Preferential over-expression of the Class Alpha rat Ya₂ glutathione S-transferase subunit in livers bearing aflatoxin-induced pre-neoplastic nodules

Comparison of the primary structures of Ya₁ and Ya₂ with cloned Class Alpha glutathione S-transferase cDNA sequences

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Normal rat liver expresses Ya (M_r 25 500), Yc (M_r 27 500) and Yk (M_r 25 000) Class Alpha glutathione S-transferase (GST) subunits. The Ya-type subunit can be resolved into two separate polypeptides, designated Ya₁ and Ya₂, by reverse-phase h.p.l.c. In rat livers that possess aflatoxin B₁-induced pre-neoplastic nodules, a marked increase is observed in the expression of Ya₁, Ya₂, Yc and Yk; of these subunits, Ya₂ exhibited the greatest increase in concentration. The Ya₁ and Ya₂ subunits isolated from nodule-bearing livers were cleaved with CNBr, and the purified peptides were subjected to automated amino-acid-sequence analysis. Differences in the primary structures of the two Ya GST subunits were found at positions 31, 34, 107 and 117. These data demonstrate that Ya₁ and Ya₂ are distinct polypeptides and are the products of separate genes. The amino acid sequences obtained from Ya₁ and Ya₂ were compared with the cloned cDNAs pGTB 38 [Pickett, Telakowski-Hopkins, Ding, Argenbright & Lu (1984) *J. Biol. Chem.* **259**, 4112–4115] and pGTR 261 [Lai, Li, Weiss, Reddy & Tu (1984) *J. Biol. Chem.* **259**, 5182–5188], which encode rat Ya-type subunits. From these comparisons it appears probable that Ya₁ represents the GST subunit encoded by pGTR 261, whereas Ya₂ represents the subunit encoded by pGTB 38. It is likely that the over-expression of Ya₁ and Ya₂ in nodule-bearing livers is of major significance in the acquired resistance of nodules to aflatoxin B₁, since previous work [Coles, Meyer, Ketterer, Stanton & Garner (1985) *Carcinogenesis* **6**, 693–697] has shown that the Ya-type GST subunit has high activity towards aflatoxin B₁ 8,9-epoxide.

INTRODUCTION

In rodents the production of experimental liver cancer by chemical carcinogens is preceded by the formation of pre-neoplastic, or hyperplastic, nodules (Farber, 1984a,b). These nodules have been the subject of considerable interest. The paradoxical facts that carcinogens inhibit cell proliferation but nodules arise from a carcinogen-containing environment suggested to Solt & Farber (1976) that either the pre-neoplastic nodules or their precursors would, of necessity, be drug-resistant. Consistent with other examples of drug-resistant mammalian tumour cells, the pre-neoplastic hepatocyte nodules display changes in the expression of detoxication enzymes and the transmembrane drug-efflux pump, P-glycoprotein (Cowan *et al.*, 1986; Fairchild *et al.*, 1987; Hayes & Wolf, 1988). The changes in expression are complex and include down-regulation of cytochrome P-450 and the induction of both glutathione S-transferases (GSTs) and P-glycoprotein.

The GST enzymes are of particular interest in experimental carcinogenesis, not only because they play a pivotal role in detoxication, but because one of the GST enzymes provides the most sensitive marker of hepatic pre-neoplastic changes that is currently available (Kitahara *et al.*, 1984; Satoh *et al.*, 1985). This marker GST has been variously called YfYf, YpYp, 7-7 or P21 (Eriksson *et al.*, 1983; Satoh *et al.*, 1985; Mannervik *et al.*, 1985;

Hayes & Mantle, 1986b) and is over-expressed approx. 40-fold in pre-neoplastic nodules (Sugioka *et al.*, 1985; Neal *et al.*, 1988; Sakai *et al.*, 1988). The expression of this marker GST appears to be associated with the step of malignant transformation, rather than cell immortalization or division (Power *et al.*, 1987).

Many GST exist, and these have been subdivided into four classes, designated Alpha, Mu, Pi and microsomal (Mannervik *et al.*, 1985; Morgenstern *et al.*, 1988). Investigations into the changes in gene expression that accompany pre-neoplasia have focused largely on the Yf subunit, which is a Pi Class polypeptide. By contrast, relatively little is known about the expression of the Class Alpha GST subunits, Ya (M_r 25 500), Yc (M_r 27 500) and Yk (M_r 25 000), or the Class Mu GST subunits, Yb₁ (M_r 26 300), Yb₂ (M_r 26 300) and Yn (M_r 26 000), in this pathological situation (for subunit designation, see Hayes & Mantle, 1986b,c).

Certain GSTs detoxify carcinogens, such as the 8,9-epoxide metabolite of aflatoxin B₁ (for a review, see Ketterer *et al.*, 1988), and might therefore play a causative role in the drug resistance exhibited by hepatocyte nodules. Neal *et al.* (1981) had previously suggested that an altered capacity for the conjugation of aflatoxin B₁ with glutathione played a central role in the resistance to aflatoxin B₁ cytotoxicity which precedes hepatocarcinogenesis. However, GST YfYf possesses a relatively low activity towards aflatoxin B₁ 8,9-epoxide and, although the expression of the Yf subunit is markedly elevated in pre-neoplastic nodules, there is

no evidence that the over-expression of Yf in aflatoxin-induced nodules confers resistance to this carcinogen. Coles *et al.* (1985) demonstrated that the rat YaYa, YaYc and YcYc GSTs possess high activity towards aflatoxin B₁ 8,9-epoxide, and it is probable that the Class Alpha enzymes, not the Class Pi GSTs, are responsible for acquired resistance to aflatoxin in hepatocyte nodules.

Because of the lack of information about the involvement of Class Alpha GSTs in hepatocarcinogenesis, we have undertaken a study into the expression of Ya, Yc and Yk subunits in aflatoxin-induced pre-neoplastic nodules. During this work we found that, by using the reverse-phase-h.p.l.c. strategy described by Ostlund Farrants *et al.* (1987), the Ya-type subunit could be resolved into two chromatographically distinct polypeptides; these have been designated Ya₁ and Ya₂. The Ya₂ polypeptide was found to be induced preferentially in nodule-bearing rat livers. Sequence analysis of peptides obtained from CNBr cleavage of Ya₁ and Ya₂ has been undertaken, and their relationship to the two published Ya cDNA sequences is discussed.

MATERIALS AND METHODS

Chemicals

These were all of analytical grade and were readily available commercially. The h.p.l.c. solvents were from Rathburn Chemicals, Walkerburn, Peeblesshire, Scotland, U.K.

Buffers

These were prepared at room temperature, but the pH values quoted are those obtained at the temperature at which they were used.

Tissue

Livers were from 18-week-old male Fischer F344 rats and were frozen at -85 °C until use. The pre-neoplastic nodule-bearing livers were obtained by feeding aflatoxin B₁-containing diets as described by Neal *et al.* (1987). The nodules that are induced by this schedule are detectable macroscopically. However, all the livers used in the present study were examined by a pathologist using optical microscopy to provide an independent assessment of the extent of the disease process. For this purpose, portions (approx. 5%) of all livers were formalin-fixed immediately after removal. An immunohistochemical examination of the GST subunits in these livers has been undertaken and has shown that none of the control livers contained microscopic foci of altered cells (Harrison *et al.*, 1990).

Analytical

Enzyme assays were performed at 37 °C by using methods described by Habig & Jakoby (1981). Protein determinations were carried out by the method of Bradford (1976). SDS/PAGE was performed in 12%-(w/v)-acrylamide resolving gels that contained 0.32% (w/v) methylenebisacrylamide (Hayes & Mantle, 1986c). Reverse-phase h.p.l.c. was carried out by using linear 40–58% gradients of acetonitrile in aq. 0.1% (v/v) trifluoroacetic acid; the system employed, as well as further experimental details, have been described elsewhere (Hayes *et al.*, 1989).

Purification of GST

The GSTs in both control and nodule-bearing livers were isolated using a GSH-agarose matrix (Simons & Vander Jagt, 1977) as described previously (Hayes, 1986). The individual GST isoenzymes eluted from the GSH-agarose columns were purified as shown in Scheme 1. The initial cation-exchange-chroma-

Scheme 1. Purification of GST from nodule-bearing rat livers

tography step, which resulted in GST being resolved into four peaks, was carried out on a Protein PAK Glass SP-5PW column (0.8 cm × 7.5 cm) using 10 mM-sodium phosphate, pH 6.7, as running buffer and 10 mM-sodium phosphate/400 mM-NaCl, pH 6.7, as limit buffer. The peaks indicated in the purification scheme are numbered according to their order of elution; peak 1 represents 'flow-through' material. Peaks 2 and 3, which were retained by the cation-exchanger and were eluted by the salt gradient, contained YaYa and YaYc respectively. However, both of these preparations were found to be contaminated with small amounts of Yb-type subunits, and this material was removed by anion-exchange chromatography on a Protein PAK Glass DEAE-5PW column (0.8 cm × 7.5 cm), which was equilibrated with 25 mM-Tris/HCl, pH 9.2; the Ya and Yc subunits were eluted in the flow-through fractions from this anion-exchanger, whereas the Yb-type subunit was retained. The Protein PAK Glass ion-exchange columns were used in a Waters Advanced (650 E) protein-purification system, which comprised a system controller, sample injector, pump and tunable absorbance detector.

Amino acid sequencing

This was performed on purified CNBr fragments of the Ya₁ and Ya₂ GST subunits using an Applied Biosystems (Warrington, Cheshire, U.K.) 477A instrument with a 120A on-line phenylthiohydantoin analyser as described previously (Hayes *et al.*, 1989).

RESULTS

Histology

Each of the control livers was histologically normal. There was no architectural or cytological atypia, and no evidence of any focal disease process. Livers from the pre-neoplastic group showed a variety of changes, ranging from focal necrosis and inflammation to expanded, ill-defined, nodular areas of proliferation. There was frequently marked nuclear pleomorphism, and hyperchromatism. Some areas of compressed histologically normal liver parenchyma were identified.

Induction of GST activities in nodule-bearing livers

Table 1 shows the level of GST activity in cytosols from the livers of control rats and nodule-bearing rat livers. Comparisons between the nodule-bearing livers and their controls revealed significant differences in activities towards a number of substrates which display specificity for different GST subunits. The specific activity towards the general GST substrate 1-chloro-2,4-dinitrobenzene (CDNB) was increased about 3-fold in the cytosol from nodule-bearing livers. The specific activity towards 1,2-dichloro-4-nitrobenzene, a substrate which is specific for the Yb₁ subunit, was increased about 4-fold. By contrast, activity towards the substrate *trans*-4-phenylbut-3-en-2-one, which is relatively specific for Yb₂, was unchanged in the livers from aflatoxin-treated

Table 1. Levels of GST activity in cytosol from control and pre-neoplastic nodule-bearing rat livers designated (PN 1–4).

All analyses were performed at 37 °C. All results are expressed as means $\pm$ s.d. for four determinations. Abbreviations: DCNB, 1,2-dichloro-4-nitrobenzene; tPBO, *trans*-4-phenylbut-3-en-2-one; EA, ethacrynic acid; Δ^5 -Add, Δ^5 -androstene-3,17-dione; CuOOH, cumene hydroperoxide.

		Specific activity (nmol · min ⁻¹ · mg ⁻¹)					
Rat liver		CDNB	DCNB	tPBO	EA	Δ ⁵ Add	CuOOH
Control	1	1910 ± 40	41.2 ± 4.2	16.1 ± 0.4	52.2 ± 3.0	14.9 ± 0.2	1038 ± 15
	2	2010 ± 40	43.6 ± 1.4	16.0 ± 0.9	49.2 ± 2.1	16.4 ± 1.1	1374 ± 12
	3	1500 ± 20	40.4 ± 1.0	10.4 ± 0.5	38.7 ± 0.5	10.6 ± 0.6	1118 ± 5
PN	1	4270 ± 90	139.3 ± 5.4	18.3 ± 1.7	146.2 ± 20.9	21.0 ± 0.6	1116 ± 8
	2	5450 ± 30	172.7 ± 7.8	17.5 ± 1.6	119.2 ± 15.0	24.5 ± 0.1	1352 ± 5
	3	5370 ± 140	159.7 ± 3.3	17.0 ± 1.4	136.4 ± 23.6	24.0 ± 0.7	1430 ± 8
	4	6130 ± 130	183.2 ± 9.4	17.5 ± 2.2	155.4 ± 31.0	30.7 ± 1.0	1337 ± 9

animals. The ethacrynic acid–GSH-conjugating activity was elevated about 2.7-fold in cytosol from nodule-bearing livers, but ethacrynic acid is not specific and serves as a substrate for Yc (Class Alpha), Yf (Class Pi) and Yk (Class Alpha) subunits (Hayes, 1986). Interestingly, the specific activity towards the compound Δ^5 -androstene-3,17-dione, which is highly selective for the Ya subunit (Benson *et al.*, 1977; Beale *et al.*, 1982), showed a significant, but modest, increase of about 1.8-fold in the cytosols from nodule-bearing livers.

Induction of GST subunits in nodule-bearing livers

To allow characterization of the over-expressed GST in nodule-bearing livers, the cytosols, prepared from PN3 and PN4 (see Table 1), were combined and, along with a pool of control rat livers (i.e. control 3, see Table 1), were subjected to affinity chromatography on columns of GSH–agarose. For this purpose, cytosol from five control rat livers was used because, although the rats were age-matched, the livers from aflatoxin-treated animals were twice the weight of those from normal animals; using five control-rat livers allowed equal amounts of protein to be applied to the GSH–agarose columns. To ensure comparability between the two GST preparations, two affinity columns were run in parallel. In both cases it was found that the columns retained about 90% of the catalytic activity towards CDNB. However, as would be predicted from the difference in cytosolic activity for CDNB (Table 1), elution of the matrix with 200 mM-Tris/HCl, pH 9.5, containing 50 mM-GSH resulted in substantially more GST being purified from the nodule-bearing livers than from controls. On the basis of the elution profiles from the affinity column (results not shown), it was calculated that, whereas GST represented 3.6% of the cytosolic protein in the control livers, it represented as much as 11.9% of the cytosolic protein from nodule-bearing livers. Therefore there is an approx. 3-fold increase in total GST recovered from the livers of aflatoxin-treated animals.

The cytosols and affinity-purified material were subjected to SDS/PAGE to allow the subunit compositions of the various preparations to be assessed. Fig. 1 shows an obvious increase in the Yb-type polypeptides in the hepatic cytosol from aflatoxin-treated rats. In common with other studies (Kitahara *et al.*, 1984; Rushmore *et al.*, 1987), SDS/PAGE also revealed the presence of the Yf subunit in nodule-bearing livers and its absence from normal livers. However, most significantly, examination of the lanes containing whole cytosol showed a large increase in the level of Ya-type subunits in the nodule-bearing livers. Electrophoresis demonstrated that little, if any, Ya-type subunit has been eluted in the ‘flow-through’ fractions from the GSH–agarose columns. Moreover, the material which was eluted

Fig. 1. SDS/PAGE of hepatic fractions obtained after affinity chromatography

This was performed as described in the text. Samples were run from the cathode (top) to the anode (bottom), and the positions of the Ya, Yb, Yc and Yf subunits are indicated. The fractions are as follows: 1, liver cytosol from normal rats; 2, liver cytosol from nodule-bearing rats; 3, the hepatic cytosol from normal rats which was not retained by GSH–agarose; 4, the hepatic cytosol from aflatoxin B₁-treated rats which was not retained by GSH–agarose; 5, affinity-purified GST from normal rat liver; 6, affinity-purified GST from rat livers that contained aflatoxin-induced pre-neoplastic nodules.

specifically from the matrix by GSH was considered to represent essentially pure GST subunits; the only peptides detected had *M_r* values between 24800 and 27500.

To allow a more accurate estimate of the changes in expression of Class Alpha GST in nodule-bearing livers, the affinity-purified material was subjected to reverse-phase h.p.l.c. By using a 40–58% (v/v) acetonitrile gradient to develop the μ -Bondapak column, the GST were resolved into seven peaks. Each of the protein-containing peaks was collected and, after concentration by freeze-drying, the subunits were identified by SDS/PAGE. This suggested that the GST peaks from normal rat livers, which were eluted at 29, 37, 41, 46, 59, 62 and 65 min, contained Yb₁, Yb₂, Yc, Yn₁, Ya, Ya and Yk subunits respectively; to avoid confusion, the Ya subunit eluted at 59 min is designated ‘Ya₁’ and that eluted at 62 min is designated ‘Ya₂’. SDS/PAGE

Fig. 2. Reverse-phase h.p.l.c. analysis of affinity-purified GST

The relative proportion of the different subunits in the affinity-purified GST pools from (a) control livers and (b) pre-neoplastic nodule-bearing (PN) livers was determined by reverse-phase h.p.l.c., as described in the text. The column employed is a Waters μ Bondapak C₁₈ column (10 μ m particle size; column size 0.39 cm $\times$ 30.0 cm) and it was developed at 1 ml/min by a linear 40–55% acetonitrile gradient in aq. 0.1% (v/v) trifluoroacetic acid formed over 60 min; this was followed by a 55–70% acetonitrile gradient in aq. 0.1% trifluoroacetic acid formed over 5 min. The eluate was monitored continuously at 220 nm. The relative output of pump B is shown by the continuous line; pump A delivered 40% acetonitrile and pump B delivered 70% acetonitrile.

of the chromatographic peaks from the nodule-bearing livers indicated that those which were eluted at 29, 41, 46, 59, 62 and 65 min contained the same subunits as were found in the chromatographic peaks from control rats. However, in our system, the Yf subunit was co-eluted with the Yb₂ subunit and, therefore, the peak with a retention time of 37 min was found to contain both Yb₂ and Yf when the GSTs from nodule-bearing livers were examined. The subunit designations of the different h.p.l.c. peaks are indicated in Fig. 2.

Differences in the two h.p.l.c. analyses of GST are apparent. However, the profiles shown in Fig. 2 were obtained after approximately equal loadings of GST from control livers and nodule-bearing livers. When allowance is made for the 3-fold increase in total GST protein in the livers of aflatoxin-treated rats, the relative increase in some subunits becomes very marked. Although the calculation of the concentrations of individual subunits in cytosol assumes that they possess the same absorbance coefficient at 220 nm, the calculation of the relative increase of GST subunits in nodule-bearing livers does not suffer from this disadvantage. Table 2 indicates that significant increases occur in all the Class Alpha GST subunits (Ya₁, Ya₂, Yc and Yk) in the nodule-bearing livers. These data show that, among the constitutively expressed GSTs, Ya₂ is subject to a greater level of induction than are other subunits.

Purification of Ya₁ and Ya₂

The Ya₁ and Ya₂ subunits were recovered in purified preparations of GST YaYa and GST YaYc; the purification scheme employed is described in the Materials and methods section. Reverse-phase h.p.l.c. of GST YaYa and GST YaYc revealed that the relative amounts of Ya₁ and Ya₂ were the same in the two enzymes (Fig. 3), suggesting that both Ya-type subunits have an equal affinity for Yc. Both Ya₁ and Ya₂ were isolated by using the μ -Bondapak C₁₈ column and, after freeze-drying, were re-analysed on the same h.p.l.c. system. Fig. 4 shows that the Ya₁ and Ya₂ polypeptides isolated using this strategy were homo-

Table 2. Expression of GST subunits in control and pre-neoplastic nodule-bearing livers

Subunit	Approximate concentration of polypeptide (mg/g of total cytosolic protein)*		
	Control livers	Nodule-bearing livers	Increase (fold)
Ya ₁	2.6	7.2	2.8
Ya ₂	2.4	18.0	7.5
Yb ₁	8.8	32.9	3.7
Yb ₂	17.1		
(Yb ₂ + Yf)		40.6	
Yc	4.0	18.6	4.7
Yn ₁	0.05	0.06	1.2
Yk	0.1	0.2	2.0

* The calculation of the relative amounts of the different GST subunits has assumed that they all possess the same absorbance coefficient at 220 nm. The protein concentrations were estimated by the method of Bradford (1976).

geneous and, significantly, the profiles obtained provided no evidence of interconversion between the two Ya-type subunits.

Structural examination of Ya₂ and Ya₁

The Ya₁, Ya₂ and Yc subunits were subjected to CNBr cleavage and the digests analysed by reverse-phase h.p.l.c. (Fig. 5). The CNBr maps showed that Ya₁ and Ya₂ are closely related and that both are readily distinguished from the map of Yc.

Primary structures of Ya₁ and Ya₂

To allow the relationship between Ya₁ and Ya₂ to be established, the peptides obtained after CNBr digestion were subjected to automated Edman degradation. Besides comparing the amino acid sequence obtained from Ya₁ with that of Ya₂, the

primary structures of these two polypeptides were also compared with the two cloned cDNAs that code for Ya-type subunits. For this reason the sequences obtained from Ya₁ and Ya₂ that are shown in Fig. 6 are aligned with the amino acid sequences deduced from pGTR 261 (Lai *et al.*, 1984) and pGTB 38 (Pickett *et al.*, 1984). From the outset, it should be noted that the GST subunit encoded by pGTR 261 contains one more methionine residue than the subunit produced by pGTB 38. The subunits predicted by both cDNAs possess eight common methionine residues, at positions 16, 51, 57, 63, 86, 94, 105 and 208. The additional methionine residue encoded by pGTR 261 is located

at position 107. Because of this difference, a CNBr digest of the GST subunit synthesized from pGTR 261 should yield ten peptides, whereas a similar digest of the subunit produced by pGTB 38 should yield only nine. From this distribution of methionine residues it would be expected that CNBr cleavage of the subunit encoded by pGTR 261 would produce a major fragment of 101 amino acids (residues 108–208), whereas the largest peptide obtained by CNBr digestion of the subunit from pGTB 38 would be marginally larger, at 103 amino acids (residues 106–208).

Fig. 6 shows that, over the region for which we obtained sequence data for Ya₁ and Ya₂, different amino acids were identified at residues 31, 34, 107 and 117. At these positions Ya₁ contains aspartic acid, phenylalanine, methionine and lysine respectively, whereas Ya₂ contains glutamic acid, leucine, isoleucine and arginine. The Ya₁ subunit is presumed to possess a

Fig. 3. Purification of Ya₁ and Ya₂ subunits by reverse-phase h.p.l.c.

The GST enzymes YaYa (a) and YaYc (b) were isolated using, sequentially, GSH-agarose affinity chromatography, Protein PAK Glass SP-5PW and Protein PAK Glass DEAE-5PW, as described in Scheme 1. Portions of these enzymes were subjected to reverse-phase h.p.l.c. using conditions similar to those described in the legend to Fig. 2; the conditions differ in that a linear 40–58% acetonitrile gradient in aq. 0.1% (v/v) trifluoroacetic acid was employed to purify Ya₁ and Ya₂, rather than the 40–55% gradient used previously. The protein-containing eluate over each of the Ya₁, Ya₂ and Yc peaks was separately collected and freeze-dried.

Fig. 4. Reverse-phase h.p.l.c. analysis of purified Ya₁ (a) and Ya₂ (b)

After freeze-drying, portions of the Ya₁ and Ya₂ subunits that were isolated as shown in Fig. 3 were dissolved in aq. 0.1% (v/v) trifluoroacetic acid and re-analysed by reverse-phase h.p.l.c.

Fig. 5. Analytical and preparative peptide maps of Ya₁, Ya₂ and Yc subunits

CNBr digests of portions (1 mg) of Ya₁ (a), Ya₂ (b) and Yc (c) were prepared simultaneously as described in the text. The resulting CNBr fragments were dissolved in 2 ml of aq. 0.1% trifluoroacetic acid and aliquots (250 µg of protein) were applied to a Waters µ-Bondapak C₁₈ column (10 µm particle size; column size 0.39 cm × 30 cm). The column was developed with a 0–70% acetonitrile gradient in aq. 0.1% trifluoroacetic acid that was formed over 60 min. The continuous straight line represents the output of pump B; pump A delivered aq. 0.1% trifluoroacetic acid and pump B delivered 70% acetonitrile (also in aq. 0.1% trifluoroacetic acid).

Ya ₁ -CNBr2	(M)	E C I R W L L A A A G V E F D E K F I	*	*
Ya ₂ -CNBr2	(M)	E C I R W L L A A A G V E F E E K L I Q S P E D L E K L K K D G N L		
pGTB 38	M	E C I R W L L A A A G V E F E E K L I Q S P E D L E K L K K D G N L		
pGTR 261	M	E C I R W L L A A A G V E F D E K F I Q S P E D L E K L K K D G N L		
	16	26	36	46
Ya ₂ -CNBr3+4	(M)	F D Q V P M V E I D G		
pGTB38	M	F D Q V P M V E I D G		
pGTR261	M	F D Q V P M V E I D G		
	51	61		
Ya ₁ -CNBr5	(M)	K L A Q T R A I L		
Ya ₂ -CNBr5	(M)	K L A Q T R A I L N Y I A T K Y D L Y G K D		
pGTB 38	M	K L A Q T R A I L N Y I A T K Y D L Y G K D		
pGTR 261	M	K L A Q T R A I L N Y I A T K Y D L Y G K D		
	63	73	83	
Ya ₁ -CNBr7	(M)	Y T E G I L D - T E	*	
pGTB 38	M	Y S E G I L D L T E		
pGRT 261	M	Y T E G I L D L T E		
	94	104		
Ya ₁ -CNBr9	(M)	Q L V I - P P D Q K E A K T A L A K D - T K N - Y L P A F E K V L	*	*
Ya ₂ -CNBr8	(M)	I I Q L V I C P P D Q R E A K T A L A K D R T K N R Y L P A F E K V L		
pGTB 38	M	I I Q L V I C P P D Q R E A K T A L A K D R T K N R Y L P A F E K V L		
pGTR 261	M	I M Q L V I C P P D Q K E A K T A L A K D R T K N R Y L P A F E K V L		
	105	115	125	135
Ya ₂ -CNBr9	(M)	D A K Q I E E A R K V F K F	*	
pGTB 38	M	D A K Q I E E A R K V F K F		
pGTR 261	M	D A K Q I E E A R K I F K F		
	208	218		

Fig. 6. Primary structure of Ya₁ and Ya₂.

Sequences of CNBr-derived peptides from Ya₁ and Ya₂ are aligned for comparison with the identical sequences deduced from the cDNAs pGTB 38 (Pickett *et al.*, 1984) and pGTR 261 (Lai *et al.*, 1984). Putative methionine residues are in parentheses. The residues marked by an asterisk are those which differ between the four sequences. It should be noted that the putative CNBr dipeptide from Ya₁ which represents residues 106–107 has not been isolated. The sequence obtained from the major Ya₁ fragment (designated Ya₁-CNBr9) starts at position 108, whereas the sequence obtained from the major Ya₂ fragment (designated Ya₂-CNBr8) starts at position 106. Sequence data are not included for Ya₁-CNBr3+4, Ya₁-CNBr, Ya₁-CNBr10 and Ya₂-CNBr7.

methionine residue at position 107, because the first Edman cycle of the major CNBr fragment (i.e. that which was eluted from the μ -Bondapak C₁₈ column at 54 min; see Fig. 5) yielded a phenylthiohydantoin derivative of glutamine. As is shown in Fig. 6, our sequencing data demonstrate that the CNBr8 fragment from Ya₂ contains two more *N*-terminal amino acids (isoleucine, isoleucine) than the equivalent peptide from Ya₁.

DISCUSSION

Aflatoxin B₁ is one of the most important hepatocarcinogens in Nature and is thought to represent a major factor contributing towards the high incidence of liver cancer in the Third World (Shank, 1972; Linsell & Peers, 1977). Aflatoxin B₁ is a mycotoxin, produced by the mould *Aspergillus flavus*, and is found to

contaminate food in certain regions of the world. It requires metabolic activation to exert its toxic effects and, following ingestion, is converted into the ultimate carcinogen, aflatoxin B₁ 8,9-epoxide, by the actions of cytochrome *P*-450. This metabolite can be detoxified by GST or by epoxide hydrolase. The ability of aflatoxin B₁ to produce genotoxic damage depends, on the one hand, on the intracellular amount of cytochrome *P*-450 that can activate it to the epoxide form and, on the other hand, on the relative activity of GST and epoxide hydrolase, which are able to deactivate aflatoxin B₁ 8,9-epoxide.

Neal *et al.* (1987) reported that the pre-neoplastic nodules induced by aflatoxin B₁ possess markedly elevated GST activity towards aflatoxin B₁ 8,9-epoxide. It is thought that the over-expression of GST is responsible for the acquired resistance of pre-neoplastic hepatocyte nodules to the toxic effects of aflatoxin

B₁ (Neal *et al.*, 1988). However, these enzymes represent a highly complex supergene family of proteins, comprising as they do of at least four classes called Alpha, Mu, Pi and microsomal GST. Coles *et al.* (1985) reported that, among GSTs, only the Class Alpha enzymes are responsible for the metabolism of activated aflatoxin B₁. It therefore appears likely that the expression of Class Alpha GST within the nodules is of critical importance in determining their ability to resist the toxic effects of aflatoxin.

Previous work has shown that the expression of Class Alpha GST is increased in both pre-neoplastic nodules (Buchmann *et al.*, 1985; Jensson *et al.*, 1985) and hepatomas (Meyer *et al.*, 1985). However, to date, the studies that have been carried out have not investigated rigorously the expression of the Class Alpha GSTs. For the most part they have either focused almost entirely on the Class Pi GSTs or have entailed only semi-quantitative measurement of Class Alpha GSTs.

The present study was undertaken to establish what changes, if any, occur in the expression of Class Alpha GST subunits (i.e. Ya, Yc and Yk) in pre-neoplastic-nodule-bearing rat livers. During this project we employed a reverse-phase h.p.l.c. system to separate and quantify the GST subunits (Ostlund Farrants *et al.*, 1987) and found that we could resolve two Ya-type subunits, which we have designated Ya₁ and Ya₂. Interestingly, the levels of Ya₁, Ya₂, Yc and Yk in the nodule-bearing livers was increased 2.8-, 7.5-, 4.7- and 2.0-fold respectively. In the aflatoxin-treated rats the Ya₂ subunit showed the greatest increase in expression of any of the constitutively expressed subunits. Only the Class Pi Yf GST showed a larger relative increase in nodule-bearing livers, but this subunit is essentially absent from normal rat hepatocytes. Amongst the Mu Class GSTs, the Yb₁ subunit was increased 3.7-fold, but the Yb₂ and Yn₁ subunits showed little significant change in expression in nodule-bearing livers.

Our data show that, by contrast with Class Mu GSTs, all the Alpha Class GST subunits are over-expressed in aflatoxin-induced carcinogenesis. It is possible that the high levels of Ya₁, Ya₂, Yc and Yk we report are a result of the selection pressure provided by the chronic administration of aflatoxin rather than a result of the carcinogenic process itself. However, this possibility has not been examined and, in this context, it would be interesting to know whether these subunits are overexpressed to a similar extent in other experimental models of hepatocarcinogenesis, such as the choline/methionine-deficient model or the chronic-enzyme-induction model. Farber (1984a,b) proposed that the biochemical changes that are observed in the resistant hepatocyte model, which he and his colleagues developed (Solt & Farber, 1976), are common to all models of liver cancer. However, this suggestion was not made on the basis of measuring individual Class Alpha or Mu GST subunits; instead, the proposal was based, among other things, on the observed large changes in expression of the Class Pi Yf subunit.

We have undertaken amino-acid-sequencing experiments on Ya₁ and Ya₂ to help establish their molecular relationship. The data obtained demonstrated that the two subunits are the products of separate genes (Fig. 6); over the regions which were successfully sequenced, differences in the primary structures of Ya₁ and Ya₂ were noted at positions 31, 34, 107 and 117. This represents the first definitive proof, at the protein level, that multiple Ya-type subunits exist. Previous molecular-cloning experiments had indicated that two genes encoding Ya subunits are expressed in rat liver (Kalinyak & Taylor, 1982; Lai *et al.*, 1984; Pickett *et al.*, 1984; Taylor *et al.*, 1984). However, it was not possible to relate these DNA-cloning data to specific Ya subunits until chromatographic methods were available that could resolve the Ya-type subunits into the individual polypeptides.

The first sequence for a Ya subunit was reported by Kalinyak & Taylor (1982) when they described the cloning of a cDNA encoding about 70% of the protein. Later, Lai *et al.* (1984) isolated a full-length clone, pGTR 261, which encompasses the cDNA described by Kalinyak & Taylor (1982). A further Ya subunit, which was deduced from the cloned cDNA pGTB 38, was described by Pickett *et al.* (1984); at about the same time, Taylor *et al.* (1984) isolated, independently, a partial-length cDNA encoding approx. 60% of the same subunit. The subunits encoded by pGTB 38 and pGTR 261 show considerable sequence homology, and over their 222-amino-acid lengths show residue differences in only eight positions. At these positions (residues, 31, 34, 96, 107, 117, 206, 207 and 219) pGTB 38 encodes glutamic acid, leucine, serine, isoleucine, arginine, proline, alanine and valine respectively, whereas pGTR 261 encodes aspartic acid, phenylalanine, threonine, methionine, lysine, leucine, proline and isoleucine at the same positions. We have obtained sequence data for Ya₁ that include five of the eight 'difference residues' (namely 31, 34, 96, 107 and 117), and at each of these positions Ya₁ shows sequence identity with pGTR 261 rather than with pGTB 38. The sequence data that we have obtained for Ya₂ also includes five of the eight difference residues (namely 31, 34, 107, 117 and 219), and at all of these positions Ya₂ contains the amino acids that would be predicted by pGTB 38.

It should be noted that Kalinyak & Taylor (1982), Pickett *et al.* (1984), Lai *et al.* (1984) and Taylor *et al.* (1984) all used mRNA from either Sprague-Dawley or Wistar rats to clone Ya cDNA, whereas in the present study we isolated the Ya subunits from Fischer 344 rats. Although small inter-strain differences may exist in the structure of the Ya-type subunits, it is highly likely that both Ya₁ and Ya₂ are represented in Wistar rats as well as in Sprague-Dawley rats. Examination of the h.p.l.c. profiles of affinity-purified hepatic GST from these two strains reveals that the Ya-containing eluate is split into two partially resolved peaks (Ostlund Farrants *et al.*, 1987; Ketterer *et al.*, 1988). In this context it should be noted that the Southern-blotting experiments described by Rothkopf *et al.* (1986) and Tu *et al.* (1987) indicate that the Ya GST family is complex, comprising more than one gene. It is therefore not unreasonable to suppose that Ya₁ and Ya₂ subunits are expressed in all rat strains.

At present, there is an obvious paucity of information at the protein level about the primary structures of GST subunits, and this lack of data makes it difficult to relate cDNA sequences to specific enzymes. For example, it is still unclear what the molecular relationship is between the enzymes GST F and GST L, both of which comprise Ya-type subunits (Sheehan & Mantle, 1984; Hayes & Mantle, 1986a). In the context of the present study it should be noted that, in an earlier paper, one of us (Hayes, 1983) used CM-cellulose to resolve the YaYa-containing GST from normal rats into three fractions, which were called peaks II α , II β and II γ . From the relative concentrations, it is probable that II α contained Ya₁Ya₁, II β contained Ya₁Ya₂ and II γ contained Ya₂Ya₂.

In conclusion, our study raises the important question of whether Ya₁ and Ya₂ possess distinct catalytic and binding (i.e. 'ligandin') activities. In particular, it will be interesting to establish whether both subunits are able to detoxify aflatoxin B₁ 8,9-epoxide. If, as seems probable from their close sequence similarity, both Ya₁ and Ya₂ are active with this carcinogen, then the over-expression of Ya₂ in nodule-bearing livers would appear to be an adaptive response to exposure to aflatoxin.

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REFERENCES

- Beale, D., Ketterer, B., Carne, T., Meyer, D. & Taylor, J. B. (1982) *Eur. J. Biochem.* **126**, 459–463
- Benson, A. M., Talalay, P., Keen, J. H. & Jakoby, W. B. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 158–162
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Buchmann, A., Kuhlmann, W., Schwarz, M., Kunz, W., Wolf, C. R., Moll, E., Friedberg, T. & Oesch, F. (1985) *Carcinogenesis* **6**, 513–521
- Coles, B., Meyer, D. J., Ketterer, B., Stanton, C. A. & Garner, R. C. (1985) *Carcinogenesis* **6**, 693–697
- Cowan, K. H., Batist, G., Tulpule, A., Sinha, B. K. & Myers, C. E. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 9328–9332
- Eriksson, L. C., Sharma, R. N., Roomi, M. W., Ho, R. K., Farber, E. & Murray, R. K. (1983) *Biochem. Biophys. Res. Commun.* **117**, 740–745
- Fairchild, C. R., Ivy, S. P., Rushmore, T., Lee, G., Koo, P., Goldsmith, M. E., Myers, C. E., Farber, E. & Cowan, K. H. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7701–7705
- Farber, E. (1984a) *Biochim. Biophys. Acta* **738**, 171–180
- Farber, E. (1984b) *Can. J. Biochem. Cell Biol.* **62**, 486–494
- Habig, W. H. & Jakoby, W. B. (1981) *Methods Enzymol.* **77**, 398–405
- Harrison, D. J., May, L., Hayes, J. D. & Neal, G. E. (1990) *Carcinogenesis*, in the press
- Hayes, J. D. (1983) *Biochem. J.* **213**, 625–633
- Hayes, J. D. (1986) *Biochem. J.* **233**, 789–798
- Hayes, J. D. & Mantle, T. J. (1986a) *Biochem. J.* **233**, 407–415
- Hayes, J. D. & Mantle, T. J. (1986b) *Biochem. J.* **233**, 779–788
- Hayes, J. D. & Mantle, T. J. (1986c) *Biochem. J.* **237**, 731–740
- Hayes, J. D. & Wolf, C. R. (1988) in *Glutathione Conjugation – Mechanism and Biological Significance* (Sies, H. & Ketterer, B., eds.), pp. 315–355, Academic Press, London
- Hayes, J. D., Kerr, L. A. & Cronshaw, A. D. (1989) *Biochem. J.* **264**, 437–445
- Jensson, H., Eriksson, L. C. & Mannervik, B. (1985) *FEBS Lett.* **187**, 115–120
- Kalinyak, J. E. & Taylor, J. M. (1982) *J. Biol. Chem.* **257**, 523–530
- Ketterer, B., Meyer, D. J. & Clark, A. G. (1988) in *Glutathione Conjugation – Mechanisms and Biological Significance* (Sies, H. & Ketterer, B., eds.), pp. 73–135, Academic Press, London
- Kitahara, A., Satoh, K., Nishimura, K., Ishikawa, T., Ruike, K., Sato, K., Tsuda, H. & Ito, N. (1984) *Cancer Res.* **44**, 2698–2703
- Lai, H.-C. J., Li, N.-q., Weiss, M. J., Reddy, C. C. & Tu, C.-P. D. (1984) *J. Biol. Chem.* **259**, 5536–5542
- Linsell, C. A. & Peers, F. G. (1977) in *Origins of Cancer* (Hiatt, H. H., Watson, J. D. & Wurst, J. A., eds.), pp. 549–556, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Mannervik, B., Alin, P., Guthenberg, C., Jensson, H., Tahir, M. K., Warholm, M. & Jornvall, H. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7202–7206
- Meyer, D. J., Beale, D., Tan, K. H., Coles, B. & Ketterer, B. (1985) *FEBS Lett.* **184**, 139–143
- Morgenstern, R. & De Pierre, J. W. (1988) in *Glutathione Conjugation – Mechanisms and Biological Significance* (Sies, H. & Ketterer, B., eds.), pp. 157–174, Academic Press, London
- Neal, G. E., Metcalf, S. A., Legg, R. F., Judah, D. J. & Green, J. A. (1981) *Carcinogenesis* **2**, 457–461
- Neal, G. E., Nielsch, U., Judah, D. J. & Hulbert, P. B. (1987) *Biochem. Pharmacol.* **36**, 4269–4276
- Neal, G. E., Moss, E. J. & Manson, M. M. (1988) in *Glutathione Conjugation – Mechanisms and Biological Significance* (Sies, H. & Ketterer, B., eds.), pp. 281–314, Academic Press, London
- Ostlund Farrants, A.-K., Meyer, D. J., Coles, B., Southan, C., Aitken, A., Johnson, P. J. & Ketterer, B. (1987) *Biochem. J.* **245**, 423–428
- Pickett, C. B., Telakowski-Hopkins, C. A., Ding, G. J.-F., Argenbright, L. & Lu, A. Y. H. (1984) *J. Biol. Chem.* **259**, 5182–5188
- Power, C., Sinha, S., Webber, C., Manson, M. M. & Neal, G. E. (1987) *Carcinogenesis* **8**, 797–801
- Rothkopf, G. S., Telakowski-Hopkins, C. A., Stotish, R. L. & Pickett, C. B. (1986) *Biochemistry* **25**, 993–1002
- Rushmore, T. H., Sharma, R. N. S., Roomi, M. W., Harris, L., Satoh, K., Sato, K., Murray, R. K. & Farber, E. (1987) *Biochem. Biophys. Res. Commun.* **143**, 98–103
- Sakai, M., Okuda, A. & Muramatsu, M. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 9456–9460
- Satoh, K., Kitahara, A., Soma, Y., Inaba, Y., Hatayama, I. & Sato, K. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3964–3968
- Shank, R. C., Bhamarapravati, N., Gordon, J. E. & Wogan, G. N. (1972) *Food Cosmet. Toxicol.* **10**, 171–179
- Sheehan, D. & Mantle, T. J. (1984) *Biochem. J.* **218**, 893–897
- Simons, P. C. & Vander Jagt, D. L. (1977) *Anal. Biochem.* **82**, 334–341
- Solt, D. B. & Farber, E. (1976) *Nature (London)* **263**, 702–703
- Sugioka, Y., Kano, T., Okuda, A., Sakai, M., Kitagawa, T. & Muramatsu, M. (1985) *Nucleic Acids Res.* **13**, 6049–6057
- Taylor, J. B., Craig, R. K., Beale, D. & Ketterer, B. (1984) *Biochem. J.* **219**, 223–231
- Tu, C.-P. D., Lai, H.-C. J. & Reddy, C. C. (1987) in *Glutathione S-transferases and Carcinogenesis* (Mantle, T. J., Pickett, C. B. & Hayes, J. D. eds.), pp. 87–110, Taylor and Francis, London

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