± 0.81	2.95 ± 2.54
trans,trans-Hexa-2,4-dienal	0.15 ± 0.05	0.17 ± 0.02	0.03 ± 0.01	0.004 ± 0.001
trans,trans-Hepta-2,4-dienal	0.34 ± 0.04	0.43 ± 0.03	0.07 ± 0.06	0.02 ± 0.001
trans,trans-Deca-2,4-dienal	0.36 ± 0.005	0.52 ± 0.01	0.08 ± 0.03	0.03 ± 0.01
trans-Hex-2-enal	0.58 ± 0.02	0.12 ± 0.02	0.35 ± 0.01	0.01 ± 0.001
trans-Oct-2-enal	1.07 ± 0.20	0.24 ± 0.01	0.54 ± 0.03	0.09 ± 0.07
trans-Non-2-enal	1.48 ± 0.05	0.31 ± 0.04	0.75 ± 0.18	0.10 ± 0.02

Vorachek et al. (1991) and was previously thought to be muscle-specific. Two additional clones have been shown to represent transcripts of a novel gene to be known as *GSTM4*. The nucleotides encoding exons 3, 4 and 5 from the GSTM4 cDNAs show complete identity with the partial genomic sequence termed *mu2* by Taylor et al. (1991). This identity suggests that the partial gene cloned by Taylor et al. (1991) represents the gene encoding λGST-T and λT7.3 and that these clones do not represent an allelic variant of a previously described locus. Northern blots have confirmed that this gene is transcribed in testis (Taylor et al., 1991), but further studies are required to determine whether it is transcribed in other tissues. The deduced amino acid sequence of the GST subunit encoded by λGST-T and λT7.3 shows greatest similarity with that of GSTM1 and it is clearly of the Mu class. It is therefore evident that λGST-T and λT7.3 cDNAs represent transcripts encoding a new Mu class subunit.

Splicing of the GST transcript

Notably, both GSTM4 cDNA clones show evidence of alternative splicing at different sites. The excision of exon 4 in λGST-T changes the reading frame so that no meaningful GST sequence would be translated after the end of exon 3. This gross truncation and change in the C-terminal sequence is likely to result in a catalytically inactive protein, as we have previously demonstrated the inactivation of an Alpha class GST by a similar deletion (Board and Mannervik, 1991). In λT7.3 the sequence encoding exon 8 has been replaced by another sequence that is possibly intronic in origin. The inclusion of this new sequence in the subunit would substitute six new amino acids and a new C-terminus in place of the 23 amino acids lost by the removal of exon 8. It is highly likely that a subunit derived from this transcript would also lack GST catalytic activity, as the truncation of GSTA1-1 (formerly GST2) by 12 residues resulted in a substantial loss of activity (Board and Mannervik, 1991). Although both transcripts are unlikely to encode catalytically active GSTs, they may still encode proteins with other properties such as ligand binding.

In other studies of GST cDNAs from a variety of tissues, we have not observed any other cases of alternative splicing and we are not aware of any other published examples within this large family of genes. cDNA clones of other GST genes isolated from the same library, including two GSTM2 clones, have not shown similar evidence of alternative splicing, suggesting that it is not a tissue-specific phenomenon but may be a property of the gene. Porter and Mintz (1991) have recently reported extensive alternative splicing of transcripts of the mouse tyrosinase gene in several tissues, suggesting in that case that the events were gene-specific. There is a growing body of evidence suggesting that alternative splicing of transcripts provides an important mechanism for gene regulation (Smith et al., 1989; Maniatis, 1991; McKown, 1992). Most GST genes studied so far appear to be predominantly regulated by the initiation of transcription (Daniel et al., 1989; Okuda et al., 1989; Rushmore and Pickett, 1990). Morrow et al. (1992) have suggested that the *GSTP1* gene may be regulated by post-transcriptional mechanisms. It is possible that the alternative splicing of *GSTM4* transcripts represents a form of gene regulation not previously noted in the GST gene family.

Alternatively, studies have shown that cells may permit a limited amount of illegitimate transcription (Chelly et al., 1989; Fonknechten et al., 1992), or there may be an inherent variability in the splicing process as a means of generating new gene products without the requirement for permanent change to the nucleotide sequence (Andreadis et al., 1987; Porter and Mintz, 1991). Both of these may be part of a continuum of permissive transcription allowing novel products to be available to cells for eventual evolutionary selection. Thus the GST-T and T7.3 transcripts of *GSTM4* may represent either illegitimate transcription or examples of 'experimental' alternative splicing in the testis. Further studies are required to determine which tissues express *GSTM4* and if full-length or truncated forms of the protein are detectable.

It is interesting to note that Taylor et al. (1991) have recently speculated that the human Mu class genes may be subjected to a novel form of regulation by somatic gene conversion where intronic sequences containing elements that influence transcription may convert a related gene and alter its regulation. If this proposed mechanism does function within the Mu class genes, it is not immediately evident what role, if any, alternative splicing would play.

Catalytic properties

The different classes of GST enzymes have characteristic but overlapping substrate specificities. Previously, it has been difficult to directly compare the catalytic properties of a range of isoenzymes from within a class. By the use of the heterologous expression in *E. coli*, we have been able to prepare recombinant forms of all known human Mu class isoenzymes and compare their properties under the same conditions.

The marked specificity of GSTM1-1 for *trans*-stilbene oxide is clear. In comparison, the other isoenzymes show no similar dramatic substrate preferences. This suggests that the primary substrate specificities of the other enzymes may not have been included in the study and have yet to be identified. Previous studies by Seidegård et al. (1984) have suggested that individuals with GSTM1 deficiency can be identified by assaying blood monocyte GST activity with *trans*-stilbene oxide as a substrate. Seidegård et al. (1984) have also shown that Alpha and Pi class isoenzymes do not utilize this substrate. The present results show for the first time that other human Mu class isoenzymes do not utilize *trans*-stilbene oxide at a significant rate. This study therefore confirms the value of *trans*-stilbene oxide for the detection of GSTM1 deficiency.

The specific activity of GSTM4-4 was low with all the substrates tested in this study. Its low activity with CDNB possibly explains why this enzyme has not been detected in previous studies.

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