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Molecular cloning and heterologous expression of a cDNA encoding a mouse glutathione *S*-transferase Yc subunit possessing high catalytic activity for aflatoxin B_1-8,9-epoxide

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Resistance to the carcinogenic effects of aflatoxin B_1 (AFB_1) in the mouse is due to the constitutive expression of an Alpha-class glutathione *S*-transferase (GST), YcYc, with high detoxification activity towards AFB_1-8,9-epoxide. A cDNA clone (pmusGST Yc) for a murine GST Yc polypeptide has been isolated. Sequencing has shown the cDNA insert of pmusGST Yc to be 922 bp in length, with an open reading frame of 663 bp that encodes a polypeptide of M_r 25358. The primary structure of the murine GST Yc subunit predicted by pmusGST Yc is in complete agreement with the partial amino acid sequence of the aflatoxin-metabolizing mouse liver GST described previously [McLellan, Kerr, Cronshaw & Hayes (1991) Biochem. J. **276**, 461–469]. A plasmid, termed pKK-musGST Yc, which permits the expression of the murine Yc subunit in *Escherichia coli*, has been constructed. The murine GST expressed in *E. coli* was purified and found to be catalytically active towards several GST substrates, including AFB_1-8,9-epoxide. This enzyme was also found to possess electrophoretic and immunochemical properties closely similar to those of the GST Yc subunit from mouse liver. However, the GST synthesized in *E. coli* and the constitutive mouse liver Alpha-class GST exhibited small differences in their chromatographic behaviour during reverse-phase h.p.l.c. Automated Edman degradation revealed alanine to be the *N*-terminal amino acid in the GST Yc subunit expressed in *E. coli*, whereas the enzyme in mouse liver possesses a blocked *N*-terminus. Although sequencing showed that the purified Yc subunit from *E. coli* lacked the initiator methionine, the amino acid sequence obtained over the first eleven *N*-terminal residues agreed with that predicted from the cDNA clone, pmusGST Yc. Comparison of the deduced amino acid sequence of the mouse Yc polypeptide with the primary structures of the rat Alpha-class GST enzymes revealed that it is more closely related to the ethoxyquin-induced rat liver Yc_2 subunit than to the constitutively expressed rat liver Yc_1 subunit. The significance of the fact that both mouse Yc and rat Yc_2 exhibit high catalytic activity towards AFB_1-8,9-epoxide, whereas rat Yc_1 possesses little activity towards this compound, is discussed in terms of structure/function.

INTRODUCTION

Aflatoxin B_1 (AFB_1) is one of a group of difuranocoumarin mycotoxins produced by the mould *Aspergillus flavus*. It is widely encountered in Nature as a contaminant of cereal crops and nuts, particularly in areas of high humidity. AFB_1 is a potent hepatocarcinogen, but its toxicity varies substantially in different species (Newberne & Butler, 1969). For example, the Fischer 344 rat, the guinea pig, the trout and the duck are very sensitive to AFB_1, whereas many strains of mouse can tolerate exposure to AFB_1 (Heathcote & Hibbert, 1978; Bailey *et al.*, 1984; Neal, 1990). In man, the acute toxicity of AFB_1 has been documented (Krishnamachari *et al.*, 1975), but the effects of chronic exposure to AFB_1 are less certain (Campbell *et al.*, 1990).

Several research groups have investigated the basis for the selective toxicity of AFB_1, as such studies can yield important information about cellular resistance mechanisms and may, in addition, provide clues about effective chemoprotective strategies in sensitive species (Degen & Neumann, 1981; O'Brien *et al.*, 1983; Monroe & Eaton, 1987; Neal *et al.*, 1987; Lotlikar, 1989). These workers have shown that, although the harmful effects of AFB_1 are a consequence of its metabolism to the 8,9-epoxide, the ability of liver fractions from different species to catalyse this reaction does not correlate with their sensitivity to AFB_1. The toxicity of this compound is determined by the relative amounts of the cytochrome *P*-450 enzymes involved in the epoxide formation, as well as the levels of the detoxification proteins involved in providing protection against AFB_1 and the 8,9-epoxide. Such detoxification systems include the glutathione *S*-transferases (GSTs), epoxide hydrolase, UDP-glucuronyltransferase, P-glycoprotein and DNA repair enzymes (for a review of resistance mechanisms, see Hayes & Wolf, 1990).

In rodents it appears that the GSTs may play a more important role in providing protection against AFB_1 than other detoxification proteins. Acute toxicity testing has shown that mice can tolerate approx. 10-fold greater amounts of AFB_1 than Fischer 344 rats. Despite the intrinsic resistance of the mouse to AFB_1, murine liver possesses about 3-fold greater capacity than the rat to form the highly reactive 8,9-epoxide. However, murine liver cytosol exhibits between 12- and 50-fold greater AFB_1–GSH-conjugating activity than rat liver cytosol (O'Brien *et al.*, 1983; Monroe & Eaton, 1987; Neal *et al.*, 1987) and it therefore appears that resistance to AFB_1 in the mouse is achieved through high detoxification activity, not low activation capacity. The hypothesis that GST-mediated AFB_1–GSH conjugation represents the resistance mechanism in the mouse is supported by

Abbreviations used: GST, glutathione *S*-transferase; AFB_1, aflatoxin B_1; IPTG, isopropyl thio-β-D-galactoside.

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The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under accession no. X65021.

the observation that depletion of hepatic GSH, by treatment with buthionine-*S*-sulphoximine and diethyl maleate, can result in a 30-fold increase in the covalent binding of AFB_1 to murine liver DNA (Monroe & Eaton, 1988).

In mouse liver the GST that can deactivate AFB_1-8,9-epoxide is the constitutively expressed Alpha-class transferase (Quinn *et al.*, 1990; Ramsdell & Eaton, 1990; Hayes *et al.*, 1991*a*). This enzyme is a homodimer comprising subunits of M_r 25800, as calculated by SDS/PAGE (Hayes & Mantle, 1986*b*), that has been variously designated GT-10.6 (Pearson *et al.*, 1983; Benson *et al.*, 1989), M1 or N4-4 (Warholm *et al.*, 1986), YaYa (Hayes *et al.*, 1987) or Ya_3Ya_3 (McLellan & Hayes, 1989). More recently, McLellan *et al.* (1991) have determined the amino acid sequence of about 45% of this mouse GST and shown that it possesses greater than 85% sequence identity with the constitutively expressed rat GST Yc_1 subunit. Throughout the present paper the constitutive mouse liver Alpha-class enzyme is therefore referred to as YcYc, to reflect the fact that it is a member of the Yc sub-family of Alpha-class GSTs.

It is interesting to note that, despite the close structural similarity between mouse YcYc and rat Yc_1Yc_1, the murine GST exhibits about 80-fold greater activity towards AFB_1-8,9-epoxide than does rat Yc_1Yc_1 (Hayes *et al.*, 1991*a*). In the present study we describe the cloning and expression in *Escherichia coli* of a cDNA encoding a murine GST Yc subunit as an initial step in the examination of the relationship between the structure and function of the mammalian Alpha-class GST.

MATERIALS AND METHODS

Materials

The chemicals used to make bacterial culture media or buffers were from Becton–Dickinson (Cockeysville, MD, U.S.A.) and Sigma Chemical Co. (St. Louis, MO, U.S.A.). The cDNA encoding the rat GST Yc_1 subunit (pGTB 42) was kindly donated by Dr. C. B. Pickett, Merck Frosst Canada Inc. A λgt 11 mouse liver cDNA library, which was constructed using mRNA from male BALB/c mice, was purchased from Clontech Laboratories (Palo Alto, CA, U.S.A.), and the plasmid pGEM-7z (+) was obtained from Promega (Madison, WI, U.S.A.). The expression vector pKK 233-2 and T7 Sequencing Kit were from Pharmacia (Canada) Inc. (Baie d'Urfe, Quebec, Canada). Restriction endonucleases, with accompanying buffers, were purchased from Boehringer Mannheim Canada Inc. (Laval, Quebec, Canada), New England Biolabs (Beverley, MA, U.S.A.) or Pharmacia (Canada) Inc. Nitrocellulose filters (0.45 μm size) were from Schleicher & Schuell (Keene, NH, U.S.A.). [α-^{32}P]dATP (3000 Ci/mmol) was from NEN Research Products (Mississauga, Ontario, Canada). The nick translation system, DNA-sequencing gel-electrophoresis apparatus model S2 and isopropyl thio-β-D-galactoside (IPTG) were from Bethesda Research Laboratories (Gaithersburg, MD, U.S.A.). Glutathione–agarose, prepared by the method of Simons & Vander Jagt (1977), was from Sigma Chemical Co.

Cloning of a cDNA encoding a murine GST Yc subunit

The cDNA for the constitutively expressed murine liver Alpha-class GST was obtained from a mouse liver λgt 11 library which was screened using the rat GST Yc_1 cDNA, pGTB 42, isolated previously by Telakowski-Hopkins *et al.* (1985).

E. coli (LE 392), in Luria–Bertani broth containing 0.2% (w/v) maltose, was infected with the λgt 11 mouse liver cDNA library and plated at a density of 50000 plaque-forming units/150 mm-diam. in NZYM (containing, in 1 litre, 10 g of NZ amine, 5 g of yeast extract, 5 g of NaCl and 4 g of $MgSO_4$) plates, as described by Sambrook *et al.* (1989). The plates were incubated at 37 °C for 16 h and the resulting plaques were screened by the filter hybridization method of Benton & Davis (1977). During plaque purification the nitrocellulose filters were probed with the 570 bp *Pst*I fragment (nucleotides 71–643) of pGTB 42; this fragment was isolated by electrophoresis in 1% agarose and labelled by nick translation (according to the instructions supplied by Bethesda Research Laboratories) before being purified on G-25 Sephadex Quick Spin columns. The hybridization reaction between the denatured probe and the immobilized DNA on the nitrocellulose filters was allowed to proceed for 16 h at 42 °C before the filters were washed in several changes, each of 300 ml, of 2 × SSC (30 mM-sodium citrate, 0.3 M-NaCl, pH 7.0) containing 0.1% (w/v) SDS for a total of 60 min at 62 °C. The purified plaques were finally screened with the 120 bp 5′-end *Pst*I fragment of pGTB 42 to help identify those clones with intact 5′-ends. Following digestion of λgt 11 DNA with *Eco*RI, the DNA inserts from hybridization-positive plaques were sub-cloned into pGEM-7z through the *Eco*RI restriction site in its multiple cloning region.

DNA sequence analysis

Nucleotide sequences of the cDNA inserts were determined using the T7 Sequencing Kit (Pharmacia) which was developed around the dideoxyribonucleotide method of Sanger *et al.* (1977). Sequencing was performed using plasmid DNA purified by caesium chloride–ethidium bromide gradient ultracentrifugation (Garger *et al.*, 1983). Both DNA strands were sequenced using T7 and other oligonucleotide primers that were made on an Applied Biosystems 380B DNA synthesizer (Applied Biosystems Inc., Foster City, CA, U.S.A.). The sequencing reactions were resolved in 6% polyacrylamide gels as described by Sambrook *et al.* (1989).

Expression of the mouse GST Yc subunit in *E. coli*

The plasmid pKK 233-2 (Pharmacia) was chosen as the vector to allow expression of the cDNA encoding mouse Yc because it contains a *Nco*I site at the start codon regulated by the *trp–lac* fusion promoter (Amann & Brosius, 1985). Construction of the expression plasmid was achieved by digestion of pmusGST Yc with *Hin*dIII, followed by limited restriction with *Nco*I. After digestion with *Nco*I, the reaction products were subjected to electrophoresis in low-melting-point agarose and the fragment which contained the cDNA encoding the entire mouse Yc subunit (about 930 bp including the 3′ non-coding region) was excised from the gel and the DNA recovered by phenol extraction and ethanol precipitation. Following digestion of the expression vector with *Nco*I and *Hin*dIII, the coding nucleotide sequence for mouse Yc was ligated into the *Nco*I and *Hin*dIII sites of pKK 233-2. Screening for pKK 233-2 which contained the cDNA insert was done by electrophoretic analysis of restriction digests of Mini-Prep plasmid DNA from transformed *E. coli* JM 107. The construct that contains the complete coding sequence for mouse Yc is called pKK-musGST Yc.

Selected isolated colonies that contained either pKK 233-2 or pKK-musGST Yc were inoculated and grown at 37 °C in dYT broth (containing, in 1 litre, 10 g of Bacto-yeast, 16 g of tryptone and 5 g of NaCl). Once the attenuance of the culture at 600 nm was about 0.6, IPTG was added to a concentration of 2 mmol/l and the cells were harvested at various time intervals thereafter.

Western blot analysis

The expression of GST in the lysates of *E. coli* that had been transformed with pKK-musGST Yc was studied by immunoblotting using a modified version of the protocol described previously by Hayes & Mantle (1986*a*); during the present study

^{125}I-Protein A was used to locate the primary antibody rather than goat anti-(rabbit IgG) antibody–horseradish-peroxidase. SDS/PAGE was performed by the method of Laemmli (1970) in 12% (w/v) polyacrylamide resolving gels that contained 0.32% (w/v) *NN'*-methylenebisacrylamide (for details about the effect of the cross-linker on the electrophoretic behaviour of GST subunits, see Hayes & Mantle, 1986*b*). The gels were calibrated using a commercially available (Bio-Rad Laboratories, Richmond, CA, U.S.A.) protein mixture containing phosphorylase *b* (M_r 97400), BSA (M_r 66200), ovalbumin (M_r 42700), bovine carbonic anhydrase (M_r 31000), soybean trypsin inhibitor (M_r 21500) and lysozyme (M_r 14400). The authentic mouse and rat liver GSTs which were included as standards in the immunoblotting experiments were purified as described elsewhere (McLellan & Hayes, 1987; Hayes *et al.*, 1987, 1991*b*). Antibodies were obtained against the mouse Alpha-class GST Yc subunit of M_r 25800 (previously called Ya_3) as described by McLellan & Hayes (1989).

Purification of GST from *E. coli*

GST in *E. coli* JM 107 that had been transformed with either pKK 233-2 or pKK-musGST Yc was purified by affinity chromatography on glutathione–agarose. The bacterial cultures, each of 1.5 litres, were grown to a D_{600} of approx. 0.6 before IPTG was added to give a concentration of 2 mmol/l. The cultures were then left to grow at 37 °C for a further 5–8 h before the cells were harvested and washed twice in 20 mM-sodium phosphate/150 mM-NaCl buffer, pH 7.4, to remove the dYT broth. Finally, the cells were resuspended in 200 ml of 50 mM-Tris/250 mM-NaCl/0.5 mM-dithiothreitol, pH 7.8, and lysed at 4 °C using a Bead Beater (Biospec Products, Bartlesville, Okla, Canada). Cellular debris was removed from the bacterial extract by centrifugation at 10000 *g* (4 °C) for 10 min, and the resulting supernatant was centrifuged at 100000 *g* (4 °C) for 60 min. The 100000 *g* supernatant was applied immediately to a 1.5 cm × 13.0 cm column of glutathione–agarose which was equilibrated and eluted at 60 ml/h with 50 mM-Tris/250 mM-NaCl/0.5 mM-dithiothreitol, pH 7.8. The column was washed with approx. 500 ml of this buffer to remove non-specifically absorbed material. The affinity matrix was washed finally with 50 ml of 250 mM-Tris/HCl, pH 9.4, before the bound protein was eluted with 200 mM-glutathione in the same buffer. The protein obtained from the column was dialysed against two changes, each of 4 litres, of 25 mM-sodium phosphate buffer, pH 7.4, containing 0.2 mM-dithiothreitol before being frozen at −70 °C until required. The purified GST obtained was examined by SDS/PAGE (Hayes & Mantle, 1986*b*), using Coomassie Brilliant Blue R to visualize the protein.

Enzyme assays

These were performed as described previously (Moss *et al.*, 1983; Hayes *et al.*, 1991*b*).

Reverse-phase h.p.l.c.

The method used to resolve murine GST subunits [devised originally by Ostlund Farrants *et al.* (1987)] has been described elsewhere (Hayes *et al.*, 1991*c*). During the present study, 40–55% acetonitrile gradients, rather than 30–60% acetonitrile gradients, were employed to resolve and prepare GST.

Amino acid sequencing

This was carried out with the use of an Applied Biosystems 477A instrument with a 120A on-line phenylthiohydantoin analyser (Warrington, U.K.), as reported previously (Hayes *et al.*, 1989).

RESULTS AND DISCUSSION

GST activity of mouse liver cytosol towards AFB_1-8,9-epoxide

The ability of hepatic cytosol from BALB/c mice to catalyse the conjugation of AFB_1-8,9-epoxide was investigated before undertaking molecular cloning of the murine Alpha-class GST Yc cDNA from a commercially available BALB/c liver cDNA library. Using the assay system devised by Moss *et al.* (1983), BALB/c liver cytosol was found to possess GST activity towards AFB_1-8,9-epoxide of 0.44 ± 0.02 nmol/min per mg of protein. This value compares favourably with the GST activity for AFB_1-8,9-epoxide of 0.27 ± 0.04 nmol/min per mg of protein in hepatic cytosol from C57/Bl mice reported previously by Neal *et al.* (1987). The BALB/c liver cDNA library therefore appeared to be a suitable source of cDNA clones encoding the murine AFB_1-metabolizing Alpha-class GST.

Isolation and characterization of a cDNA clone encoding mouse GST Yc

Amino acid sequencing experiments have shown that the constitutively expressed Alpha-class GST in mouse liver, which has high catalytic activity for AFB_1-8,9-epoxide, comprises Yc-type subunits (McLellan *et al.*, 1991). Molecular cloning of the cDNA for this enzyme was therefore undertaken using pGTB 42, which encodes rat Yc_1, to screen a mouse liver λgt11 cDNA library. Of the 5×10^5 plaques that were screened, about 30 gave a positive hybridization signal when probed with the 570 bp *Pst*I fragment of pGTB 42. Six of the 30 plaques were purified by three rounds of screening. Each of the six pure clones hybridized to the 570 bp *Pst*I and the 3′ 170 bp *Pst*I fragments from pGTB 42; however, only two gave a positive hybridization signal with the 120 bp 5′-end *Pst*I fragment of pGTB 42. Analytical agarose-gel electrophoresis revealed that after digestion with *Eco*RI one of these two clones contained a cDNA insert of about 900 bp, whereas the cDNA insert from the other clone was not released by *Eco*RI from the bacteriophage DNA. It was concluded that one of the two *Eco*RI sites flanking the cDNA insert is absent in the second clone, which was therefore not studied further. The cDNA insert which was successfully cut out of the remaining

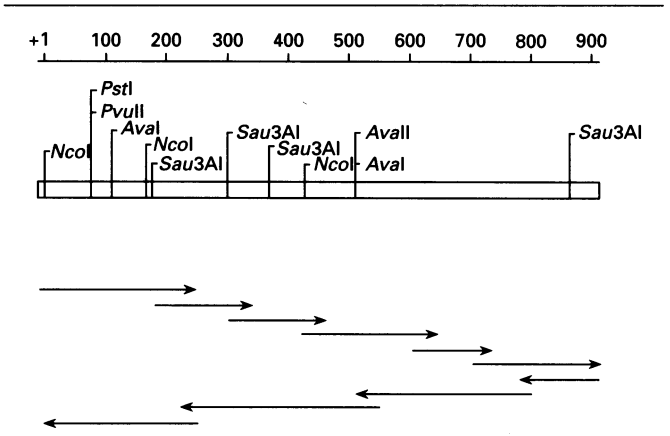

Fig. 1. Restriction endonuclease map and sequencing strategy of the murine GST Yc subunit cDNA

The solid bar indicates the size of the cDNA clone; the relative positions of *Nco*I and certain other restriction sites are shown by the open box. The arrows represent the direction and extent of the 10 sequencing reactions used to determine the sequence of pmusGST Yc.

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                                                                -12        -1
                                                                  GCAACTGCTGCC
+1
 M   A   G   K   P   V   L   H   Y   F   D   G   R   G   R   M   E   P   I   R    20
ATG GCG GGG AAG CCA GTC CTT CAT TAC TTT GAT GGC AGG GGA AGA ATG GAG CCT ATC CGG   60

 W   L   L   A   A   A   G   V   E   F   E   E   K   F   L   K   T   R   D   D    40
TGG CTC TTG GCT GCA GCT GGT GTG GAG TTT GAA GAA AAA TTT CTG AAA ACT CGG GAT GAC  120

 L   A   R   L   R   S   D   G   S   L   M   F   Q   Q   V   P   M   V   E   I    60
CTG GCA AGG TTA CGA AGT GAT GGG AGT CTG ATG TTC CAG CAA GTG CCC ATG GTA GAG ATC  180

 D   G   M   K   L   V   Q   T   K   A   I   L   N   Y   I   A   S   K   Y   N    80
GAC GGG ATG AAA CTG GTG CAG ACC AAA GCC ATT CTC AAC TAC ATT GCC TCC AAA TAC AAC  240

 L   Y   G   K   D   M   K   E   R   A   I   I   D   M   Y   T   E   G   V   A   100
CTC TAT GGG AAG GAC ATG AAG GAG AGA GCC ATC ATT GAC ATG TAC ACA GAA GGA GTG GCG  300

 D   L   E   I   M   I   L   Y   Y   P   H   M   P   P   E   E   K   E   A   S   120
GAT CTG GAG ATA ATG ATT CTC TAT TAC CCC CAC ATG CCC CCT GAG GAG AAA GAG GCA AGC  360

 L   A   K   I   K   E   Q   T   R   N   R   Y   F   P   A   F   E   K   V   L   140
CTT GCC AAG ATC AAG GAA CAA ACC AGG AAC CGT TAC TTC CCT GCC TTT GAA AAG GTG TTG  420

 K   S   H   G   Q   D   Y   L   V   G   N   R   L   S   R   A   D   I   A   L   160
AAG AGC CAT GGA CAA GAT TAT CTC GTT GGC AAC AGG CTG AGC AGG GCT GAT ATT GCC CTG  480

 V   E   L   L   Y   H   V   E   E   L   D   P   G   V   V   D   N   F   P   L   180
GTT GAA CTC CTC TAC CAT GTG GAA GAG CTG GAC CCG GGC GTT GTG GAC AAC TTC CCT CTC  540

 L   K   A   L   R   S   R   V   S   N   L   P   T   V   K   K   F   L   Q   P   200
CTG AAA GCG CTG AGA AGC AGA GTC AGC AAC CTC CCC ACA GTG AAG AAG TTT CTT CAA CCT  600

 G   S   Q   R   K   P   F   D   D   A   K   C   V   E   S   A   K   K   I   F   220
GGC AGC CAG AGG AAG CCT TTT GAT GAC GCA AAA TGT GTT GAG TCA GCA AAG AAG ATT TTC  660

 S   *                                                                          221
AGT TAA TTCAGGCAACTATAAGTACATAGCCCCCACAAAGCCAACCTTCTAAAATTTTGCATCACATTGAAGTGTTT  737

TGACTAAGTGTTGACCCTACTTAGAAAGCTAACAAGTTTTCTAAGGCGTCTGTGTCAATTCAGGTAGACATGACTGACG  816

AGGAACGGCCGGATGCTCTCTAGTTGTAGTTAAAATTGAAATCATGATCACTTCCTCGGATATTTTCTTACTGTTCAAT  895

AAAACAAAACAAGCT                                                               910
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Fig. 2. Nucleotide sequence of murine GST Yc cDNA and deduced amino acid sequence of the Yc subunit

The nucleotide sequence of the mouse GST cDNA was determined, after sub-cloning into pGEM-7z, by the dideoxynucleotide chain termination method. The deduced amino acid sequence is shown in single letter format above the cDNA sequence. Position +1 is the first base in the ATG initiation codon; the twelve bases 5′ to the ATG codon are designated −12 to −1. Numbering of the amino acid residues includes the initiator methionine. The asterisk, shown after the *C*-terminal serine, indicates the TAA termination codon. The AATAAA polyadenylation signal sequence is underlined.

λgt11 clone was sub-cloned into the *Eco*RI site of pGEM-7z. Recombinant pGEM-7z plasmids were transformed into competent *E. coli* strain JM 107.

The restriction map of the murine GST cDNA, together with the sequencing strategies employed, are shown in Fig. 1. The DNA sequence of the insert along with the deduced amino acid sequence encoded is presented in Fig. 2. This shows that the cDNA insert is 922 nucleotides in length. The first ATG triplet from the 5′-end is believed to represent the translation start site and therefore the first nucleotide of this codon is designated as +1. The insert contains an open reading frame of 663 bp flanked at the 3′-end by 247 bases of non-coding sequence.

The nucleotide sequence of the cDNA insert of pmusGST Yc is distinct from that of pGT 41, a mouse Alpha-class GST cDNA clone described by Pearson *et al.* (1988), and from λmYa1, a genomic clone of mouse Alpha-class GST described by Daniel *et al.* (1987). Moreover, pmusGST Yc is separate from any of the mouse Mu-class GST cDNA clones (Pearson *et al.*, 1988; Townsend *et al.*, 1989) or the Pi-class GST cDNA clone (Hatayama *et al.*, 1990) described to date.

Including the initiator methionine residue, the open reading frame of the cDNA insert of pmusGST Yc codes for a protein comprising 221 amino acids with an M_r of 25358. This value compares reasonably well with the M_r value of 25800 that has been estimated from SDS/PAGE of the constitutive hepatic mouse Alpha-class GST subunit (Hayes & Mantle, 1986*b*). Our contention that the mouse liver cDNA clone codes for the constitutive Alpha-class GST is strongly supported by the fact that no differences exist between the amino acid sequence reported by McLellan *et al.* (1991) for this enzyme (referred to previously as Ya_3Ya_3) and that deduced from pmusGST Yc. McLellan *et al.* (1991) sequenced amino acid residues 16–56, 63–73, 94–110 and 112–142, and over these regions the sequence obtained directly from cyanogen bromide-derived fragments of the constitutive Alpha-class liver GST and that predicted from pmusGST Yc are in complete agreement.

Relationship between mouse Yc and rat GST

A comparison of the amino acid sequence of the protein encoded by pmusGST Yc with the primary structures of the rat Alpha-class GST shows that the mouse enzyme is more closely related to the rat Yc-type subunits than either the rat Ya or Yk subunits. The murine Yc subunit shares greater than 85% sequence identity with rat Yc-type subunits (Telakowski-Hopkins *et al.*, 1985; Hayes *et al.*, 1991*b*), but less than 70% sequence identity with rat Ya-type subunits (Lai *et al.*, 1984; Pickett *et al.*, 1984) and less than 60% sequence identity with the rat Yk subunit (Ålin *et al.*, 1989).

Table 1. Structural comparison between GST Yc-type subunits

Data for mouse Yc are taken from Fig. 2. The data for rat Yc_1 and Yc_2 subunits are from Telakowski-Hopkins *et al.* (1985) and Hayes *et al.* (1991*b*) respectively. The complete primary structure of Yc_2 has not been determined, and this is the reason for the absence of data for this subunit at residues 46, 91, 104, 158–163 and 207–217. At residues 136 and 170 mouse Yc and rat Yc_1 possess identical amino acids but differ from rat Yc_2. At all other positions listed, mouse Yc and rat Yc_1 contain different amino acids.

Residue	GST subunit...	Mouse Yc	Rat Yc_1	Rat Yc_2
2		Ala	Pro	Pro
33		Lys	Gln	Asn
46		Ser	Asn	–
69		Lys	Arg	Lys
77		Ser	Thr	Thr
91		Ile	Leu	–
96		Thr	Ala	Ala
103		Glu	Asp	Glu
104		Ile	Glu	–
105		Met	Ile	Met
106		Ile	Val	Val
108		Tyr	His	Tyr
111		His	Tyr	Tyr
112		Met	Ile	Met
115		Glu	Gly	Gly
126		Glu	Asp	Asp
127		Gln	Lys	Lys
128		Thr	Ala	Ala
136		Phe	Phe	Tyr
158		Ile	Val	–
159		Ala	Tyr	–
162		Glu	Gln	–
163		Leu	Val	–
170		Leu	Leu	Met
173		Gly	Ser	Gly
174		Val	Ala	Ile
175		Val	Leu	Val
176		Asp	Ala	Asp
186		Ser	Thr	Thr
207		Phe	Leu	–
208		Asp	Glu	–
210		Ala	Glu	–
217		Lys	Val	–

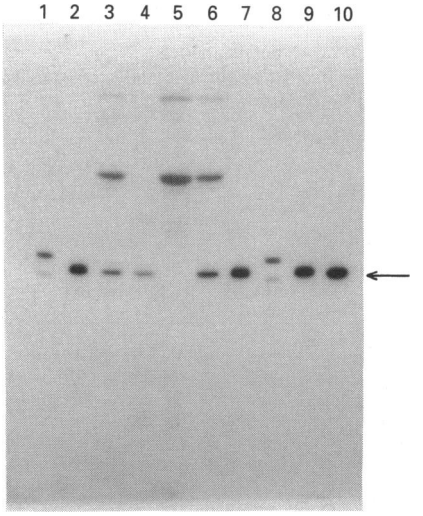

Fig. 3. Expression of the murine GST Yc subunit in *E. coli*

Bacterial extracts from *E. coli* transformed with either pKK-musGST Yc or pKK 233-2 were prepared by sonication. The samples, which were subjected to SDS/PAGE prior to immunoblotting, were as follows: track 1, 0.3 μg of rat liver GST Ya_1Yc_1; track 2, 0.3 μg of mouse liver GST YcYc; track 3, 40 μg of protein from *E. coli* transformed with pKK-musGST Yc and grown in dYT medium containing 2 mM-IPTG for 2 h; track 4, 40 μg of protein from *E. coli* transformed with pKK-musGST Yc and grown in minimal salts (M9 medium) plus 0.2% glucose containing 2 mM-IPTG for 2 h; track 5, 40 μg of protein from *E. coli* transformed with pKK 233-2 and grown in dYT media containing 2 mM-IPTG for 2 h; track 6, 40 μg of protein from *E. coli* transformed with pKK-musGST Yc and grown in dYT media containing 2 mM-IPTG for 6 h; track 7, 0.3 μg of mouse liver GST YcYc; track 8, 0.3 μg of rat liver GST Ya_1Yc_1; track 9, 0.3 μg of mouse liver GST YcYc; track 10, 0.3 μg of mouse GST Yc subunit purified from *E. coli* transformed with pKK-musGST Yc (see the text and Fig. 4). Note that the rat Ya_1 (apparent M_r 25500) and rat Yc_1 (apparent M_r 27500) subunits, analysed in tracks 1 and 8, have SDS/PAGE mobilities distinct from that of the constitutive mouse liver Alpha-class GST Yc subunit (apparent M_r 25800); the electrophoretic mobility of mouse Yc is indicated by the horizontal arrow. The identity of the immunoreactive band of M_r approx. 40000, seen in tracks 3, 5 and 6, is not known (see the text).

Two distinct rat Yc-type subunits, Yc_1 and Yc_2, which differ in their catalytic activity towards AFB_1-8,9-epoxide, have been identified (Hayes *et al.*, 1991*b*). Unfortunately, the entire primary structure of rat Yc_2 has not been described, but over the region for which amino acid sequence data are available the mouse GST possesses greater identity with rat Yc_2 than with Yc_1. Mouse Yc and rat Yc_1 share 86% sequence identity and, as Table 1 demonstrates, a total of 31 amino acid differences exists between these two polypeptides. The amino acid sequence of 145 residues of Yc_2 has been reported (Hayes *et al.*, 1991*b*) and over this region 14 amino acid differences exist between mouse Yc and rat Yc_2. Over the same region, 20 amino acid differences exist between mouse Yc and rat Yc_1.

Expression of mouse GST Yc in *E. coli*

The ability of the protein encoded by pmusGST Yc to metabolize GST substrates, and in particular to detoxify AFB_1-8,9-epoxide, was assessed by expressing the cDNA in *E. coli*. *E. coli* JM 107 cells were transformed with the plasmid pKK-musGST Yc as described in the Materials and methods section. Western blotting (Fig. 3) showed the presence of a polypeptide in *E. coli* transformed with pKK-musGST Yc which not only cross-reacted with antibodies raised against mouse GST YcYc but also co-migrated during SDS/PAGE with the murine enzyme. This immunoreactive polypeptide of apparent M_r 25800 was absent from extracts of *E. coli* which had been transformed with pKK 233-2 (i.e. vector without insert), shown in track 5 of Fig. 3. The identity of the bacterial protein of M_r approx. 40000 which cross-reacted with antiserum raised against mouse Alpha-class GST is not known. This immunoreactive band (seen in tracks 3, 5 and 6 of Fig. 3) does not appear to arise from dimerization of the Yc subunit, since it is absent from the tracks containing GST standard alone, and its presence probably reflects lack of specificity of the polyclonal antibodies. Interestingly, the M_r 40000 protein does not appear to be expressed in *E. coli* grown in M9 media (Fig. 3, track 4).

The mouse GST was found to be expressed at modest levels in *E. coli* cells transformed with pKK-musGST Yc that were grown in M9 medium containing 20% (w/v) glucose. When the transformed *E. coli* cells were grown in dYT medium, no change in expression of mouse Yc was observed. However, following treatment with 2 mM-IPTG an approx. 2-fold increase in expression of mouse Yc was noted. This increase in GST expression was found 90 min after IPTG treatment and was maintained for at least 18 h.

Table 2. Specific activities of murine GST YcYc

Enzyme activity was determined at 37 °C. The results represent mean values ± S.D. for either triplicate or quadruplicate determinations. n.d., not detected.

Substrate	GST source...	Specific activity (nmol/min per mg) *E. coli* transformed with pKK-musGST Yc	Specific activity (nmol/min per mg) Mouse liver
AFB_1-8,9-epoxide		15.03 ± 2.14	21.86 ± 3.98
1-Chloro-2,4-dinitrobenzene		10380 ± 1520	15100*
Cumene hydroperoxide		23690 ± 770	12700*
Hydrogen peroxide		n.d.	n.d.

* Data from Hayes *et al.* (1991*a*).

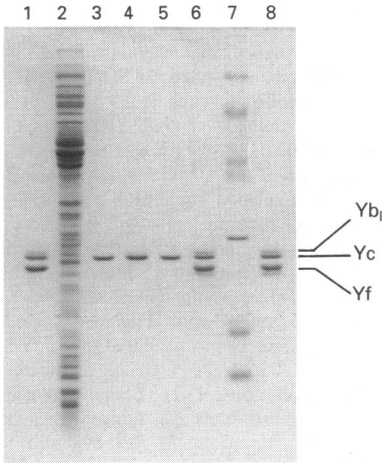

Fig. 4. SDS/PAGE of purified mouse GST Yc obtained from *E. coli*

The samples, which were analysed by SDS/PAGE in a 12% polyacrylamide resolving gel, were applied as follows: tracks 1, 6 and 8, hepatic GST pool (Yb_1 Yc and Yf subunits) from BALB/c mice purified by glutathione–agarose affinity chromatography; track 2, extract of *E. coli* containing pKK-musGST Yc grown in the presence of 2 mM-IPTG; tracks 3 and 5, mouse GST Yc subunit affinity-purified from the extract shown in track 2; track 4, GST YcYc purified from BALB/c mouse liver; track 7, M_r standards [in order of increasing mobility towards the anode: phosphorylase *b*, BSA, ovalbumin (two bands), carbonic anhydrase, trypsin inhibitor, lysozyme]. Following electrophoresis, proteins were stained with Coomassie Brilliant Blue R. The mobilities of the murine Yf (apparent M_r 24800), Yc (apparent M_r 25800) and Yb_1 (apparent M_r 26400) subunits are shown.

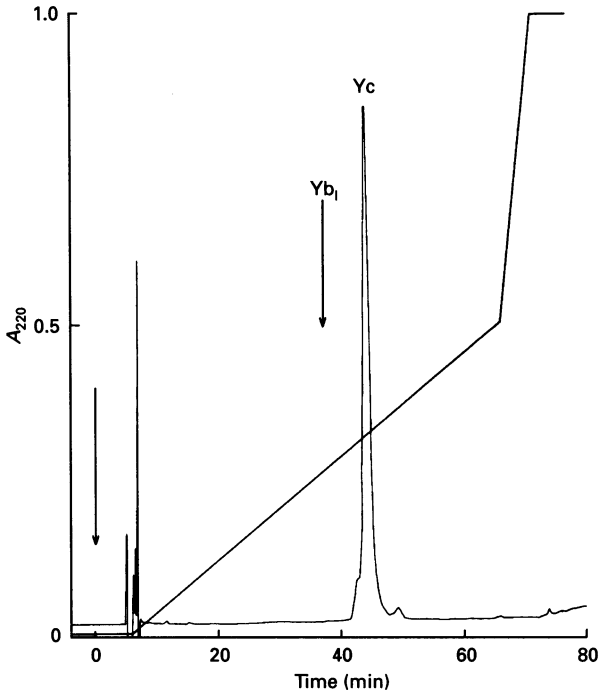

Fig. 5. Reverse-phase h.p.l.c. of the murine GST Yc subunit

The GST subunit synthesized by *E. coli* transformed with pKK-musGST Yc was isolated by glutathione–agarose affinity chromatography, as shown in Fig. 4. Portions (about 150 μg) of the purified material were applied to a Waters μ-Bondapak C_{18} column (10 μm particle size; column size 0.39 cm × 30.0 cm). The column was equilibrated with 40% acetonitrile in aq. 0.1% trifluoroacetic acid, delivered by pump A. Over the first 5 min of the run the sample was loaded isocratically, during which time the flow rate from pump A was increased from 0.1 ml/min to 1.0 ml/min. The column was subsequently developed at 1.0 ml/min by a 40–55% acetonitrile gradient, formed over 60 min, which was followed by a 55–70% gradient, formed over 5 min; the acetonitrile gradients contained 0.1% trifluoroacetic acid throughout. The eluate was monitored continuously at 220 nm. The relative output of pump B, which delivered 70% acetonitrile in aq. 0.1% trifluoroacetic acid, is shown by the continuous straight line. Under these chromatographic conditions the Yb_1 subunit was found to elute at 37 min (shown by the vertical arrow), whilst the Yf and Yc subunits from mouse liver both eluted at 45 min (i.e. 1 min later than the elution position of the GST subunit synthesized by *E. coli* from pKK-musGST Yc).

Characterization of mouse GST Yc expressed in *E. coli*

The murine GST was purified from the 100000 *g* supernatant of *E. coli* cells using 1.5 cm × 13.0 cm columns of glutathione–agarose. Typically, about 0.5 mg of GST was isolated from 1.8 g of cytosolic protein obtained per litre of overnight *E. coli* culture that had been exposed to 2 mM-IPTG for 4–6 h. The purified enzyme was found to be active with AFB_1-8,9-epoxide as well as with cumene hydroperoxide, an Alpha-class GST substrate, and 1-chloro-2,4-dinitrobenzene, a GST substrate of broad specificity. A comparison of the catalytic properties of the murine GST expressed in *E. coli* with those of the Alpha-class GST isolated form normal mouse liver is shown in Table 2.

Fig. 4 shows that the enzyme purified from *E. coli* has a closely similar electrophoretic mobility to that of the Alpha-class GST from normal mouse liver, but is resolved during SDS/PAGE from the Yf (Pi-class) and Yb_1 (Mu-class) subunits of the mouse. As was to be expected, the enzyme purified from *E. coli* cross-reacted with antibodies raised against the constitutive mouse liver Alpha-class GST (see Fig. 3).

The enzyme purified from *E. coli* was also found to have chromatographic properties similar, but not identical, to those of the constitutive mouse liver Alpha-class GST when analysed by reverse-phase h.p.l.c. Using the conditions outlined in Fig. 5, the GST Yc subunit synthesized by *E. coli* was eluted from the μ-Bondapak C_{18} column at 44 min, whereas the GST Yc subunit from mouse liver was eluted at 45 min. When equal amounts of the polypeptide from the two sources were mixed before application to the column, two partially resolved peaks (one eluting at 44 min and the other eluting at 45 min) were obtained, indicating that the difference in retention time is not artefactual. The GST Yc subunit that was expressed in *E. coli* was eluted from the μ-Bondapak C_{18} column and subjected to automated amino acid sequencing. The data presented in Table 3 indicate that the Yc subunit from *E. coli* is amenable to Edman

Table 3. Comparison of the N-terminal amino acid sequence of mouse GST Yc expressed in *E. coli* with that predicted from pmusGST Yc

Murine GST Yc isolated from *E. coli* transformed with pKK-musGST Yc			Amino acid predicted from DNA sequence	
Edman degradation cycle no.	Amino acid	Amount (pmol)	Codon no.	Amino acid
1	Ala	2965	2	Ala
2	Gly	2305	3	Gly
3	Lys	3607	4	Lys
4	Pro	1851	5	Pro
5	Val	1935	6	Val
6	Leu	1560	7	Leu
7	His*	–	8	His
8	Tyr	1457	9	Tyr
9	Phe	1697	10	Phe
10	Asp	1266	11	Asp
11	Gly	1789	12	Gly

* Not quantified by sequencer.

degradation, whereas previous work has shown that the Yc subunit isolated from mouse liver has a blocked *N*-terminus (Mannervik *et al.*, 1985; Warholm *et al.*, 1986). The Yc subunit was subjected to eleven Edman cycles and the sequence of amino acids obtained was found to correspond exactly to that predicted from DNA sequencing of codons 2–12 of pKK-musGST Yc. It is therefore probable that differences in the *N*-termini of the Yc subunit from the two sources are responsible for their distinct chromatographic behaviour.

Comments about structure/function of GST Yc subunits

The fact that mouse Yc appears to share more sequence identity with rat Yc_2 than with rat Yc_1 is of interest, because mouse Yc and rat Yc_2 exhibit about 50-fold greater activity towards AFB_1-8,9-epoxide than rat Yc_1 (Hayes *et al.*, 1991*a*). This marked difference in the activity of Yc subunits appears to be due to relatively minor differences in their primary structures. The mouse Yc and rat Yc_1 subunits differ at 31 of their 221 amino acid residues (see Table 1). Although it has not been demonstrated which of these 31 residues are responsible for the difference in activity towards AFB_1-8,9-epoxide, Hoesch & Boyer (1989) have presented data from photoaffinity labelling experiments suggesting that, in the Yc_1 subunit, amino acids between residues 91 and 110 as well as amino acids between residues 206 and 218 may be involved in the active centre. More recent X-ray crystallography data (Reinemer *et al.*, 1991) are consistent with the peptides identified by photoaffinity labelling being involved in the hydrophobic ligand-binding domain of GST. Over these two regions 11 amino acid differences exist between mouse Yc and rat Yc_1; between amino acids 91 and 110 seven different residues exist and between amino acids 206 and 218 four different residues exist.

Whilst any of these 11 residues may be implicated in metabolizing AFB_1-8,9-epoxide the availability of amino acid sequence data for about 65% of rat Yc_2 (Hayes *et al.*, 1991*b*) can help identify which residues are involved in this catalytic reaction. At residues 103, 105 and 108 the same amino acids are found in mouse Yc and rat Yc_2, namely, glutamic acid, methionine and tyrosine respectively, whereas rat Yc_1 contains aspartic acid, isoleucine and histidine at the same positions. Unfortunately, no sequence data are available for rat Yc_2 for residues 206–218, and therefore no comment can be made about the catalytic significance of the differences between mouse Yc and rat Yc_1 at residues 207, 208, 210 and 217. Although the available data are incomplete, it is interesting to suggest that residues 103–108 are of importance in determining the ability of these GSTs to metabolize AFB_1-8,9-epoxide. Clearly, further studies, involving site-directed mutagenesis, are required to test this hypothesis.

Concluding remarks

During the present study a cDNA for a mouse GST Yc subunit with high detoxification activity for AFB_1-8,9-epoxide has been isolated, characterized and expressed in *E. coli*. Whilst we have emphasized the ability of this enzyme to confer resistance to AFB_1, it is apparent that the Yc-type subunits are also involved in resistance to nitrogen mustards. Tew and his colleagues (Wang & Tew, 1985; Buller *et al.*, 1987) as well as Robson *et al.* (1986, 1987) and Schecter *et al.* (1991) have described the over-expression of Yc-type subunits in cell lines selected for resistance to nitrogen mustards. More recently, the murine Yc subunit has been shown to catalyse the conjugation of GSH with chlorambucil (Ciaccio *et al.*, 1990) as well as with L-phenylalanine mustard (Bolton *et al.*, 1991). It therefore appears likely that the nitrogen-mustard-resistant cell lines which have been described (Tew *et al.*, 1990) will be cross-resistant to AFB_1.

Fahl and his colleagues (Manoharan *et al.*, 1987; Puchalski & Fahl, 1990) and Black *et al.* (1990) have used transfection experiments to examine the ability of several rat and human GST cDNAs to confer resistance to alkylating agents, but this technique has not been employed to study the role of Yc in chemotherapeutic drug resistance. The availability of a cDNA encoding mouse GST Yc will allow such transfection experiments to be undertaken.

Note added in proof (received 27 March 1992)

Recently, Buetler & Eaton (1992) have also described a cDNA clone for the mouse GST Yc subunit. Differences between their cDNA and that characterized by ourselves exist at nucleotide positions 516, 828, 857 and 886.

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