Cloning and characterization of the major hepatic glutathione S-transferase from a marine teleost flatfish, the plaice (*Pleuronectes platessa*), with structural similarities to plant, insect and mammalian Theta class isoenzymes

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A cDNA clone (PLGSTA) of 896 bp, containing an open reading frame encoding a 225-amino-acid polypeptide of M_r 25 723, was isolated from a cDNA library constructed in λ gt11 from the liver of a marine teleost flatfish, the plaice (*Pleuronectes platessa*). The identification of this cDNA as that coding for the subunit of the major cytosolic glutathione S-transferase of plaice liver, GST-A, was supported by its heterologous expression in and purification of its protein product from *Escherichia coli*. The recombinant-derived protein exhibited identical M_r and immunoreactivity and a similar substrate specificity to GST-A previously isolated from plaice liver. Comparison of the deduced amino acid sequence of the plaice GST-A polypeptide with the primary structures of

GSTs from other Phyla revealed that it showed the greatest similarity to plant, insect and mammalian Theta class GSTs. Southern blot analysis of plaice DNA hybridized to the PLGSTA cDNA showed a banding pattern indicative of the presence of a single gene. Northern blot analysis of a variety of plaice tissues showed hybridizing bands of approx. 1100 nucleotides in all tissues tested, with the highest relative amounts in liver and intestinal mucosa. A marked increase in hybridization intensity was observed in hepatic RNA samples from plaice treated with *trans*-stilbene oxide, suggesting that GST-A is induced by epoxides in this species.

INTRODUCTION

Different roles for the glutathione S-transferases (GSTs; EC 2.5.1.18) and the UDP-glucuronosyltransferases (EC 2.4.1.17) have been implicated in the selective toxicity of certain toxins such as aflatoxin B₁ (Goeger et al., 1986) and of xenobiotics such as the lampreyicide, tetrafluoromethylnitrophenol (Lech and Statham, 1975) in different fish species. Many benthic Pleuronectid flatfish exhibit a high incidence of hepatic neoplasia (Malins et al., 1987; Baumann, 1989), and it has been suggested that this may be attributed to the relative activities of various components of the phase I and II xenobiotic biotransformation systems (Varanasi et al., 1987; Leaver et al., 1992). In previous enzymological studies of the Pleuronectid flatfish, the plaice (*Pleuronectes platessa*), we have demonstrated the presence of multiple isoforms of UDP-glucuronosyltransferases (Clarke et al., 1992a) and GSTs (George and Buchanan, 1990), and we have shown that they are differentially induced by xenobiotic chemicals (George and Buchanan, 1990; Clarke et al., 1992b). Our aims are to describe the mechanisms of this regulation by xenobiotics and to explain the molecular basis for the differential toxicities of these chemicals in fish. To this end, knowledge of the gene structure and regulation of the enzymes is necessary.

The GSTs are a multi-gene family of dimeric multifunctional proteins found in all organisms and they are believed, at least in vertebrates, to play major roles in the following: (1) the biosynthesis of certain leukotrienes and prostaglandins and the isomerization of steroids, (2) Se-independent GSH peroxidase activity towards organic peroxides including lipid, steroid and

DNA hydroperoxides, (3) intracellular transport (high-affinity binding) of endogenous compounds such as haem, bilirubin and steroid hormones, (4) irreversible binding of reactive electrophilic xenobiotics such as azo dye carcinogens and polyaromatic hydrocarbons, and (5) catalysis of glutathione (GSH) conjugation with electrophilic centres in a variety of compounds (e.g. epoxides) as the first step in the formation of mercapturic acids (Jakoby, 1978; Mannervik, 1985; Hayes et al., 1990). Thus, apart from their role in the metabolism and transport of endogenous compounds, GSTs play an essential role in protecting the organism from peroxidative damage (Mannervik, 1985), and have also been implicated in acquired drug (Hayes and Wolf, 1990), herbicide (Tu et al., 1990) and pesticide (Clark, 1990) resistance.

In mammals such as the rat, at least 12 GST subunits have been characterized and they have been divided on a structural basis into five classes, the cytosolic Alpha, Mu, Pi and Theta classes and the single microsomal enzyme (Mannervik et al., 1985; Hayes et al., 1990; Meyer et al., 1991). Multiple GST isoforms have been purified from two species of Elasmobranch and four species of Teleost fish (Sugiyama et al., 1981; Foureman and Bend, 1984; Ramage and Nimmo, 1984; Dierickx, 1985; Ramage et al., 1986; George and Buchanan, 1990; Dominey et al., 1992). Binding and substrate specificity studies have shown marked differences in the expression of GST isoforms between these species, and an apparent lack of 'ligandin'-like activity and Se-independent peroxidase activity towards cumene hydroperoxide indicates marked differences between fish and mammals. Immunochemical studies have demonstrated that one

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; GST, glutathione S-transferase; IPTG, isopropyl β -D-thiogalactopyranoside; SSC, 0.15 M NaCl/0.015 M sodium citrate; DTT, dithiothreitol.

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of the plaice enzymes is related to the rat Alpha class GSTs and that several fish species contain a Pi class GST (George et al., 1989). Indeed, recent protein sequencing studies have confirmed that the major salmon GST is a Pi class GST orthologue (Dominey et al., 1992). Previously we purified and characterized two major GST isoforms from the liver of the plaice (*Pleuronectes platessa*). The major homodimeric isoform, GST-A, shows no common immunoreactive epitopes with the rat Alpha, Mu or Pi class GSTs (George and Buchanan, 1990). In the present paper we describe the characterization of a full-length cDNA for this isoform, its heterologous expression in *Escherichia coli*, the expression of GST-A in different organs of the plaice, and the effect of epoxide administration on hepatic GST-A mRNA levels.

MATERIALS AND METHODS

Animals and inducer treatment

Plaice were obtained from an unpolluted area of the Scottish coast and held for 2 weeks in recirculating filtered seawater before use. For induction studies, fish were injected intraperitoneally with 0.5 ml of corn oil per kg body weight (control) or *trans*-stilbene oxide in corn oil at 200 mg per kg body weight 3 days before they were killed.

Materials

All chemicals were of analytical, electrophoretic or molecular biology grade from Sigma (Poole, Dorset, U.K.) or BDH (Poole, Dorset, U.K.). SDS and electrophoresis chemicals were from Bio-Rad (Watford, Herts., U.K.). Ampicillin, 5-bromo-4-chloroindol-3-yl β -D-galactopyranoside, isopropyl β -D-thiogalactopyranoside (IPTG) and Nitro Blue Tetrazolium were purchased from IBI-Kodak (Cambridge, U.K.). Bacterial growth media were prepared with tryptone and yeast extract from Oxoid (Unipath Limited, Basingstoke, Hants., U.K.). S-Hexylglutathione was synthesized and used as described by Mannervik and Guthenberg (1981). *Eco*RI adaptors, oligo(dT)-cellulose, the cDNA synthesis kit, the vectors λ gt11, M13mp18, M13mp19, pT7T318U and pKK223-3, the bacterial strains *E. coli* Y1088, Y1090 and NM522, and the chromatography media agarose, Sephadex and Sepharose were obtained from Pharmacia LKB Biotechnology (Milton Keynes, U.K.). All restriction and DNA-modifying enzymes used were from Pharmacia Life Sciences (Paisley, Scotland, U.K.) or Boehringer (Lewes, E. Sussex, U.K.) and were used according to the manufacturer's instructions. Radioactive nucleotides, *in vitro* packaging extracts, nylon (Hybond N) and nitrocellulose (Hybond C) membranes were supplied by Amersham International (Amersham, Bucks., U.K.).

The carp actin probe was a gift from Professor I. Johnston, University of St Andrews.

RNA purification and cDNA library preparation

Total RNA was extracted from an immature male plaice liver using the guanidinium isothiocyanate method of Chirgwin et al. (1979), and poly(A)⁺ RNA was prepared by a single round of binding, washing and elution from oligo(dT)-cellulose (Aviv and Leder, 1972).

First- and second-strand cDNA was synthesized using oligo(dT) primers from poly(A)⁺ RNA by the method of Gubler and Hoffman (1983), using a commercially supplied kit (Pharmacia). *Eco*RI adaptors were ligated to cDNA, oligonucleotides were removed by separation on a Sepharose 400

column and the cDNA was ligated to *Eco*RI-cut dephosphorylated λ gt11. This DNA was packaged by the method of Hohn (1979) using commercially supplied packaging extracts (Amersham), titred (5×10^4 recombinants) and amplified in *E. coli* Y1088.

Library screening and clones

A total of 5×10^4 plaques from the amplified library were plated with *E. coli* Y1090, induced with IPTG, transferred to nitrocellulose (Hybond C) and screened with an antibody to plaice GST-A (George and Buchanan, 1990) as described by Huynh et al. (1985). Two positive plaques were taken through four rounds of plaque purification and designated λ PLGSTA1 and λ PLGSTA2.

Nucleic acid analysis

Subcloning into pT7T318U, pKK223-3 and M13 vectors was carried out by standard methods (Maniatis et al., 1982). Sequencing reactions were performed using T7 DNA polymerase and purified single-stranded M13 by the dideoxynucleotide chain termination method with [³²P]dCTP (Sanger et al., 1977; Yanisch-Perron et al., 1985). Total RNA for hybridization was blotted directly on to nylon membranes (Hybond N) from glyoxalated samples prepared as described by McMaster and Carmichael (1977) and separated on 1% agarose gels in 40 mM Mops, pH 7.0, 10 mM sodium acetate and 1 mM EDTA. After baking the membranes at 80 °C for 1 h, hybridization was accomplished using a PLGSTA cRNA probe generated by *in vitro* transcription in the presence of [³²P]UTP from linearized PLGSTA clones in pT7T318U (Melton et al., 1984). Plaice genomic DNA was isolated from plaice liver by the method of Blin and Stafford (1976). Samples were digested with restriction enzymes (4 units/ μ g of DNA) for 16 h according to the manufacturer's instructions. A 10 μ g portion of each digested DNA was electrophoresed in 0.6% agarose in 40 mM Tris/acetate, pH 8.0, and 1 mM EDTA. Gels were vacuum-blotted to nylon membranes (Hybond N) using 0.4 M NaOH and 1.5 M NaCl after brief treatment with 0.25 M HCl, then hybridized at 42 °C to PLGSTA cDNA and labelled with [³²P]dCTP by the random priming method (Feinberg and Vogelstein, 1983), in 0.1 M Na₂HPO₄/NaH₂PO₄, pH 7.6, 0.75 M NaCl, 0.2% (w/v) BSA, 0.2% (w/v) Ficoll 400, 0.2% (w/v) polyvinylpyrrolidone, 0.1% SDS, 100 μ g/ml denatured herring sperm DNA and 50% formamide for 24 h. The membranes were then washed extensively in $2 \times$ SSC/0.1% SDS at 42 °C, before a final wash for 5 min in $0.1 \times$ SSC. Autoradiography was performed at -70 °C for 24 h with an intensifying screen.

Heterologous expression and characterization of plaice GST-A in *E. coli*

The cDNA insert was removed from λ PLGSTA by digestion with *Eco*RI and subcloned into the unique *Eco*RI restriction site of pT7T318U. A fragment containing the entire coding and 3' non-coding regions was then generated by digestion with *Nco*I and *Hind*III and ligated into pKK223-3 (Amman et al., 1983). The ligation mixture was used to transform *E. coli* NM522, and plasmid-bearing colonies were selected on ampicillin-containing LB agar plates. Several individual colonies were picked, grown and tested for the presence of PLGSTA cDNA by restriction digestion of plasmid minipreps.

Individual recombinant plasmid-containing clones and a parent non-recombinant plasmid-containing clone were grown

Figure 1 Restriction endonuclease map and sequencing strategy of the PLGSTA cDNA clone

The relative positions of the *NcoI* and certain other restriction sites are shown. The arrows represent the direction and length of sequencing. The stippled region represents the coding sequence.

in small-scale cultures (1 ml volume containing 2.0 mM IPTG), centrifuged and sonicated in 50 mM Tris/HCl, pH 7.6, and 2 mM dithiothreitol (DTT), and assayed for 1-chloro-2,4-dinitrobenzene (CDNB)-conjugating activity with CDNB as substrate, or analysed for GST-A by immunoblotting. After heating at 90 °C for 5 min in SDS/PAGE sample buffer, the proteins were separated on SDS/12.5% PAGE, electrophoretically transferred to a nitrocellulose membrane and GST-A was visualized by immunostaining using a rabbit anti-(plac GST-A) primary antiserum, an anti-(rabbit IgG)-alkaline phosphatase-labelled reporter antibody conjugate and the chromogenic substrates Nitro Blue Tetrazolium and bromo-4-chloroindol-3-yl β -D-galactopyranoside as described previously (George and Buchanan, 1990).

		<i>NcoI</i>	
		G CAG CUU CUC UCU GAA ACC	-1
	M A K D M T L L W G S G S P P C W R V M		20
+1	<u>AUG</u> GCC AAG GAC AUG ACU CUG CUG UGG GGC UCC GGC UCU CCU CCC UGC UGG AGG GUG AUG		60
	I V L E E K N L Q A Y N S K L L S F E K		40
	AUC GUG CUG GAG GAG AAG AAC CUG CAG GCG UAC AAC AGC AAA UUG CUC UCC UUC GAG AAA		120
	G E H K S A E V M S M N P R G Q L P S F		60
	GGG GAG CAC AAG UCA GCC GAG GUG AUG AGC AUG AAC CCC AGG GGU CAG CUC CCU UCC UUC		180
	K H G S K V L N E S Y A A C M Y L E S Q		80
	AAA CAU GGC AGC AAA GUU CUG AAU GAA UCC UAC GCC GCC UGC AUG UAC CUG GAG AGC CAG		240
	F K S Q G N K L I P D C P A E Q A M M Y		100
	UUC AAG UCC CAG GGA AAC AAG CUG AUC CCC GAC UGC CCC GCU GAG CAG GCC AUG AUG UAC		300
	Q R M F E G L T L A Q K M A D V I Y Y S		120
	CAG CGC AUG UUU GAG GGU CUC ACG CUC GCC CAG AAA AUG GCG GAU GUU AUC UAC UAC AGC		360
	W K V P E A E R H D S A V K R N K E N V		140
	UGG AAG GUC CCG GAG GCA GAG AGA CAC GAC UCU GCU GUG AAG AGA AAC AAA GAG AAU GUG		420
	S T D V K L S Q E Y L O K T S G S F V A		160
	AGC ACU GAC GUG AAG CUG UCC CAG GAA UAU CUG CAG AAG ACG UCC GGC UCG UUC GUG GCA		480
	G K S F S L A D V S V F P G V A Y L F R		180
	GGA AAG AGC UUC UCC CUG GCU GAU GUG UCG GUU UUC CCA GGC GUG GCU UAU CUC UUC CGU		540
	F G L T E E R Y P Q L T A Y Y N S L K E		200
	UUU GGG UUG ACU GAA GAG CGU UAC CCC CAA CUG ACG GCG UAC UAC AAC UCU CUG AAG GAG		600
	R P S I K A S W P P T W L E S P Q G Q D		220
	AGA CCC AGC AUC AAA GCC AGC UGG CCC CCC ACC UGG CUG GAG AGU CCA CAG GGU CAA GAC		660
	M L K D V *		225
	AUG CUG AAA GAC GUC UGA GAUUAACAAACGUGCACCCUCCGCCUUGUUGUUAUAUGUAACCAUCCUCCGAA		733
	CUGCACUACUGGGCUGACAGGAACAUGAAUUUCUGUAACUGGCUUGUGCUUGUUAUUUUAUUUAUUUAUUUGUA		812
	UGUGAGUUAAACGUGGAUGUUUAUCCUGUAAGAAAGCU <u>UAAAA</u> UCUGACUUCAAAAAA		877

Figure 2 Nucleotide and deduced amino acid sequence of PLGSTA

The deduced amino acid sequence is shown in single letter format above the nucleotide sequence. Position +1 is the first base of the AUG start codon and the untranslated 5' bases are designated -19 to -1. Numbering of the amino acid residues includes the initiator methionine. The termination codon and putative polyadenylation signal are underlined.

Figure 3 Heterologous expression of plaice GST-A subunit in *E. coli*

Immunoblot analysis of bacterial lysates and plaice cytosol. Samples of 10 μ l were electrophoresed on SDS/12.5%-PAGE, transferred to nitrocellulose and immunostained with anti-(plaice GST-A) serum as described in the Materials and methods section. Lanes 1–4, individual bacterial lysates containing pKK-PLGSTA; lane 5, bacterial lysate containing pKK223-2; lane 6, plaice cytosol. Numbers below each lane represent the total CDNB conjugating activity in each sample (nmol/min per sample).

For purification of the expressed protein, an individual recombinant was grown in large-scale culture (500 ml) until mid-exponential phase and then induced for 3 h at 28 °C by the addition of IPTG to 2.0 mM. Cells were then collected by centrifugation (2000 *g* for 10 min at 4 °C), then lysed at 4 °C in 50 mM Tris/HCl, pH 7.6, 2 mg/ml lysozyme, 2 mg/ml DNAaseI and 1 mM phenylmethanesulphonyl fluoride for 15 min, followed by the addition of Nonidet P40 to 0.2% and incubation for a further 10 min at 4 °C. The lysate was then diluted 5-fold with 50 mM Tris/HCl, pH 7.6, 2 mM DTT, 1 mM EDTA and 1 mM phenylmethanesulphonyl fluoride (buffer A), centrifuged at 15000 *g* for 15 min and loaded on to an *S*-hexylglutathione affinity column (1.5 cm $\times$ 10 cm) equilibrated with buffer A (Mannervik and Guthenberg, 1981). After washing extensively with buffer A containing 200 mM NaCl, specifically bound material was eluted with 10 mM GSH in buffer A.

Enzyme activities

GST activities were measured as described previously (George and Buchanan, 1990), glutathione peroxidase activity was measured by the method of Wendel (1981) using cumene hydroperoxide as substrate, and protein concentrations were measured by the method of Bradford (1976), using BSA standards.

RESULTS

Characterization of the λ PLGSTA clone

Two positive plaques, designated λ PLGSTA1 and λ PLGSTA2, were selected from a plaice liver cDNA library in λ gt11 by screening with a specific anti-(plaice GST-A) serum. These plaques were then purified by a further three rounds of screening. Identical results were subsequently obtained with both isolates, indicating that the same clone had been amplified. Hereafter the numerical designation will be omitted. Digestion of the λ PLGSTA clone with *Eco*RI showed it to contain an insert of approx. 900 bp and this was subcloned into M13mp18 and M13mp19, either directly or after further restriction digestion, and sequenced according to the strategy shown in Figure 1. The PLGSTA cDNA (Figure 2) has 19 bp of 5' non-coding sequence, 198 bp of 3' non-coding sequence containing a typical poly(A) addition signal, flanking an open reading frame coding for 225 amino acids coding for a protein of M_r 25723, a molecular size

Figure 4 Immunoblot and SDS/PAGE of purified GST-A

Samples were separated on 12.5% gels and immunoblotted as described in the Materials and methods section. (a) Coomassie Blue-stained gel. Lane 1, M_r standards (205 000, 116 000, 97 400, 66 000, 45 000 and 29 000); lane 2, plaice cytosol; lane 3, recombinant GST-A; lane 4, *S*-hexylglutathione-agarose affinity-purified plaice cytosol; lane 5, glutathione-agarose affinity-purified plaice cytosol. (b) Immunoblot developed with anti-(plaice GST-A). Lane 1, plaice cytosol; lane 2, recombinant GST-A; lane 3, *S*-hexylglutathione-agarose affinity-purified plaice cytosol; lane 4, glutathione-agarose affinity-purified plaice cytosol.

Table 1 Specific activities of plaice GST-A

Enzyme activity was determined at 25 °C. The results represent mean values for triplicate determinations (ND, not detectable).

Substrate	Specific activity (μ mol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$)	
	GST-A from plaice liver*	GST-A recombinant
CDNB	35	25.5
Ethacrynic acid	ND	0.81
1,2-Dichloro-4-nitrobenzene	0.13	0.23
<i>p</i> -Nitrobenzyl chloride	0.01	1.12
1,2-Epoxy-3-(<i>p</i> -nitrophenoxy)propane	ND	ND
Bromosulphophthalein	ND	ND
Cumene hydroperoxide	ND	0.13
<i>t</i> -Butylhydroperoxide	—	ND

* Data from George and Buchanan (1990).

consistent with that of the purified plaice GST-A polypeptide on SDS/PAGE (apparent M_r 27 000; George and Buchanan, 1990).

Expression of PLGSTA cDNA in *E. coli*

The first AUG codon of PLGSTA was, fortuitously, contained within an *Nco*I restriction site, enabling the insert to be ligated directly to the first initiation codon downstream of the *trc* promoter and ribosome binding site of pKK223-3. The induction with IPTG of small-scale cultures of *E. coli* NM522 containing the recombinant plasmid showed that the specific CDNB-conjugating activity (76–184 nmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$) in lysates of these cultures was elevated 40–100-fold compared with a culture containing the parent pKK223-3 plasmid (< 2 nmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$). Immunoblot analyses of these lysates with the specific GST-A antiserum showed that only those cultures containing the recombinant PLGSTA cDNA-containing plasmid expressed a protein with the same electrophoretic mobility and immunoreactivity as GST-A from plaice cytosol (Figure 3).

The expressed protein was purified from large-scale induced

Figure 5 Southern blot analysis of restriction-digested plaice DNA

Samples of DNA (10 μ g) were electrophoresed in a 0.6% agarose gel, transferred to a nylon membrane and hybridized to 32 P-labelled PLGSTA cDNA as described in the Materials and methods section. Lanes B, *Bam*HI digestion; E, *Eco*RI digestion; H, *Hind*III digestion; P, *Pst*I digestion; S, *Sst*I digestion. The sizes of *Hind*III-digested λ phage standards are noted in bp.

Figure 6 Northern blot of plaice tissue RNA

Total RNA was extracted from different plaice tissues, separated by electrophoresis, transferred to nylon membranes and hybridized to 32 P-labelled PLGSTA cRNA as described in the Materials and methods section. Lane 1, brain; lane 2, ovarian wall; lane 3, gill; lane 4, heart; lane 5, intestinal mucosa; lane 6, kidney; lane 7, liver; lane 8, spleen. A 10 μ g sample of total RNA was loaded per lane. Positions of size standards are given in nucleotides.

culture of one of these PLGSTA-containing clones (pKK PLGSTA) by affinity chromatography on *S*-hexylglutathione. A heterologously expressed enzyme displaying a similar specific activity towards CDNB to that of the purified plaice liver enzyme was only obtained after inducing the cultures at 28 °C and by using relatively gentle lysis procedures. Sonication or growth at higher temperatures resulted in markedly lower specific activities. Optimal activity and yield was obtained by induction at 28 °C and treatment with lysozyme and DNAase I, followed by detergent disruption under reducing conditions (2 mM DTT). Two major polypeptides of plaice liver cytosol with apparent M_r values of 26000 and 24000, designated GST-A and GST-B respectively (George and Young, 1988; George and Buchanan,

Table 2 Tissue distribution of GST-A in plaice

Tissues from eight sexually immature fish of 98–226 g (mean 157 g) were dissected and a sample taken for Northern blot analysis. Cytosol was prepared from the remainder for determination of total GST activity (with CDNB as substrate) and for Western immunoblot analysis with a specific antiserum to plaice GST-A, as described in the Materials and methods section. *Pooled tissue from all animals fractionated.

Tissue	GST specific activity		GST-A Western blot	GST-A mRNA Northern blot
	(μ mol/g)	Relative to liver (100)		
Liver	74 $\pm$ 19	100	+	+
Trunk kidney	4.4 $\pm$ 1.6	5.9	+	+
Intestinal mucosa	8.4 $\pm$ 1.4	11.4	+	+
Gill filaments	1.1 $\pm$ 0.6	1.5	+	+
Spleen*	1.06	1.4	?	+
Heart*	0.6	0.8	+	+
Brain*	0.99	1.3	+	+

Figure 7 Northern blot of hepatic RNA from plaice treated with *trans*-stilbene oxide

Fish were treated with *trans*-stilbene oxide (or corn oil for vehicle controls). Total RNA was isolated from livers at 72 h post-injection, and 10 μ g samples were separated by electrophoresis, transferred to nylon membranes and hybridized to a 32 P-labelled PLGSTA cRNA probe as described in the Materials and methods section. (a) Lanes 1–3, untreated fish; lanes 4–6, *trans*-stilbene oxide-treated fish; (b) Identical samples as in (a) hybridized to a carp actin probe. Positions of size standards are given in nucleotides.

1990), bind to GSH affinity matrixes: GST-A preferentially binds to an *S*-hexylglutathione matrix and GST-B to a GSH affinity matrix (Figure 4). The heterologously expressed protein exhibited the same preference as GST-A for the *S*-hexylglutathione matrix rather than GSH-agarose, displayed the same M_r and a similar immunoreactivity with anti-GST-A antibody as GST-A from plaice cytosol, and a comparable activity towards CDNB and similar substrate specificity (Table 1) to that reported for GST-A purified from plaice liver cytosol (George and Buchanan, 1990).

We conclude that the PLGSTA cDNA insert encodes the subunit of the homodimeric plaice GST-A enzyme, and we have designated it as subunit A.

Genomic complexity detected by PLGSTA cDNA

Southern blot analysis of plaice genomic DNA digested with various restriction enzymes using the PLGSTA cDNA insert as a probe revealed between one and four bands in the various restriction digestions, excluding the bands at the origin and at 23 kb, which are present in all digests (Figure 5). Given the small number of bands present in all digests and that the three (faint) bands at 4.5, 2.3 and 0.8 kb in the *Pst*I digest are the minimum predictable from the two *Pst*I restriction sites in the cDNA, we would suggest that there is only a single gene for plaice GST subunit A and no closely related isoforms.

Tissue expression of GST-A mRNA

Northern blot analysis of total RNA from plaice liver with ³²P-labelled PLGSTA cRNA (Figure 6) detected an RNA species of approx. 1100 nucleotides. Similar analysis of total RNA from various plaice tissues indicated that the PLGSTA cRNA probe visualized a single hybridizing band of 1100 bp in all plaice tissues tested, including brain, gill, gonad, intestine, kidney and spleen. Equal amounts of total RNA were electrophoresed and blotted for each tissue, indicating from the intensity of signal that the relative abundance of GST-A mRNA in each tissue was in the order liver > intestine = gill > kidney > brain = gonad > heart = spleen. These are compared with the total CDNB-conjugating activities and GST-A protein levels in Table 2.

Inducibility of GST-A mRNA by *trans*-stilbene oxide

Previous studies have indicated that total CDNB-conjugating activity of plaice liver can be induced by treatment of fish with various agents, including 3-methylcholanthrene, a commercial polychlorinated biphenyl mixture (Aroclor 1254), ethoxyquin and *trans*-stilbene oxide (George and Buchanan, 1990). Total RNA was prepared from the livers of three plaice 72 h after intraperitoneal injection of *trans*-stilbene oxide (200 mg/kg). Northern blot analysis demonstrated that GST-A mRNA levels were induced several-fold relative to those in vehicle (corn oil) injected fish (Figure 7), indicating that this isoform was induced by epoxide treatment.

DISCUSSION

Using an antibody to purified plaice GST-A, we have isolated and characterized a cDNA clone encoding the plaice GST subunit A of *M*, 25723, comprising 225 amino acids. The CDNB conjugating activity (25.5 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) of the recombinant enzyme, purified from induced cultures of a construct of this cDNA in the expression vector pKK233-2, exhibits a comparable specific activity towards CDNB conjugation to that of the

homodimeric GST-A of plaice liver as characterized previously (George and Buchanan, 1990). The recombinant plaice enzyme has a comparatively narrow substrate specificity towards the common GST substrates; especially notable are the apparent lack of significant peroxidase activity towards cumene hydroperoxide and the low rate of conjugation of ethacrynic acid.

In previous studies we demonstrated a complete lack of cross-reactivity between antibodies raised against rat GST of the Alpha (subunits 1, 2 and 8), Mu (subunit 3) or Pi (subunit 7) classes and plaice GST-A, or between a specific antiserum raised towards GST-A and rat subunits 1, 2, 3, 4, 6, 7, 8 or 9 (George et al., 1989; George and Buchanan, 1990). This lack of common immunoreactive epitopes is borne out by the lack of similarity when the amino acid sequences are compared, confirming that plaice GST-A is not structurally homologous to the mammalian Alpha, Mu or Pi class GSTs. Intriguingly, the deduced amino acid sequence of plaice GST-A, the first full-length sequence obtained for a GST from a lower vertebrate, bears closer structural similarities to GSTs which have been sequenced from non-vertebrates (insects and plants) than those from terrestrial vertebrates. On the basis of sequence similarities it has been suggested that GSTs from the fruit fly (Toung et al., 1990) and maize (Grove et al., 1988) might be assigned to another class of GSTs, the Theta class, of which rat subunits 5 and 12 (Meyer et al., 1991) and Yrs (Ogura et al., 1991) have been sequenced. Whilst there is no reciprocal immunological cross-reaction between the rat Theta class subunit 5 and plaice GST-A (results not shown), when the amino acid sequences of GSTs from plaice, fruit fly (Toung et al., 1990) and maize (Grove et al., 1988) are aligned with the mammalian Theta class enzymes (Figure 8), there is significant similarity between residues 1 and 100, indicating that they may all belong to a primitive GST class. Arg-18, implicated in GSH binding (Reinemer et al., 1991), can be aligned in all sequences, and Tyr-8, which is also implicated in GSH binding, is present in all of these GSTs except the plaice form.

GST-A mRNA was detectable in all plaice tissues and this suggests that GST-A may have a cellular 'housekeeping' function, although steady-state mRNA levels were much higher in liver, intestine, gills and kidney than, for example, in brain, consistent with a possible role in the metabolism of harmful electrophiles or in protection from their action. The possibility that there may be other related isoenzymes in different plaice tissues, as seen for mammalian Alpha class GSTs (Pickett, 1987), is unlikely as the Southern blotting experiment revealed a simple banding pattern consistent with the existence of a single gene. Indeed, the presence of only three bands in the *Pst*I digest is the minimum number predictable from the cDNA sequence.

Administration of the epoxide *trans*-stilbene oxide resulted in an increase in GST-A mRNA levels, a result which we have also

Figure 8 Alignment of deduced amino acid sequences of GSTs

PLGSTA, plaice GST-A (this work); DMEGST1, *Drosophila melanogaster* GST 1-1 (Toung et al., 1991); RATYRS, rat GST Yrs subunit (Ogura et al., 1991); ZMAGST3, maize GST IIIA0 (Grove et al., 1988). Only the first 100 amino acids are shown. Residues which are identical or equivalent to the plaice GST-A sequence are boxed.

observed in the closely related flounder, *Platichthys flesus* (Scott et al., 1992). We have also observed in the flounder that inducers such as the polyaromatic hydrocarbon 3-methylcholanthrene, which act at the [*Ah*] locus (Nebert et al., 1990), do not affect or may even suppress mRNA for GST-A in this species, whereas *trans*-stilbene oxide induces GST-A mRNA in both plaice and flounder (Scott et al., 1992). This spectrum of inducer action differs from that observed in mammals, where both *trans*-stilbene oxide and polyaromatic hydrocarbons induce the Alpha class GST-1 of rats and Mu class GST of mouse (DiSimplicio et al., 1989; Hayes et al., 1991; Rushmore et al., 1991), again supporting the conclusion from the sequence, substrate specificity, ligand binding and immunological data (above and George and Buchanan, 1990; George et al., 1989) that plaice GST-A is not an Alpha or Mu class orthologue. GST-A is also induced by various antioxidants such as butylated hydroxyanisole and ethoxyquin (George and Buchanan, 1990; Leaver et al., 1992), as well as by *t*-butylhydroperoxide (M. J. Leaver, K. Scott and S. G. George, unpublished work), indicating a possible commonality of mechanism of induction with *trans*-stilbene oxide. Such a mechanism has been demonstrated for the rat GST-1 gene by the characterization of an antioxidant responsive element (ARE) (Rushmore et al., 1991). The possibility that a similar system operates in plaice for GST-A expression is currently under investigation.

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