Biochem. J. (1991) 276, 461–469 (Printed in Great Britain)

Regulation of mouse glutathione *S*-transferases by chemoprotectors

Molecular evidence for the existence of three distinct Alpha-class glutathione *S*-transferase subunits, Ya_1, Ya_2 and Ya_3, in mouse liver

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Liver cytosol from mice fed on a normal diet contains Alpha-class glutathione *S*-transferase (GST) subunits of M_r 25800, Mu-class GST subunits of M_r 26400 and Pi-class GST subunits of M_r 24800. Feeding female mice with a diet containing the anticarcinogenic antioxidant butylated hydroxyanisole (BHA) causes induction of the constitutively expressed Mu-class and Pi-class subunits. BHA also induces an Alpha-class GST comprising subunits of M_r 25600, which is not expressed at detectable levels in normal mouse liver [McLellan & Hayes (1989) Biochem. J. **263**, 393–402]. Data are now presented that show that administration of the anticarcinogen β-naphthoflavone (BNF), like BHA, induces the Alpha-class 25600-M_r subunits but not the constitutive Alpha-class GST with subunits of M_r 25800. The effects of BNF on expression of hepatic GST were studied in both DBA/2 and C57BL/6 mice; these studies revealed a preferential induction of the Alpha-class 25600-M_r subunits and of the Pi-class 24800-M_r subunits in those mice in possession of a functional Ah receptor. The BHA/BNF-inducible Alpha-class GST can be resolved into two separate, non-interconvertible peaks by reverse-phase h.p.l.c. Automated amino acid sequence analysis of CNBr-derived peptides from each of these h.p.l.c.-purified peaks showed that the peaks contained at least two very similar subunits. These have been named Ya_1 and Ya_2. The amino acid sequence of the Ya_1 subunit was compared with sequences deduced from a genomic clone, λmYa1 (Daniel, Sharon, Tichauer & Sarid (1987) DNA **6**, 317–324], and a cDNA clone, pGT41 [Pearson, Reinhart, Sisk, Anderson & Adler (1988) J. Biol. Chem. **263**, 13324–13332]. Our data suggest that the Ya_1 subunit represents the subunit encoded by the genomic clone, λmYa1. Sequence analysis of the constitutive Alpha-class Ya_3 subunit (M_r 25800) shows that, although it is a member of the same gene family as the Ya_1 and Ya_2 subunits, it represents a distinct sub-family of Alpha-class GST, containing subunits that are more similar to rat Yc. Our data indicate that, of these Alpha-class GST subunits, the two with M_r 25600 (Ya_1 and Ya_2) are selectively induced by BHA or BNF in mouse liver; neither BHA nor BNF induces significantly the GST subunit with M_r 25800 (Ya_3).

INTRODUCTION

Glutathione *S*-transferases (GSTs), a large and varied group of phase II xenobiotic-metabolizing enzymes, are believed to play a critical role in the protection of cellular macromolecules from attack by reactive electrophiles. Multiple isoenzymes have been described, and the majority can be classified as belonging to the cytosolic multigene families, Alpha, Mu and Pi, or to the membrane-bound microsomal GST family (Morgenstern & DePierre, 1983; Mannervik *et al.*, 1985).

Hepatic cellular concentrations of several cytosolic GSTs in rodents are known to be readily increased in response to a wide range of exogenous compounds (Kaplowitz *et al.*, 1975; Hayes *et al.*, 1979; Sparnins *et al.*, 1982; Benson & Barretto, 1985; Ding & Pickett, 1985; Talalay *et al.*, 1988; Benson *et al.*, 1989). Of particular interest is the ability of anticarcinogens, compounds that protect against chemical carcinogenesis, to induce GSTs. Anticarcinogens, or chemoprotectors, are a diverse group of chemicals that can exert their anticarcinogenic effects in many different ways [for reviews see Wattenberg (1978, 1985) and Talalay (1989)], but the induction of phase II and, in certain instances, phase I drug-metabolizing enzymes (defined by Williams, 1971), which results in an increase in the capacity of cells to eliminate alkylating toxins, is thought to be a major mechanism by which anticarcinogens act.

Chemoprotective inducing agents fall into two broad categories, described as type A and type B (Wattenberg, 1985) or monofunctional and bifunctional inducers (Prochaska & Talalay, 1988). Monofunctional inducers, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene, can markedly increase the expression of phase II drug-metabolizing enzymes (e.g. GST and UDP-glucuronyltransferase), whereas their effects on phase I metabolism are less obvious. Bifunctional inducers, which are typified by planar aromatic hydrocarbons and which include flavonoids such as β-naphthoflavone (BNF), increase cellular concentrations of both phase II enzymes and certain phase I enzymes, most notably those of the cytochrome *P*-450 I gene family.

Induction of cytochrome *P*-450 IA1 involves the interaction of planar aromatic hydrocarbons with cytosolic receptor proteins, Ah or dioxin receptors, which can subsequently bind to specific xenobiotic-regulatory elements in the 5′ flanking region of the *P*-450 IA1 gene, thereby promoting transcription [for reviews see Whitlock (1986) and Nebert & Jones (1989)]. Less is known about the regulation of phase II xenobiotic-metabolizing enzymes by planar aromatic hydrocarbons, although recently it has been

Abbreviations used: GST, glutathione *S*-transferase; BNF, β-naphthoflavone; BHA, 2(3)-t-butyl-4-hydroxyanisole.
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shown that the 5′ flanking region of one of the rat Ya genes contains a region of DNA that has a sequence identical with the core sequence of the xenobiotic-regulatory elements found on the 5′ flanking sequence of the cytochrome *P*-450 IA1 gene (Rushmore & Pickett, 1990; Rushmore *et al.*, 1990). Rushmore *et al.* (1990) also identified a second distinct regulatory element in the 5′ flanking region of the rat GST Ya gene that is responsive to BNF.

Most of the studies into chemoprotectors have used the mouse as an animal model. However, much less is known about regulation of mouse GST genes (see Pearson *et al.*, 1988; Daniel *et al.*, 1989) than those in the rat. Although Telakowski-Hopkins *et al.* (1988) implicated the Ah receptor in the control of expression of the rat Ya genes, it is at present unclear whether or not the Ah receptor is directly involved in the induction of mouse GSTs (see Felton *et al.*, 1980). Unlike rats, certain inbred strains of mice possess defective Ah receptors with a low affinity for BNF and other planar aromatic hydrocarbons. These mouse strains have proved valuable in dissecting the mechanisms by which genes can be activated by xenobiotics (Nebert *et al.*, 1982; Eisen *et al.*, 1983). During the present study we have exploited the fact that DBA/2 mice (unlike C57BL/6 mice) lack a functional Ah receptor, in order to investigate the possible involvement of this receptor in the induction of GST by the chemoprotector BNF in these two strains.

The livers from normal untreated male mice express Alpha-class GST (Ya_3Ya_3, subunit M_r 25800), Mu-class GST (Yb_1Yb_1, subunit M_r 26400), Pi-class GST (YfYf, subunit M_r 24800) and microsomal GST (subunit M_r 17300) (Hayes *et al.*, 1987; McLellan & Hayes, 1987, 1989); by comparison with male liver, the female mouse liver contains little GST YfYf. Feeding female mice with BHA causes hepatic induction of several Mu-class GSTs (Pearson *et al.*, 1983; DiSimplicio *et al.*, 1989; Hayes *et al.*, 1991), Pi-class GST and a novel Alpha-class GST (subunit M_r 25600) not expressed at appreciable levels in livers from untreated mice (McLellan & Hayes, 1989). Pearson *et al.* (1988) described a cDNA clone, pGT41, which encodes a mouse Alpha-class GST. The coding region of this clone differs from that of a genomic clone of mouse Alpha-class GST, λmYa1, described by Daniel *et al.* (1987). The deduced amino acid sequences of these two DNA clones are very similar, but it is unclear which GST isoenzymes they encode; on the basis of physical and catalytic differences between the Ya-type subunits of M_r 25600 and M_r 25800, it seems unlikely that they are both encoded by pGT41 and λmYa1 (McLellan & Hayes, 1989).

In the present study, of which a preliminary account has been reported elsewhere (McLellan & Hayes, 1990), we have resolved the inducible GST of subunit M_r 25600 into two fractions by reverse-phase h.p.l.c. and have used amino acid sequencing to demonstrate the existence of at least three genetically distinct Alpha-class GST subunits, Ya_1 (M_r 25600), Ya_2 (M_r 25600) and Ya_3 (M_r 25800). These subunits are all expressed in the livers of mice treated with BHA and BNF. The subunits Ya_1 and Ya_2, which possess similar electrophoretic and immunochemical properties, display close sequence similarity and are more distantly related to the Ya_3 subunit than to each other. The relationship between the Ya_1, Ya_2 and Ya_3 subunits and the two mouse DNA clones, pGT41 and λmYa1, is discussed.

MATERIALS AND METHODS

Chemicals

β-Naphthoflavone (BNF), 2(3)-t-butyl-4-hydroxyanisole (BHA) and CNBr were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). The h.p.l.c. solvents were from Rathburn Chemicals (Walkerburn, Borders, Scotland, U.K.). All other chemicals were of analytical grade and were obtained from BDH Chemicals (Poole, Dorset, U.K.).

Animals

Mice were purchased from Bantin and Kingman, Hull, U.K. Animals, which were age-matched, were received at 7–9 weeks old and all mice were female. They were acclimatized for about 1 week before commencement of treatment with either (*a*) BHA or (*b*) BNF.

(*a*) Mice were fed on an unrestricted diet (ground Rat and Mouse no. 3 Breeding Diet; SDS, Stetfield, Witham, Essex, U.K.) containing BHA (0.75% by wt.) for 14 days, with free access to drinking water.

(*b*) Alternatively, the mice were injected intraperitoneally with BNF (200 mg/kg body wt.) in corn oil. This was followed by a second injection 24 h later, of the same dose. Control mice were injected with corn oil only, and all animals were killed 48 h after the second injection. Food and water were given *ad libitum*.

Analytical

Enzyme assays were performed at 37 °C. Those with 1-chloro-2,4-dinitrobenzene as substrate were carried out in a Cobas Fara centrifugal analyser (Roche Diagnostics, Welwyn Garden City, Herts., U.K.) by a method similar to that described by Hayes & Clarkson (1982). Up to 29 samples were pre-incubated simultaneously with GSH and the reactions were started by the addition of 1-chloro-2,4-dinitrobenzene. The final substrate concentrations were 2 mM and 1 mM for GSH and 1-chloro-2,4-dinitrobenzene respectively in a reaction volume of 0.25 ml containing 0.1 M-sodium phosphate buffer, pH 6.5. The reactions were monitored by initial measurement of the absorbance at 340 nm, 10 s after mixing, followed by seven absorbance measurements at 5 s intervals. Reaction rates were determined by an integral kinetic data analysis program, which performed linear-regression analysis on the absorbance readings from each cuvette.

Glutathione peroxidase activity was determined at pH 7.2 by a method similar to that of Reddy *et al.* (1981), modified for use on the centrifugal analyser (Howie *et al.*, 1990). All other enzyme assays were performed as described by Habig & Jakoby (1981).

Determination of cytosolic protein concentrations was by the method of Bradford (1976). Protein in unsolubilized microsomal fractions was measured by the biuret method.

Statistical analyses of enzymic rates were performed by the Mann–Whitney U test.

Electrophoresis and immunoblotting

The methods described by Hayes & Mantle (1986*a*,*b*) were used. Gels for SDS/PAGE routinely contained 12% or 15% acrylamide (C_{bis} 2.6%) for cytosolic and microsomal proteins respectively.

Subcellular fractionation

Cytosols and microsomal fractions were prepared from individual mouse livers (average wt. 1.2 g) by homogenizing with 4 ml of ice-cold 50 mM-sodium phosphate buffer, pH 7.4, containing 200 mM-NaCl in a Potter-type homogenizer. The homogenate was centrifuged at 10000 ***g*** for 20 min at 4 °C and the pellet was discarded. The 10000 ***g*** supernatant was diluted to a final volume of 10 ml with the same buffer and centrifuged at 100000 ***g*** for 1 h (4 °C). The buoyant fatty layer was discarded from each sample and the remaining supernatant was retained as the cytosolic fraction. The 100000 ***g*** pellet was re-suspended in 10 ml of ice-cold 50 mM-sodium phosphate buffer, pH 7.4, containing 200 mM-NaCl using a Potter-type homogenizer and

Table 1. Effect of BNF on hepatic cytosolic GST activity in DBA/2 and C57BL/6 mice

Data were compiled from duplicate measurements of samples from 16 individual mice (eight strain DBA/2 and eight strain C57BL/6), which were either treated with BNF in corn oil (four of each strain) or corn oil alone (remaining animals). Standard deviations are indicated. Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; CuOOH, cumene hydroperoxide; EA, ethacrynic acid.

		Specific activity (μmol/min per mg)				
Strain	Treatment	CDNB	DCNB	CuOOH	H_2O_2	EA
DBA/2	Corn oil	2.25±0.15	0.068±0.009	1.25±0.07	0.60±0.03	0.035±0.006
DBA/2	BNF	4.20±0.56	0.150±0.025	1.37±0.07	0.61±0.04	0.040±0.002
C57BL/6	Corn oil	3.11±0.27	0.110±0.010	1.38±0.04	0.64±0.02	0.027±0.004
C57BL/6	BNF	5.90±0.50	0.230±0.034	1.16±0.07	0.50±0.03	0.056±0.015

centrifuged again at 100000 *g*. This procedure was repeated once. The twice-washed 100000 *g* pellet was suspended in 1.5 ml of 50 mM-sodium phosphate buffer, pH 7.4, containing 250 mM-sucrose and retained as the microsomal fraction.

Enzyme purification

Purification of mouse Ya_3Ya_3 was performed as described previously (Hayes *et al.*, 1987; McLellan & Hayes, 1987). The BHA-inducible Alpha-class GST was purified from BHA-fed female Balb/c mouse liver as described by McLellan & Hayes (1989).

Resolution of GST subunits by the use of reverse-phase h.p.l.c. (Ostlund Farrants *et al.*, 1987) was performed by the method of Hayes *et al.* (1989).

Separation of proteins in cytosol by reverse-phase h.p.l.c.

Portions of hepatic cytosol (1.5 ml; approx. 10 mg of protein/ml) from BNF-treated mice and BHA-treated mice were applied separately to a column (1.0 cm × 50.0 cm) of Sephadex G-75 (superfine grade) equilibrated with 50 mM-sodium phosphate buffer, pH 7.4. The column was developed under gravity with the same buffer. The fractions that contained GST activity were collected and pooled separately. After dilution to 1 mg/ml, trifluoroacetic acid was added to the cytosolic material to a final concentration of 0.1 % (v/v). Finally, 1 mg portions of the gel-filtered cytosolic proteins were applied to reverse-phase h.p.l.c. columns, the same conditions as those used for separation of GST subunits (see above) being used; 0.5 ml fractions were collected.

Peptide preparation

Peptides were generated from cleavage of proteins with CNBr and isolated by reverse-phase h.p.l.c. for amino acid sequencing, as described previously (Hayes *et al.*, 1989).

Amino acid sequencing

Automated amino acid sequencing of purified CNBr-cleavage fragments was performed with an Applied Biosystems (Warrington, Cheshire, U.K.) 477A sequencer with a 120-A on-line phenylthiohydantoin analyser, as described by Hayes *et al.* (1989).

RESULTS

Effects of BNF on the expression of mouse hepatic GST

To determine if induction of mouse hepatic GST is associated with an active Ah receptor, mice from the strains DBA/2 and C57BL/6 were treated with BNF; the DBA/2 strain lacks a functional high-affinity Ah receptor. Specific activities of the cytosol towards a variety of substrates were determined (Table 1). BNF was found to increase the specific activities towards 1-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene about 2-fold in both strains. The activity towards cumene hydroperoxide was not significantly altered by BNF treatment in the DBA/2 strain but was decreased in the C57BL/6 strain ($P < 0.05$). A similar trend was observed when H_2O_2 was the substrate. Activity with ethacrynic acid as substrate was not significantly changed in the DBA/2 strain after BNF treatment, but was increased in the C57BL/6 strain ($P < 0.05$).

The cytosolic and microsomal fractions from each liver were examined by Western blotting (Fig. 1). The microsomal fractions were also probed with antiserum raised against a 3-methylcholanthrene-inducible cytochrome *P*-450, MC_{1b} (Wolf, 1986); this is equivalent to cytochrome *P*-450 IA1, and the antiserum was used to confirm that this enzyme is induced by BNF treatment in C57BL/6 mice (which possess a high-affinity Ah receptor) but not in DBA/2 mice (which do not possess a functional receptor). The non-constitutive BHA-inducible Alpha-class GST subunit and the Pi-class GST subunit were preferentially induced in C57BL/6 mice; therefore their pattern of induction correlated with the pattern of induction of cytochrome *P*-450 IA1. Slight induction of these subunits was observed in the DBA/2 strain but to a much smaller extent than in the C57BL/6 strain. Preferential induction of the Yf subunit in C57BL/6 mice agrees with the increase in specific activity with ethacrynic acid as substrate in this strain (Table 1); ethacrynic acid is a 'diagnostic' substrate for mouse GST YfYf (Warholm *et al.*, 1986; McLellan & Hayes, 1987; Hayes *et al.*, 1987). Hepatic contents of the constitutive Alpha-class GST subunit Ya_3 and of microsomal GST were unaffected by treatment with BNF. The Yb subunit appeared to be induced to a greater extent in the C57BL/6 strain, but this was more difficult to determine as there is a high basal concentration of this subunit in mouse liver.

Regulation of two Alpha-class GSTs with subunit M_r of 25600

When purified BHA-inducible Alpha-class GST was subjected to reverse-phase h.p.l.c. on the μBondapak C_{18} column, it was resolved into two separate peaks (Fig. 2). It is unknown whether both forms of this GST are induced by BNF as well as by BHA.

Portions of hepatic cytosol from individual BNF-treated female C57BL/6 mice or pooled liver cytosols from five BHA-fed female Balb/c mice were subjected to reverse-phase h.p.l.c. Fractions eluted between 40 and 50 min after application were analysed by Western blotting with antiserum raised against the inducible Alpha-class 25600-M_r subunits. Fig. 3(*a*) shows Western-blot analysis of the profile obtained from BHA-treated mice, where two areas of cross-reactivity can be seen that correspond to the two peaks of induced Alpha-class GST. The

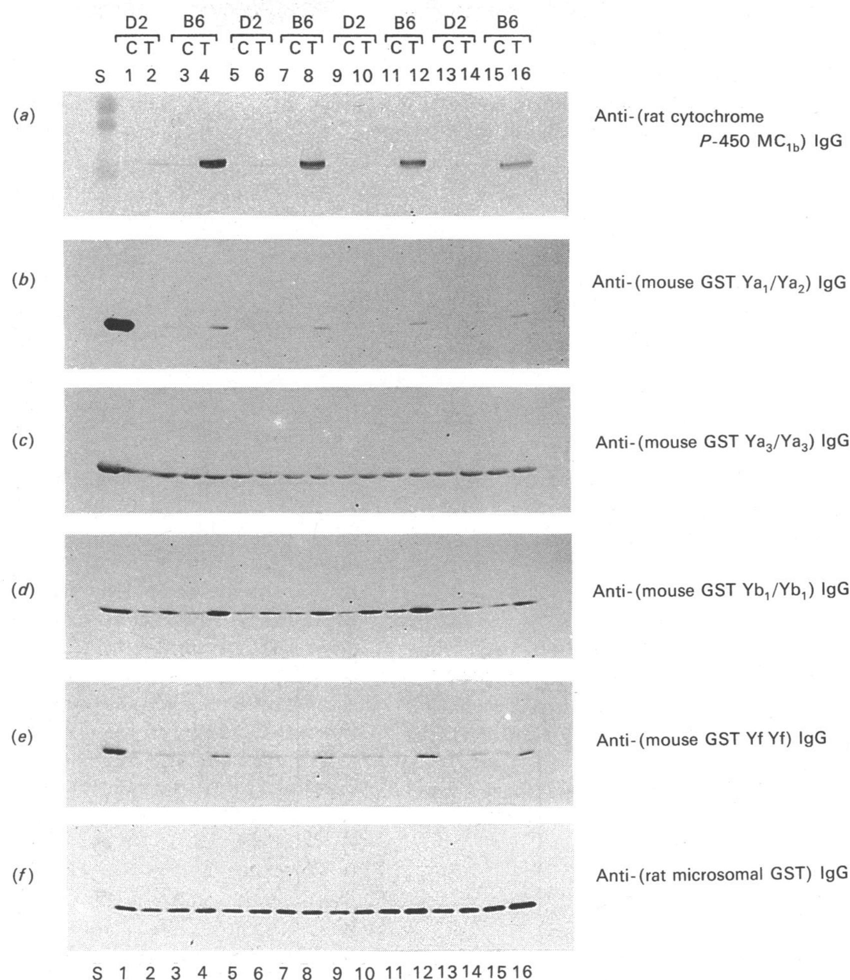

Fig. 1. Effect of BNF on GST isoenzyme contents in mouse liver

Contents of hepatic GST in 16 individual female mice (eight strain DBA/2 and eight strain C57BL/6) were determined by Western blotting. Four mice of each strain had been injected intraperitoneally with BNF (T) in corn oil and four of each strain with corn oil alone (C) as described in the text. The effect of BNF on a 3-methylcholanthrene-inducible cytochrome *P*-450 MC_{1b}, was also measured. Protein [30 μg for both cytosolic (panels *b–e*) and microsomal fractions (panels *a* and *f*)] was subjected to SDS/PAGE as follows: lanes 1, 5, 9 and 13, control samples from four DBA/2 mice; lanes 2, 6, 10 and 14, samples from four BNF-treated DBA/2 mice; lanes 3, 7, 11 and 15, control samples from four C57BL/6 mice; lanes 4, 8, 12 and 16, samples from four BNF-treated C57BL/6 mice; lane S, the antigen against which the relevant antibody was raised (except for panels *a* and *f*), where pre-stained M_r markers were used. The resolved proteins were probed for cross-reactivity with antisera raised against (*a*) rat cytochrome *P*-450 MC_{1b}, (*b*) mouse GST Ya_1/Ya_2, (*c*) mouse GST Ya_3Ya_3, (*d*) mouse GST Yb_1Yb_1, (*e*) mouse GST YfYf and (*f*) rat microsomal GST.

25600-M_r subunits were induced to a much smaller extent when mice were treated with BNF as compared with BHA. However, a similar result was seen when BNF was the inducing agent, with the subunits from both peaks being induced (Fig. 3*b*). The experiment was performed with three individual livers from C57BL/6 mice that had been treated with BNF, and similar results were obtained from each liver.

Evidence for the existence of three genetically distinct Ya-type subunits

The two peaks obtained from reverse-phase h.p.l.c. of the inducible Alpha-class GST were collected separately and named peak 1 (45 min) and peak 2 (48 min) in order of their elution (Fig. 2). The constitutively expressed Alpha-class GST with subunit M_r 25800, Ya_3, was isolated as described elsewhere (Hayes *et al.*, 1987, 1991; McLellan & Hayes, 1987, 1989). Material (1–5 mg of protein) from peaks 1 and 2 and the Ya_3 subunit was cleaved with CNBr, and the peptides generated were isolated by using reverse-phase h.p.l.c. Peptides were collected and sequenced by automated Edman degradation.

The peptide sequences from peak 1 were aligned with the deduced sequences from the clones encoding the Alpha-class GST described by Daniel *et al.* (1987) and Pearson *et al.* (1988) (Fig. 4). The amino acid sequences of the peptides from peak 1 were found to be identical with the amino sequence deduced from the genomic clone, λmYa1, described by Daniel *et al.* (1987). It is thus reasonable to predict that the Ya gene described by Daniel *et al.* (1987) is functional and codes for the inducible Alpha-class polypeptide that comprises peak 1. The amino acid sequences obtained from the CNBr-derived peptides from peak 1 were unambiguous, indicating that peak 1 contained pure homogeneous protein. Henceforth this GST subunit is referred to as Ya_1.

The BHA-inducible Alpha-class GST with subunit M_r 25600 has previously been named Ya_1Ya_1 and the constitutive Alpha-class GST with subunit M_r 25800 was named Ya_3Ya_3, on the supposition that multiplicity exists in mouse hepatic BHA-inducible Alpha-class GST (McLellan & Hayes, 1989). This premise is now supported by evidence for the existence of a second genetically distinct inducible Alpha-class GST, recovered

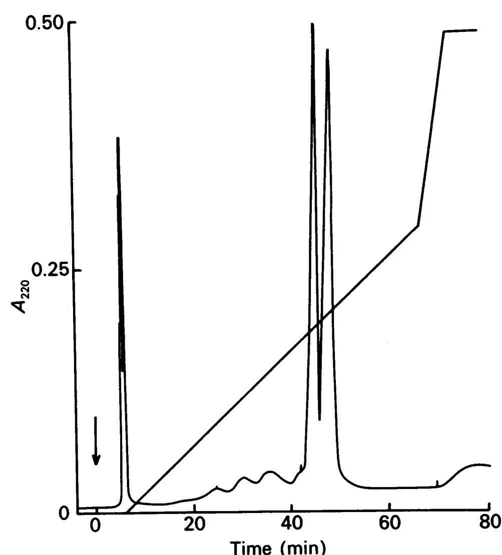

Fig. 2. Resolution of inducible Alpha-class GST subunits

The inducible Alpha-class GST from mouse liver was isolated as described by McLellan & Hayes (1989). Portions of the GST were subjected to reverse-phase h.p.l.c. on a Waters μBondapak C_{18} column (particle size 10 μm; column size 0.39 cm × 30.0 cm). The column was developed with a linear gradient of 40–58% (v/v) acetonitrile in aq. 0.1% (v/v) trifluoroacetic acid over 60 min, followed by 58–70% (v/v) acetonitrile in aq. 0.1% (v/v) trifluoroacetic acid over 5 min. Pump A delivered 40% acetonitrile and pump B delivered 70% acetonitrile, both at 1 ml/min over the gradient. The output of pump B is depicted by the straight line. The eluate was monitored continuously at 220 nm.

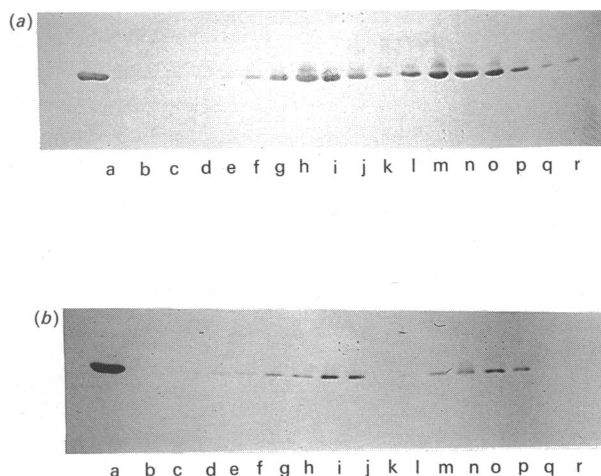

Fig. 3. Analysis of BNF-mediated induction of mouse GST Ya subunits

Hepatic cytosols from BHA-treated mice and BNF-treated mice were subjected to gel filtration and portions of the GST-containing fractions were subjected to reverse-phase h.p.l.c. as described in the text. Conditions for reverse-phase h.p.l.c. chromatography were identical with those described in the legend to Fig. 2. Fractions were collected every 0.5 min throughout the gradient and were freeze-dried separately. The fractions eluted in the region where subunits Ya_1 and Ya_2 are resolved (Fig. 2) were subjected to SDS/PAGE in order of their elution from the reverse-phase column. The resolved proteins were examined by Western blotting with antiserum raised against mouse Ya_1/Ya_2. Lane a contains purified mouse Ya_1/Ya_2. Lanes b–r contain the fractions taken at 0.5 min intervals from 41 min to 49 min. Panel (*a*) shows the Western blot obtained where dietary BHA was used as the inducing agent. Panel (*b*) shows the Western blot obtained where BNF given intraperitoneally was the inducing agent.

from peak 2 of the reverse-phase-h.p.l.c.-resolved Alpha-class GST. The amino acid sequences obtained for peptides generated from CNBr cleavage of protein from peak 2 were identical with the sequences obtained from peak 1, but were heterogeneous at specific residues. At the regions of heterogeneity (amino acid residues 66, 96 and 158), approximately equimolar amounts of two alternative amino acids were found, one of which always corresponded to the sequence obtained from Ya_1; the other was equivalent to the sequence that would be expected from the polypeptide encoded by pGT41, described by Pearson *et al.* (1988) (valine-66, threonine-96 and valine-158). The reverse was not true, as the Ya_1 subunit gave a unique unambiguous sequence with only alanine, serine and isoleucine being recovered at residues 66, 96 and 158 respectively; neither valine nor threonine was observed at these residues. The amino acid sequence of the CNBr-6 fragment from peak 2 was not sufficiently extensive to cover the other potential difference residue in this peptide between pGT41 and Ya_1, residue 163. It is also worth noting that the amino acid substitution at residue 208 is methionine to leucine in pGT41, and so it would be expected that the protein encoded by pGT41 would yield a different CNBr-cleavage pattern from Ya_1; peptide CNBr-7 should not have been recovered from the CNBr digest of a polypeptide encoded by the cDNA clone, pGT41. The CNBr-derived peptide maps from peak 1 and peak 2 were, however, closely similar, although peak 2 yielded an apparently smaller quantity of peptide CNBr-7 than did peak 1. The separated peaks, 1 and 2, were each estimated to be approx. 95% pure by re-applying the purified material to reverse-phase h.p.l.c. Furthermore, their relative elution positions remained constant and they did not interconvert. Peak 2 may thus comprise equal amounts of two different unassociated subunits, both of which are distinct from Ya_1, that are not resolved by the h.p.l.c. system. Alternatively, although Ostlund Farrants *et al.* (1987) report that this chromatographic system separates GST subunits, it may be possible that in this instance these particular Alpha-class GST subunits do not dissociate under the conditions used for reverse-phase h.p.l.c. on the μBondapak C_{18} column, and that peak 2 comprises a heterodimer of Ya_1 and a further inducible Alpha-class GST subunit. The distinct Ya subunit recovered from peak 2 that we have sequenced will now be referred to as Ya_2, although it is recognized that this subunit exhibits micro-heterogeneity. Our sequence data suggests that Ya_2 may represent the subunit encoded by the DNA clone pGT41.

The CNBr-cleavage fragments from the constitutive Alpha-class GST subunit, Ya_3, were found to have a primary structure that differed considerably from that of the Ya_1 subunit (Fig. 5). Over the region of the Ya_3 subunit that we have sequenced there is 68% amino acid sequence identity with the amino acid sequence deduced from the mouse Ya gene (Ya_1). Peptides from mouse Ya_3 subunit could be aligned most easily with the amino acid sequence deduced from the clone pGTB42 described by Telakowski-Hopkins *et al.* (1985), which codes for the Yc subunit from rat. The degree of sequence identity is 86%.

DISCUSSION

Two distinct clones encoding mouse hepatic Alpha-class GSTs have been isolated by separate laboratories. One of these is a genomic clone, λmYa1 (Daniel *et al.*, 1987), and the other a cDNA clone, pGT41, that encodes an mRNA that can be induced 50-fold in mouse liver by treating with BHA (Pearson *et al.*, 1988). The deduced amino sequences from these Alpha-class clones are very similar to each other, with only ten amino acid substitutions. This close sequence similarity suggests that it is improbable that the subunits encoded by λmYa1 and pGT41 represent the constitutive Alpha-class GST and the BHA-

Ya1-CNBr1 *Met*-Glu-Cys-Ile-Arg-Trp-Leu-Leu-Ala-Ala-Ala-Gly-Val-Glu-Phe-Glu-Glu-Lys-Phe-Ile-Gln
λmYa1 Met-Glu-Cys-Ile-Arg-Trp-Leu-Leu-Ala-Ala-Ala-Gly-Val-Glu-Phe-Glu-Glu-Lys-Phe-Ile-Gln
pGT41 Met-Glu-Cys-Ile-Arg-Trp-Leu-Leu-Ala-Ala-Ala-Gly-Val-Glu-Phe-Glu-Glu-Lys-Phe-Ile-Gln
16 21 26 31

Ya1-CNBr2 *Met*-Phe-Asp-Gln-Val-Pro-Met-Val-Glu
λmYa1 Met-Phe-Asp-Gln-Val-Pro-Met-Val-Glu
pGT41 Met-Phe-Asp-Gln-Val-Pro-Met-Val-Glu
51 56

Ya1-CNBr3 *Met*-Lys-Leu-Ala-Gln-Thr-Arg-Ala-Ile-Leu-Asn-Tyr-Ile-Ala-Thr-Lys-Tyr-Asp-Leu-Tyr-Gly-Lys-Asp
λmYa1 Met-Lys-Leu-Ala-Gln-Thr-Arg-Ala-Ile-Leu-Asn-Tyr-Ile-Ala-Thr-Lys-Tyr-Asp-Leu-Tyr-Gly-Lys-Asp
pGT41 Met-Lys-Leu-Val-Gln-Thr-Arg-Ala-Ile-Leu-Asn-Tyr-Ile-Ala-Thr-Lys-Tyr-Asp-Leu-Tyr-Gly-Lys-Asp
63 68 73 78 83

Ya1-CNBr4 *Met*-Lys-Glu-Arg-Ala-Leu-Ile-Asp
λmYa1 Met-Lys-Glu-Arg-Ala-Leu-Ile-Asp
pGT41 Met-Lys-Glu-Arg-Ala-Leu-Ile-Asp
86 91

Ya1-CNBr5 *Met*-Tyr-Ser-Glu-Gly-Ile-Leu-Asp-Leu-Thr-Glu
λmYa1 Met-Tyr-Ser-Glu-Gly-Ile-Leu-Asp-Leu-Thr-Glu
pGt41 Met-Tyr-Thr-Glu-Gly-Ile-Leu-Asp-Leu-Thr-Glu
94 99 104

Ya1-CNBr6 *Met*-Ile-Gly-Gln-Leu-Val-Leu-*Cys*-Pro-Pro-Asp-Gln-Arg-Glu-Ala-Lys-Thr-Ala-Leu-Ala-Lys-Asp-Arg-Thr-Lys-Asn-Arg-Tyr-Leu-Pro-Ala-Phe-Glu
λmYa1 Met-Ile-Gly-Gln-Leu-Val-Leu-Cys-Pro-Pro-Asp-Gln-Arg-Glu-Ala-Lys-Thr-Ala-Leu-Ala-Lys-Asp-Arg-Thr-Lys-Asn-Arg-Tyr-Leu-Pro-Ala-Phe-Glu
pGT41 Met-Ile-Gly-Gln-Leu-Val-Leu-Cys-Pro-Pro-Asp-Gln-Arg-Glu-Ala-Lys-Thr-Ala-Leu-Ala-Lys-Asp-Arg-Thr-Lys-Asn-Arg-Tyr-Leu-Pro-Ala-Phe-Glu
105 110 115 120 125 130 135

CONT.
Lys-Val-Leu-Lys-Ser-His-Gly-Gln-Asp-Tyr-Leu-Val-Gly-Asn-Arg-Leu-Thr-Arg-Val-Asp-Ile-His-Leu-Leu-Glu-Val-Leu-Leu
Lys-Val-Leu-Lys-Ser-His-Gly-Gln-Asp-Tyr-Leu-Val-Gly-Asn-Arg-Leu-Thr-Arg-Val-Asp-Ile-His-Leu-Leu-Glu-Val-Leu-Leu
Lys-Val-Leu-Lys-Ser-His-Gly-Gln-Asp-Tyr-Leu-Val-Gly-Asn-Arg-Leu-Thr-Arg-Val-Asp-Val-His-Leu-Leu-Glu-Leu-Leu-Leu
138 143 148 153 158 163

Ya1-CNBr7 *Met*-Asp-Ala-Lys-Gln-Ile-Gln-Glu-Ala-Arg-Lys-Ala-Phe-Lys-Ile-Gln
λmYa1 Met-Asp-Ala-Lys-Gln-Ile-Gln-Glu-Ala-Arg-Lys-Ala-Phe-Lys-Ile-Gln
pGT41 Leu-Asp-Ala-Lys-Gln-Ile-Glu-Glu-Ala-Arg-Lys-Val-Phe-Lys-Phe
208 213 218

Fig. 4. Primary structure of mouse GST Ya$_1$ subunit

The BHA-inducible Alpha-class subunit that was recovered from peak 1 from reverse-phase h.p.l.c. was cleaved with CNBr, and the peptides were isolated as described by Hayes *et al.* (1989). The amino acid sequences of the CNBr-derived peptides were determined by automated Edman degradation [see Hayes *et al.* (1989) for details]. The sequences obtained are shown aligned with deduced amino acid sequences of mouse Alpha-class GST subunits from the genomic clone, λmYa1 (Daniel *et al.*, 1987), and the cDNA clone, pGT41 (Pearson *et al.*, 1988). Amino acids in *italics* indicate putative assignments in the case of methionine and in certain instances cysteine. Underlined amino acids indicate residues that differ between subunit Ya$_1$ and the derived sequence from the cDNA clone, pGT41.

inducible Alpha-class GST because the constitutive and inducible Alpha-class GSTs are readily distinguishable by their catalytic and physicochemical properties. We have now demonstrated that at least three genetically distinct Alpha-class GST subunits are expressed in the livers of mice that have been treated with the chemoprotectors, BHA or BNF. Two of these, Ya$_1$ and Ya$_2$ (subunit M_r 25600), are structurally very similar and are members of the same Alpha-class sub-family, whereas subunit Ya$_3$ (M_r 25800) is a member of a distinct sub-family of Alpha-class GST.

The hepatic contents of subunits Ya$_1$ and Ya$_2$ are increased dramatically by administration of dietary BHA. The Ya$_1$ subunit

Fig. 5. Primary structure of mouse GST Ya_3 subunit

The amino acid sequences obtained from CNBr-cleavage fragments of mouse subunit Ya_3 are aligned with deduced amino acid sequences from the rat cDNA clone, pGTB42, encoding Yc (Telakowski-Hopkins *et al.*, 1985), and the mouse genomic clone, λmYa1, encoding a mouse Alpha-class GST subunit (Daniel *et al.*, 1987). Putative methionine residues are in *italics*.

has an amino acid sequence that is identical with that deduced from the genomic clone, λmYa1, described by Daniel *et al.* (1987). The Ya_2 subunit was isolated either as a mixture with a further, as yet uncharacterized, Alpha-class GST or possibly as a heterodimer with Ya_1. Although a homogeneous preparation of subunit Ya_2 was unavailable, we predict that it is possible that this subunit may represent the polypeptide that is encoded by the cDNA clone pGT41 described by Pearson *et al.* (1988).

The amino acid sequence of the third Alpha-class GST subunit, Ya_3 (subunit M_r 25800), which is constitutively expressed in mouse liver but not induced perceptibly by BHA, has 68% sequence identity with the deduced sequence from the mouse Ya_1 gene (Daniel *et al.*, 1987) over the region we have sequenced. Although this value is fairly low for a pair of GSTs within the same designated class, it approximates the degree of identity seen between the rat Ya and Yc subunits. The degree of identity between mouse Ya_3 and rat Yc subunits is 86% over the region that we have sequenced (about 45% of the predicted primary structure). The degree of sequence identity may not, however, be high enough to characterize mouse Ya_3 as the orthologue of the rat Yc subunit. Mouse Ya_1 and Ya_2 subunits have 95% amino acid sequence identity with the rat Ya_1 and Ya_2 subunits, and mouse Yb_1 and Yb_2 subunits have 93% and 95% sequence identity respectively with their counterparts in the rat (Pearson *et al.*, 1988; Townsend *et al.*, 1989). This level of conservation of subunits between these species leads to speculation that certain GSTs from rat and mouse are analogous. Where the degree of amino acid sequence identity is much less than about 95%, the relationships between the GSTs from different species is less clear. However, it is apparent from our sequencing data that mouse Ya_3 and rat Yc subunits are closely related and are members of the same Alpha-class sub-family.

The effects of the chemoprotective bifunctional inducing agent BNF on expression of the mouse Alpha-class GST was investigated. The chemoprotection provided by BNF is thought to entail GST induction, but previous work has not identified either the isoenzymes involved or the mechanisms responsible for induction. It has been reported that planar-aromatic-hydrocarbon-mediated induction of mouse hepatic GST does not involve the Ah receptor (Felton *et al.*, 1980). Unfortunately, during their study, Felton and his colleagues used mice at 4–6 weeks old of either sex. It has since been found that considerable

sex differences exist in mouse hepatic GST content (Hatayama *et al.*, 1986; McLellan & Hayes, 1987), so the involvement of the Ah receptor in the induction of mouse GST, particularly by chemoprotectors, warranted further investigation. During the present study we have shown that in female mice treated with BNF maximum induction of both the 25600-M_r Alpha-class subunits and the Pi-class subunits occurs in animals that possess a high-affinity Ah receptor. These results contrast with those reported by Felton *et al.* (1980), who predicted that an alternative mechanism to one that involved the Ah receptor was operational in GST induction by planar aromatic hydrocarbons. However, although our results indicate that the Ah receptor may be involved in induction of certain GSTs, the mechanism of the induction is still speculative. Involvement of the Ah receptor in the induction of certain phase II enzymes by planar aromatic hydrocarbons has been shown by several workers (Owens, 1977; Pickett *et al.*, 1987; Prochaska & Talalay, 1988; Telakowski-Hopkins *et al.*, 1988), but it is believed that this may occur at least partly, by a mechanism that does not entail direct transcriptional activation of these genes by binding with an activated Ah receptor (Prochaska & Talalay, 1988). It has been proposed that bifunctional inducing agents, such as BNF, can cause induction of phase II enzymes by being themselves metabolized to electrophilic products that are capable of inducing phase II enzymes. In this case the conversion of BNF into electrophilic metabolites would be catalysed by phase I enzymes that have been induced via the Ah receptor. It is possible that the BNF-mediated induction of some, or all, of the inducible mouse GSTs occurs by this latter mechanism (Prochaska & Talalay, 1988). A further alternative explanation for the differential effects of BNF on the GST complement of DBA/2 and C57BL/6 mice is that these mice contain other differences in transcriptional regulation of GST isoenzymes by xenobiotics that are unrelated to the Ah receptor. We therefore cannot conclude from our data alone that any transcriptional activation of specific mouse GST genes is mediated directly by the Ah receptor. However, the data of Daniel *et al.* (1989) and Rushmore *et al.* (1990) indicate that transcription of Ya mRNA in rodents can be controlled to some extent by the direct action of the Ah receptor. Daniel *et al.* (1989) reported that the mouse Ya_1 gene that they had cloned contains a cis-acting regulatory element responsible for planar-aromatic-hydrocarbon-mediated induction, functional only in cells with normal Ah receptors. Rushmore *et al.* (1990) have demonstrated that a single xenobiotic-regulatory element, identical with the xenobiotic-regulatory-element sequences in the cytochrome *P*-450 IA1 gene, and thus capable of binding a functionalized Ah receptor, is present in the 5′ flanking sequence of the rat Ya gene. Although specific 'drug-regulatory elements' (Sogawa *et al.*, 1986) were shown to be present in the 5′ flanking sequence of the mouse Ya gene (Daniel *et al.*, 1989), a sequence similar to that of the xenobiotic-regulatory element in the rat Ya gene was not identified in the mouse gene. The significance of this remains to be determined, and the involvement of the Ah receptor in transcription of mouse GST genes further elucidated at the molecular level.

In conclusion, we have identified three genetically distinct Alpha-class GST subunits in the livers of mice that have been treated with the chemoprotectors BHA and BNF. The Ya_1 and Ya_2 subunits are very similar and comprise a novel Alpha-class GST sub-family. The Ya_1 subunit is thought to represent the polypeptide that is encoded by the mouse genomic DNA clone, λmYa1 (Daniel *et al.*, 1987), whereas the identity of the Ya_2 subunit is more speculative. The amino acid sequence of the Ya_3 subunit shows 68% identity with the deduced sequence from the mouse Ya gene described by Daniel *et al.* (1987). The Ya_3 subunit has not been successfully subjected to amino acid sequence analysis previously, and our sequence data indicate that the cDNA encoding this GST has not, to date, been cloned. The molecular cloning of this enzyme represents an important future objective, as the Ya_3 subunit possesses high activity for aflatoxin B_1 8,9-epoxide and is thought to be responsible for the intrinsic resistance of the mouse to the carcinogenic effects of aflatoxin B_1 (D. J. Judah, J. D. Hayes, L. I. McLellan & G. E. Neal, unpublished work).

L. I. McL. thanks Dr. C. R. Wolf for generously providing antiserum raised against cytochrome *P*-450 MC_{1b} and for helpful discussion. We thank Mrs. E. Ward for excellent secretarial assistance, Mr. I. Lennox for artwork and Mr. D. Dirom for photography. Dr. A. F. Howie is thanked for advice on statistical analyses and Dr. R. Dalton and Mr. D. Henderson are thanked for their advice and assistance. We are grateful to the Medical Research Council for financial support (Grant G8622978CA). The protein sequencing facility was provided by grants from The Wellcome Trust, from the University of Edinburgh, from the Norman Salvesen Trust and from Heriot-Watt University. We gratefully acknowledge Professor L. G. Whitby for critically reading this manuscript.

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Received 9 August 1990/12 November 1990; accepted 4 December 1990