Identification of a novel murine glutathione S-transferase class mu gene

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Screening of a genomic mouse DNA library with a glutathione S-transferase class mu cDNA probe resulted in the identification of mGSTM5, a novel member of the murine glutathione S-transferase class mu gene family. Here we present the sequence of the promoter region, the exon–intron organization of the gene and the isolation and characterization of its complete cDNA. Conceptual translation of the cDNA sequence revealed that several amino acid positions have been changed in 'invariant' mu class signature sequences in mGSTM5. Reverse transcriptase polymerase chain reaction using gene specific primers revealed that mGSTM5 is uniquely expressed in mouse liver, stomach and small intestine.

INTRODUCTION

Glutathione S-transferases (GSTs, EC 2.5.1.18) are an important family of multifunctional isoenzymes, which play a role in the protection of tissues by detoxification of hazardous and carcinogenic compounds. The main function of the GSTs is the catalysis of nucleophilic addition of glutathione (GSH) at electrophilic centres of xenobiotic compounds. This is the first step in the mercapturic acid pathway, which in most cases leads to elimination of the potentially toxic compound. In addition, GSTs also serve as intracellular binding and transport proteins of hydrophobic compounds, facilitating their transport and excretion. Furthermore, it has been suggested that GSH-peroxidase activity of GSTs towards lipid hydroperoxides might protect tissues during oxidative stress [1].

GSTs are identified in all eukaryotes and are expressed in a tissue specific and developmentally regulated manner. GST-levels may be increased by exposure to foreign compounds, suggesting that these proteins form part of an adaptive response towards chemical stress [1].

Extensive study of the GST multigene family resulted in the identification of several GST isoenzyme encoding genes. Based on their structural, biochemical and immunological properties, cytosolic GSTs are divided in four classes: alpha, mu, pi and theta [1]. Each class includes one or multiple isoenzymes, consisting of two subunits, occurring as either homo- or heterodimers. In mice four mu class GST genes (mGSTM1-4) have been identified so far, based on isolated cDNA and genomic DNA sequences [2–5]. The multibanded pattern obtained after Southern hybridization of mouse genomic DNA with mouse GST mu cDNA probes [2] suggests that additional mu GST genes might exist. In the present study we report the identification of a new functional murine GST class mu gene and corresponding cDNA with a high homology to the other murine GST class mu subunit genes, but displaying a distinct tissue-specific expression.

MATERIALS AND METHODS

Materials

Primers and enzymes were purchased from either Promega (Madison, WI, U.S.A.), Life Technologies (Bethesda, MD, U.S.A.) or Boehringer Mannheim (Mannheim, Germany). Radioisotopes were procured from Amersham (Bucks, U.K.). Mouse strains C57Bl6, Balb/c and FVB were obtained from the Central Animal Laboratory, University of Nijmegen, The Netherlands.

Phage library screening

Mouse strain 129 Sv/E genomic DNA library in phage EMBL3 [6] was plated on *E. Coli* LE 392 host bacteria and 3×10^5 plaques were screened with a ^{32}P random prime labelled 886 bp cDNA fragment from pmGT10 [3] using filter hybridization [7]. Positively hybridizing phages were plaque-purified. The GST mu phage DNA inserts were subcloned in pBluescript SK phagemids (Stratagene) and tested for the presence of GST mu sequences using colony hybridization [7]. Ultimately, four positive subclones with inserts of approx. 7–8 kb were obtained. After resolving the restriction maps, the clones were subsequently subcloned and sequenced in duplicate using the Sanger dideoxynucleotide method [8].

RT PCR

After sequencing it became obvious that clone 4.6 contained part of a new GST mu gene. To determine whether this gene is functionally active a specific primer pair was designed to use in a RT PCR. Total RNA was isolated from different mouse tissues using Trizol reagent according to the manufacturers instructions (Life Technologies). RNA (1 μg) was reverse transcribed using mGSTEx5 primer, which recognizes all known GST mu mRNAs. Next, the mGSTM5Ex1 and mGSTM5Ex4/5 primer set was used for amplification of clone 4.6 specific cDNA. Therefore 3 μl of RT mix was subjected to 33 cycles of PCR (94, 48 and 72 °C for 60, 60 and 10 s. respectively) followed by 10 min extension at 72 °C in 50 μl of PCR mix (50 mM KCl, 10 mM Tris/HCl pH 9.0, 1.75 mM $MgCl_2$ and 0.2 mM dNTPs) with 2.5 U Taq polymerase in a Thermojet apparatus (Eurogentec, Seraing, Belgium). As positive control a RT PCR with mGSTEx4 and mGSTEx5 primer pair was used. RT PCR fragments were

Abbreviations used: RT PCR, reverse transcriptase PCR; ORF, open reading frame.

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The nucleotide sequence data reported will appear in EMBL and GenBank Nucleotide Sequence Databases under the accession numbers AJ 000412 and AJ 000413.

A

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-298   agatctga gcccagattc ccagagtcta ggtgtgaaaa actgatctac caggaaagga
-240 gatgggcaga acaggtccta gctggcctgt ctccagggat actgccaact tcctgagaat
-180 ccagcagggg gcagatggaa gcagggcctg ccaatcaatc actgccttcg gattagatgc
-120 tttacttgtt acagtgggtt ggctttatgg ttccacggtt ggttccttgg gagggcagag
 - 60 cagctccgga cctgttctca gactcatcag ctgagcatta cagagtcttt cccgaccagt
   1 GACTGACTGA CACAGTCTTC GAGGCTGGAA TAGCAATCAT GCCCGTGACT CTGGGATATT
                                          mGSTM5Ex1
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B

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<--------------------------------Exon1-------><--------------------
AATCATGCCC GTGACTCTGG GATATTGGGA TATCCGTGGA CTGGGTCACG CCATCCGCCT
     mGSTM5Ex1              Eco RV
-------------------------------Exon2---------------------------><---
GCTCCTGGA ATACACAGAA ACAGGCTATG AAGAGAGGAG ATACGCCATG GGGGACGCTCC
-------------------------------Exon3-----------------------------
TGACTATGA CAGAAGCCAG TGGCTGAATG ACAAGTTCAA GCTTGNCCTG GACTTTCCCAA
><---------------------------Exon4-----------------------------
TCTGCCCTA CTTAATTGAT GGGTCACACA AGGTCACCCA GAGCAATGCC ATCCTGCGCTA
                                          mGSTEx4
--------------------------><----Exon5-----------------------------
CCTTGGCCG GAAGCACAAC CTGTGTGGAG AGACAGAGGA GGAGAGGATC CGTGTGGACAT
          mGSTM5Ex4/5
-----------------------------------------------------------------
TTTGGAGAA CAGGGTCATG GACACTCGAA TTCAGATGGG CATGCTTTGC TACANCGCTGA
----><---------------------------Exon6-----------------------------
CTTTGAGAA ACGGAAGCCA GAGTTCTTGA AGGGCCTCCC AGATCAGCTG AAACTCTACTC
-----------------------------------------------><-----------------
GGAGTTCCT GGGGAAGCAG CCATGGTTTG CAGGGGACAA GATCACCTTT GCAGACTTCCT
-------------------------------Exon7-----------------------------
CGTCTATGA TGTCCTTGAT CAGCATCGAA TGTTTGAACC CACGTGCCTG GACGCCTTCCC
-----------------------------><-----------------------------------
AAACCTAAG GGACTTCATG GCCCGCTTTG AGGGCCTGAG GAAGATCTCT GCCTATATGAA
-------------------------------Exon8-----------------------------
GACCAGCCG CTTCCTTCCA AGTCCTGTGT ACTTAAAACA GGCCACGTGG GGCAATGAGTA
                                                    mGSTM5Ex8
->
Agaccatgc atggagtgcc ggtgttgtgg gagactatgc agtgtgcctg tcaccgtgaac
actgaccgg gccacagcta gctt(n)27t tcattaatct tccccatttt tttccctctgt
cttttcatt aactttccct ctacaagaac tccttgtctc cctttcactc aagccttccaa
tgtcagctc cctgtcctca gcaaagtcct ttgcttccct tgtttctttc tgccatgtttc
taaccagac aatctttcac tgcactttgn gcagcttagc ccaaagatcc atactccccag
tatgcaggc tattgtgtga gctctagtaa agtgttatcc atact(A)n
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Figure 1 Nucleotide sequences of the 5′ part of the mGSTM5 gene and mGSTM5 cDNA

(**A**) Nucleotide sequence of the 5′ part of the mGSTM5 gene, including the promoter region, as isolated from mouse strain 129Sv/E genomic mouse DNA library. The mGSTM5 was isolated and characterized up to half of exon 5. Nucleotide numbering, exon/intron boundary and position of the transcription site were based on homology to mGSTM1 and mGSTM3 gene sequences [4]. (**B**) Nucleotide sequence of mGSTM5 cDNA, including 3′UTR, as isolated and sequenced from mouse strain FVB total liver RNA. In the deduced sequence of the ORF of the cDNA only two nucleotides, indicated by a N, differed with the cDNA isolated from mouse strain C57Bl6. The names of the forward and reverse primers as used for sequencing mGSTM5 and in RT PCR and the exon–intron organization are indicated. The mGSTEx4 and mGSTEx5 primers recognize all known mouse GST mu gene sequences; mGSTM5Ex1, mGSTM5Ex4/5 and mGSTM5Ex8 specifically recognize mGSTM5. The EcoRV site located 18 nucleotides upstream of mGSTM5Ex1 primer unique for mGSTM5 is underlined. The mGSTM5 gene sequence up to exon 5 and whole cDNA sequence have been submitted to Genbank™/EMBL Data Bank under accession numbers AJ 000412 and AJ 000413, respectively.

purified on Qiagen columns, digested with EcoRV and resolved by agarose-gel electrophoresis.

All primer sequences used are indicated in Figure 1.

Isolation of whole cDNA with 3′ rapid amplification of cDNA ends (3′ RACE)

Whole mGSTM5 cDNA was amplified from total liver RNA from mouse strain FVB using 3′ RACE. For this purpose total liver RNA of FVB mice was reverse transcribed using oligodT-anchor primer of the 3′ RACE kit (Boehringer Mannheim). Subsequently the anchor primer of the same kit and the specific mGSTM5Ex1 primer was used for amplification of the whole cDNA. Therefore, 3 μl of RT mix was subjected to 33 cycles of PCR as described above. The PCR fragments were analysed on agarose gel and purified via Qiagen columns. After cloning into pGEMT (Promega) inserts were sequenced in duplicate using the dideoxynucleotide method [8]. Based on this nucleotide sequence a new primer was designed at the end of exon 8 (mGSTM5Ex8, Figure 1). This primer was subsequently used to reverse transcribe RNA from two different total liver RNA isolates of mouse strains FVB and C57Bl6. Obtained cDNAs were amplified by a PCR using primer pair mGSTM5Ex1 and mGSTM5Ex8. The resulting PCR fragments were cloned into pGEMT and sequenced in duplicate.

RESULTS

Cloning of a novel mouse GST class mu subunit gene

To isolate previously unidentified mouse GST class mu subunit genes a 129Sv/E genomic DNA library was screened with the mGSTM1 cDNA [3] using moderately stringent conditions. Two mGSTM1 positive clones, 5.14 and 4.6, were obtained. Sequencing of these clones with mGSTEx4 primer (Figure 1), and comparison with all identified murine mu class genes, demonstrated that clone 5.14 was identical to the previously reported mGSTM1 gene sequence [4]. The sequence of clone 4.6, however, did not match any of the published GST class mu genes. Based on the restriction endonuclease map of clone 4.6 (Figure 2) different deletion subclones were made in pBluescript and used for determining the entire sequence of the isolated genomic clone (Figure 1). Searches of GenBank DNA data showed that the overall sequence and gene organization matches those of other published mouse GST class mu genes. Based on the nomenclature used to classify GSTs, [9] and in consultation with Professor B. Mannervik, the gene will be referred to as mGSTM5 hereafter.

RT PCR

To investigate whether this gene was a genuine, functional active murine GST class mu gene an RT PCR was performed using the mGSTM5 specific primer pair mGSTM5Ex1 and mGSTM-

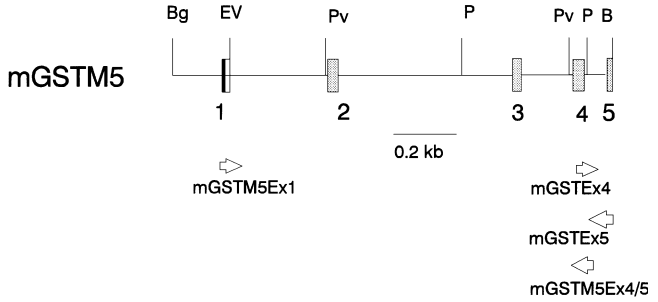

Figure 2 Restriction endonuclease map and gene organization of clone 4.6

Restriction enzymes used for gene sequencing were Bg (BglII), B (BamHI), Pv (PvuII), P (PstI) and EV (EcoRV).

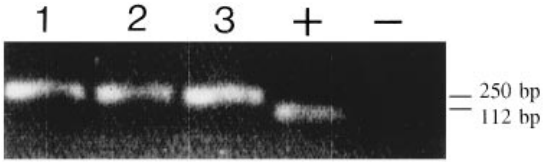

Figure 3 Expression of mGSTM5 in liver tissue of three different mouse strains

RT PCR on RNA isolated of liver tissues from mouse strains C57Bl6 (1), Balb/c (2) and FVB (3). RT PCR was performed with mGSTM5 specific primer pair mGSTM5Ex1 and mGSTM5Ex4/5, resulting in a PCR product of 250 bp. As positive control, RT PCR with GST mu primer pair mGSTEx4 and mGSTEx5 was used (+), which results in a 112 bp fragment. RT reaction without primers was used as negative control (−).

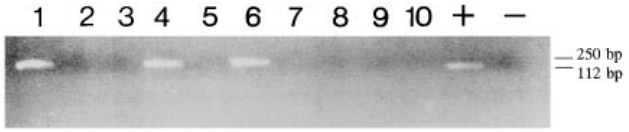

Figure 4 Expression of mGSTM5 determined by RT PCR in various tissues of mouse strain C57B16

RT PCR on total RNA isolated from liver (1), spleen (2), kidney (3), stomach (4), colon (5), ileum (6), heart (7), muscle (8), brain (9) and lung (10) from C57Bl6 mice using mGSTM5 specific primer pair mGSTM5Ex1 and mGSTM5Ex4/5, resulting in the 250 bp fragment. As positive control, RT PCR with mGSTEx4 and mGSTEx5 primers, resulting in the 112 bp fragment, was used (+). As negative control an RT reaction without primers was performed (−).

5Ex4/5 (Figure 1) on total liver RNA of mouse strains C57Bl6, FVB and Balb/c. As shown in Figure 3, PCR products were demonstrated in the above-mentioned mouse strains. To distinguish mGSTM5 products from related cDNAs a unique and specific *Eco*RV site at position 27 of the amplified segment was used (Figure 1).

To study the expression distribution of mGSTM5 in different mouse tissues an RT PCR was performed on RNA isolated from liver, spleen, kidney, stomach, colon, small intestine, heart, muscle and brain tissues of mouse strain C57Bl6. PCR products could be demonstrated in RNA isolates of liver, stomach and small intestine, but were completely absent in spleen, kidney, colon, heart, muscle, brain and lung (Figure 4), indicating that mGSTM5 expression is indeed tissue-specific, which is characteristic for GST genes.

Cloning of whole cDNA by 3′ RACE

Whole mGSTM5 cDNA of 1034 bp including polyA-tail was amplified using a 3′ RACE reaction, starting from total liver RNA of mouse strain FVB (Figure 1B). The ORF is 657 bp long and encodes a protein of 217 amino acids, with a calculated molecular mass of approx. 25.6 kDa. Based on this nucleotide sequence mGSTM5Ex8 primer (Figure 1), located at the 3′ end of the ORF, was designed and used in a second series of RT PCR reactions to amplify the mGSTM5 coding sequence from total liver RNA of mouse strains FVB and C57Bl6. Fragments obtained in this way were cloned in PgemT (Promega) and sequenced in dublicate.

DISCUSSION

Screening of a mouse genomic DNA library with mGSTM1 cDNA [3] resulted in the isolation of a previously unidentified functional active mouse GST mu class gene sequence mGSTM5. Searches of genebank DNA data showed that the overall sequence and gene organization of mGSTM5 matches those of other published mouse GST class mu genes. As already described for mGSTM3 [4], the promoter region of mGSTM5 does not contain a TATA box near the transcription initiation site. Typical consensus sequences for the xenobiotic responsive element (XRE), anti-oxidant responsive element (ARE), AP-1-binding site, glucocorticoid responsive element (GRE) and the Barbie box element, which might be involved in the regulation of GST expression [1], were also lacking in the sequence of mGSTM5.

To study the structure, function and expression of mGSTM5, whole cDNA was amplified from total liver RNA of mouse strain FVB. The ORF is 657 bp long and encodes a protein with a calculated mass of 25.6 kDa. To confirm the cDNA sequence obtained by 3′ RACE from mouse strain FVB and to demonstrate

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                10        20        30        40        50        60
mGSTM5    MPVTLGYWDIRGLGHAIRLLLEYTETGYEERRYAMGDAPDYDRSQWLNDKFKLGLDFPNL
mGSTM1    --MI----NV--------M-----DSS---K--T------F-------E----------
mGSTM2    --M----------A----------D-S--DKK-T-----------SE----------
mGSTM3    --M-----NT---T-S--------DSS---K--V-----NF------SE----------
mGSTM4MSSKS-MV---------A----M---F-DTS---K--IC-E----------DV----D------
hGSTM1    --MI---------A----------DSS---KK-T-------------E----------
hGSTM2    --M----------A--------F-D-S---K--TC-E----------DV----------
hGSTM3  MCESSMV--------A--------F-D-S---K--TC-E----------DV----D------
hGSTM4    --MI---------A----------DSS---KK-T-------------E----------
hGSTM5    --M----------A----------DSS-V-KK-T-------------E----------
rGSTM1    --MI----NV---T-P--------DSS---K-----------------E----------
rGSTM2    --M----------A-----F----D-S--DKK-S-------------SE----------
rGSTM3    --M----------A----------DSS---K--T------F-------E----------

                70        80        90        100       110       120
mGSTM5    PYLIDGSHKVTQSNAILRYLGRKHNLCGETEEERIRVDILENRVMDTRIQMGMLCYSADF
mGSTM1    ---------I----------A---H-D---------A--V--Q-----M-LI----NP--
mGSTM2    ---------I----------A-------------------QA------LA-V---P---
mGSTM3    ------------------------------------T---Q-------LIV-C-P---
mGSTM4    ---M--KN-I---------IA----M--D----K-----M--QI--F-M-LVR---NSNH
hGSTM1    ------A--I--------C-IA------------K--------QT--NHM-L---I--NPE-
hGSTM2    ------T--I----------IA--------S-K-Q--E-----QF--S-M-LAK---DP--
hGSTM3    ---L--KN-I---------I-----M-------K--------Q---F-T-LIR----S-H
hGSTM4    ------A--I--------C-IA------------K--------QT--NHM-L--I--NPE-
hGSTM5    ------A--I---------IA------------K--------Q---NHMELVR---DP--
rGSTM1    -------R-I------M---A---H----------A--V--Q---N-M--LI---NP--
rGSTM2    ---------I----------------------------V--QA----L-LA-V---P--
rGSTM3    ---------I--------------------------QL--N-MVLAR---NP--

                130       140       150       160       170       180
mGSTM5    EKRKPEFLKGLPDQLKLYSEFLGKQPWFAGDKITFADFLVYDVLDQHRMFEPTCLDAFPN
mGSTM1    --Q------TI-EKM---------R-------V-YV---A--I---Y-----K-------
mGSTM2    --K---Y-E---EKM--------------N-V-YV-----------I---K-------
mGSTM3    --Q------AI-EKM---------R-------V-YV---A--I---Y-----K-------
mGSTM4    -NL--QY-EQ---A---QF-L----FT----E-L--V---T------N-I---K---E---
hGSTM1    --L--KY-EE---EK----------R-----N----V---------L--I---N-------
hGSTM2    --L---Y-QA---EM-----Q--------L------V--IA----ERNQV---S-------
hGSTM3    --L--QY-EE--G---QF-M--W-FS----E-L--V---T--I---N-I-D-K---E---
hGSTM4    --L--KQ-EE---EK----------R-----N----V---------L--I---K-------
hGSTM5    --L--KY-EE--------------R----------V---A-----MK-I---K-----L-
rGSTM1    --Q------TI-EKM---------R-------V-YV---A--I---YHI---K-------
rGSTM2    -RK---Y-E---EKM--------------N---YV-----------I---K-------
rGSTM3    --L--GY-EQ--GMMR--------R----------V--IA----ERNQV--A--------

                190       200       210       Sequence Homology(%)
mGSTM5    LKDFMARFEGLRKISAYMKTSRFLPSPVYLKQATWGNE
mGSTM1    -R--L------K-------S--YIAT-IFS-M-H-S-K              75.7
mGSTM2    -----G-----K---D---S----SK-IFA-M-F-NPK              79.8
mGSTM3    -R--L------K-------S-----R--FT-I-Q--TD              78.4
mGSTM4    --A--C---A-E--A-FLQSD--FKM-INN-M-K---KCLC           61.9
hGSTM1    ----IS-----E-------S-----R--FS-M-V---K              77.5
hGSTM2    ----IS-----E-------S-----R--FT-M-V---K              74.7
hGSTM3    --A--C---A-E--A--LQSDQ-CKM-INN-M-Q---KPVC           66.9
hGSTM4    ----IS-----E-------S-----R--FS-M-V---K              77.5
hGSTM5    ----IS-----K-------S-Q--RGLLFG-S---NSK              74.3
rGSTM1    ----L------K-------S--Y-ST-IFS-L-Q-S-K              75.2
rGSTM2    ----V------K---D---SG---SK-IFA-M-F-NPK              78.4
rGSTM3    ----I------K---D---S-----R-LFT-M-I--SK              76.6
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Figure 5 Comparison of deduced amino acid sequence of mGSTM5 and other known mouse GST mu class subunit cDNA sequences

Identical residues are indicated by the dashes. The sequence information was obtained from following articles: mGSTM1-3, [2–4]; mGSTM4, [5]; hGSTM1, [11]; hGSTM2, [12]; hGSTM3, [13]; hGSTM4, [14]; hGSTM5, [15]; rGSTM1, [16]; rGSTM2, [17]; rGSTM3, [18].

that mGSTM5 is a genuine mouse gene, the ORF of the cDNA was also amplified from total liver RNA of mouse strain C57Bl6. Altogether two nucleotides differed in the deduced sequences in the two ORFs (Figure 1) as amplified from the two different mouse strain total liver RNA isolates. These positions may represent errors introduced by *Taq* polymerase or otherwise may indicate sequence polymorphisms between mouse strains FVB and C57Bl6. Homology search via BLAST revealed a homology of 82–85% with other mouse, rat and human GST class mu cDNA sequences. Conceptual translations revealed that several amino acids at highly conserved positions in other GST class mu isoenzymes have been changed in mGSTM5 (Figure 5). Dirr et al. [10] described invariant amino acids involved in the tertiary structure formation of the GST class mu proteins. Of these amino acids leucine-111, which is probably involved in the packaging of α4 and β5 helices, is changed into a methionine in mGSTM5. The consequence of this and other amino acid substitutions on the function and substrate specificity of mGSTM5 will hopefully be elucidated by structure–function studies, which are currently in progress, on recombinant mGSTM5.

Using RT PCR we demonstrated that mGSTM5, like other GSTs, is expressed in a tissue-specific manner. The mGSTM5 RNA transcripts are specifically found in mouse liver, stomach and small intestine. This pattern of distribution is different from those described for mGSTM1 and mGSTM2. Whereas mGSTM1

is expressed in mouse liver, kidney, lung, heart, brain and small intestine, mGSTM2 is expressed in small intestine and to a much lower extent in liver, lung, brain and heart [2,3]. This demonstrates that although there is high sequence homology between the different mouse class mu transcripts, their expression in various tissues is specifically regulated. Additional experiments are required to investigate if mGSTM5 expression, like those of mGSTM1, mGSTM2 and other GSTs, is inducible by chemical stress. This will give more insight into the function of mGSTM5 in mice.

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