The effect of hepatic regeneration on the expression of the glutathione S-transferases

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The effect of hepatic regeneration on expression of four glutathione S-transferase (GST) subunits (Ya, Yc, Yb₁, Yb₂) was examined in rats following partial hepatectomy (PH). mRNA levels of the Ya and Yc subunits (Alpha class) decreased and were 13% and 42% of levels in sham-operated animals respectively 12 h after surgery. mRNA levels for the Yb₁ subunit (Mu class) also decreased but were not maximally reduced until 24 h after PH (22% of sham-treated level). mRNA levels of the Yb₂ subunit were affected little by PH. Changes in levels of mRNA appeared to reflect a decrease in both transcriptional activity and mRNA stability. The decrease in mRNA levels was associated with a fall in enzymic activity and in protein levels of

Alpha-class GSTs. Within 48 h of surgery, levels of mRNA, protein enzymic activity and transcriptional activity had all fully recovered. GSH levels also decreased in the first 6 h after PH. However, 24 h after surgery GSH levels in animals having undergone PH exceeded those in sham-treated animals by 2-fold and this difference persisted for 72 h. These findings suggest that during the early phases of hepatic regeneration, because of decreased GST and GSH levels, the liver may be unusually susceptible to injury by toxic compounds. However, by the first round of cell division (36–48 h post-surgery) the liver has fully recovered its ability to metabolize toxic electrophiles.

INTRODUCTION

The glutathione S-transferases (GSTs) are a family of proteins. In the rat they have been separated into at least four classes, termed Alpha, Mu, Pi, and Theta, based on N-terminal sequences, substrate specificities and affinity for non-substrate ligands [1,2]. They are important enzymes of detoxification [3–6] and appear to play a role in the intracellular transport of hydrophobic molecules [7–11]. The GSTs account for 5–10% of the cytoplasmic proteins of the liver. The levels of some of the isoenzymes can be increased following treatment with phenobarbital or antioxidants, due to an increase in transcription [12–14]. However, little is known about the physiological events that may regulate expression of the GSTs. Studies using primary hepatocyte cultures have shown that the levels of GST isoenzyme changes during culture. Levels of Alpha- and Mu-class GSTs tend to fall whereas levels of GST π increase [15–17]. The latter is interesting in that GST π is a fetal form that is not expressed in adult hepatocytes but is expressed in fetal and transformed hepatocytes [18–21]. Therefore, hepatocytes in culture appear to assume a fetal GST phenotype. How the results in culture reflect changes *in vivo* are unknown.

An *in vivo* model that may be mimicked by cell culture is hepatic regeneration. Hepatocyte injury is followed by regeneration. There is increased expression of α -fetoprotein after certain types of injury, suggesting that regenerating hepatocytes assume a fetal phenotype [22]. Additionally, regeneration is associated with changes in expression of a variety of genes, including enzymes of detoxification such as the cytochrome P-450s [22–25]. If detoxification systems are uniformly reduced during hepatic regeneration then the regenerating liver may be unusually susceptible to injury by toxic compounds.

In the current study, we investigated hepatic regeneration after partial hepatectomy (PH) by monitoring the expression of the GST subunits Ya, Yc (Alpha family), Yb₁, Yb₂ (Mu family) and Yp (Pi family). (A nomenclature has been suggested for the rat

GSTs [25a]. Using this nomenclature, Ya = 1, Yc = 2, Yb₁ = 3, Yb₂ = 4 and Yp = 7.) Changes in mRNA levels were correlated with protein levels, GST activity, and rates of transcription. In addition, hepatic GSH levels were measured after PH.

MATERIALS AND METHODS

Materials

All chemicals used were of reagent grade and unless otherwise stated were purchased from Sigma Chemical Co. All restriction enzymes were purchased from Gibco-BRL. [³²P]dCTP, [³²P]UTP, 'megaprime labelling' kit and 'enhanced chemiluminescence' kit were from Amersham International (Amersham, Bucks., U.K.). Antibodies to the rat forms of GST were purchased from Biotrin, Dublin, Ireland.

PH treatment

Male Sprague–Dawley rats (250–400 g) were subjected to either PH or sham operation. Animals for each time point were of the same weight and were operated on as a group, with half undergoing PH and half sham operation. PH was performed as described by Higgins and Anderson [26] after the animals were anaesthetized with an intraperitoneal injection of pentobarbital (5 mg/100 g body wt.). Anaesthesia was maintained with diethyl ether during the surgery. A mid-line incision was made in the subxyphoid area and the median and left lateral lobes of the liver were removed, achieving a 70% PH. Sham operations were performed by externalizing the same lobes, then replacing them after a brief period of manipulation. Following surgery, animals were kept warm using a heat lamp until they regained consciousness. Water, but not food, was provided during the first 24 h following surgery, after which the animals were fed *ad libitum*. At various time points after surgery (6, 12, 24, 48 and

Abbreviations used: GST, glutathione S-transferase; PH, partial hepatectomy; SSC, 0.15 M NaCl and 0.015 M sodium citrate; DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride.

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72 h), rats were given ether anaesthesia and the liver removed as described below.

RNA preparation

Following anaesthesia the abdomen was opened and the livers perfused with 0.9% NaCl which contained 0.1% diethylpyrocarbonate (100 ml for PH-treated animals and 200 ml for sham-operated animals). The livers were rapidly removed and snap-frozen in liquid N₂. A sample (1 g) of each liver was immediately pulverized and homogenized in 10 ml of ice-cold guanidium thiocyanate solution and the RNA prepared as described previously [27]. The remainder of the liver was stored at -80 °C for further analysis.

Northern-blot analysis

The cDNA probes for GST Ya, Yc, Yb₁ and Yb₂ were a gift from Dr. Cecil Pickett [28]. The cDNA for Yp was provided by Dr. Barch [29]. The cDNA for mouse albumin was provided by Dr. E. Morgan. The cDNA fragments were cut from the plasmids with appropriate restriction enzymes and purified. The fragments were labelled with [³²P]dCTP using a random-primer kit from Amersham.

Total RNA was pooled from three to five animals which had been killed at each time point. The RNA was ethanol-precipitated and dissolved in 0.5% SDS. The quantity of RNA was estimated by measuring the A₂₆₀ value. A sample of total RNA (15 µg) was denatured in 2.2 M formaldehyde and 50% (w/v) formamide, and 1 µl of ethidium bromide was added before electrophoresis. The samples were electrophoresed on 1% (w/v) agarose gels which contained 2.2 M formaldehyde. At the completion of electrophoresis the gels were examined under u.v. light to judge the quantity of the RNA and the equality of loading. RNA was transferred to a nylon membrane (NEN, Dupont, Boston, MA, U.S.A.) in 20 × SSC (1 × SSC: 0.15 M NaCl/0.015 M sodium citrate) overnight. The membrane was then baked in an oven at 80 °C for 2 h and kept refrigerated until subjected to hybridization. The blots were hybridized at 65 °C for 8–16 h and then washed three times at 65 °C for 20 min each time in 1 × SSC. The blots were then exposed to Kodak XAR film. Each blot was initially probed with one of the GST cDNAs. The blots were then placed in boiling water to remove the GST probe and hybridized a second time with the cDNA for albumin. Conditions of hybridization were quite stringent to minimize the possibility that a GST cDNA probe would hybridize with more than one specific transcript within a class. As PH had different effects on the GSTs within the same class (see the Results section) the conditions used appeared to have minimized the possibility of the probes hybridizing with different, but related, subunits.

Semi-quantitative measurement of each transcript was performed in the following manner. Two separate gels were run for each GST cDNA. Following development, the film was scanned with a densitometer (ISCO) and the areas of the curves obtained with the GST and albumin cDNAs from the same gel determined using a computer (Beckman Instruments). The areas obtained with the GST cDNAs at each time point were corrected for variations in loading by normalizing the results using albumin. The areas obtained from the two blots for each GST cDNA were then averaged. The average values for PH- and sham-treated animals were then compared at each time point following surgery.

Nuclear run-off assay

The nuclei were isolated from freshly snap-frozen livers of both sham- and PH-treated animals 6 h and 24 h after surgery. The

snap-frozen livers were pulverized and ground to a powder using a mortar and pestle, pouring liquid N₂ on to the tissue as needed to keep it frozen. The resulting fine powder was transferred into a prechilled Dounce homogenizer containing 10 ml of buffer [0.32 M sucrose, 3 mM magnesium acetate, 1 mM Hepes (pH 6.8), 5 mM dithiothreitol (DTT), 1 mM EGTA, 1 mM spermidine, and 0.1 mM phenylmethanesulphonyl fluoride (PMSF)]. The pellet was resuspended using a loose-fitting Dounce (10 strokes) and then homogenized fully using a second 10 strokes. The homogenate was transferred to polypropylene tubes and centrifuged at 1000 g for 10 min at 4 °C. The upper layer was removed using a pipette and the pellet resuspended in 8.5 ml of 1.65 M sucrose buffer that contained 5 mM DTT, 5 mM magnesium acetate, 1 mM Hepes, 1 mM EGTA, 1 mM spermidine and 0.1 mM PMSF. The resultant solution was layered over 4 ml of a 2 M sucrose buffer (2 M sucrose, 5 mM DTT, 3 mM magnesium acetate, 1 mM Hepes) and centrifuged for 1 h at 30000 g. The nuclei were recovered and resuspended in 250 µl of buffer containing 40% (v/v) glycerol, 5 mM magnesium acetate, 0.1 mM EDTA, 50 mM Tris/HCl, 1 mM EGTA, 1 mM spermidine, 0.1 mM PMSF and 5 mM DTT, snap-frozen with liquid N₂ and kept at -80 °C until used (1–7 days). An aliquot was saved and the number of nuclei counted using a haemocytometer.

Nuclei were thawed on ice and 5 × 10⁸ nuclei were added to transcription buffer which contained [³²P]dUTP. The mixture was incubated at 26 °C for 45 min. The reaction was stopped by lysing the nuclei. The labelled RNA was extracted with CHCl₃-phenol and precipitated with ethanol. Following digestion with DNAase and proteinase, unincorporated isotope was removed with multiple ethanol precipitations. cDNA samples (5 µg) for Ya, Yc, Yb₁, Yb₂, albumin and pBR322 were transferred on to nitrocellulose filters which were then baked at 80 °C for 2 h. Aliquots of labelled RNA (5 × 10⁷ c.p.m.) were hybridized with the cDNA-containing filters at 65 °C for 36 h in 2 ml of hybridization buffer which contained 10 mM Tris/HCl, 10 mM EDTA, 300 mM NaCl, 1 × Denhardt's solution, and 0.25 mg/ml tRNA. The blots were washed twice for 20 min each time in 2 × SSC at 65 °C, treated with RNAase A (10 µg/ml) for 30 min at 37 °C, washed a third time in 1 × SSC for 1 h at 37 °C and then exposed to film for 2–3 days. The autoradiograms were subjected to densitometry. Each nuclear run-off was performed twice at each time point and the areas were averaged.

Western blot and protein assay

Portions (0.5 g) of each rat liver from each time point were combined, homogenized in 0.25 M sucrose and the cytosol prepared as described previously [30]. The protein concentration was measured using the Bio-Rad assay with BSA as the standard. Standard curves had *r* values of ≥ 0.99. The pooled cytosols were used for Western blots and measurement of enzymic activity. The Western blots were performed using a 10% (w/v) acrylamide gel and 2 µg of protein in each lane. Proteins were transferred to nitrocellulose paper and incubated with anti-Ya antiserum. The enhanced chemiluminescence detection system (Amersham) was employed to identify positive reactions. The resultant autoradiograms were scanned as described above to estimate the amount of protein present at each time point in PH- and sham-treated animals

Measurement of GST activity and GSH concentration

GST activity in cytosol was measured using chlorodinitrobenzene or cumene hydroperoxide in a recording spectrophotometer as described previously [30]. Each assay was performed in triplicate

on pooled cytosol from three to five animals. Coefficients of variation for assays with chlorodinitrobenzene and cumene hydroperoxide were $11.9 \pm 6.4\%$ and $9.6 \pm 6.0\%$ respectively. Proteins were precipitated from the pooled cytosols with 5% (v/v) trichloroacetic acid, removed by centrifugation, and the resultant supernatant was assayed for GSH as described previously [31]. Each assay was performed in triplicate on two separate occasions.

RESULTS

Effects of PH on mRNA levels

Levels of mRNA for each of the GST subunits (Ya, Yc, Yb₁, Yb₂ and Yp) and albumin following PH were compared with those in sham-operated animals at each time point. Use of sham-treated

Figure 1 Northern blot of RNA prepared from PH- and sham (S)-treated animals: the filters were probed with Ya, Yc, Yb₁, Yb₂ and albumin (Alb) cDNAs

RNA was prepared from animals killed 6, 12, 24, 48 and 72 h post-surgery. Each lane was loaded with 15 µg of total RNA.

Table 1 Relative changes in mRNA levels of GSTs after PH

Data were obtained from densitometric scans of duplicate gels. Areas were normalized using albumin mRNA from the same gel as described in the Materials and methods section. The normalized areas were averaged and the average areas used to determine the relative amounts of each transcript in PH-treated compared with sham-operated animals.

Time after surgery (h)	GST subunits ...	mRNA level (% of level in sham-operated control)			
		Ya	Yc	Yb ₁	Yb ₂
6		66	94	62	150
12		13	42	59	141
24		39	69	22	94
48		129	82	88	165
72		89	110	129	130

animals is essential to act as controls for the effects of anaesthesia and surgery on gene expression independent of PH. Minimal and inconsistent signals were obtained using the GST π cDNA in both sham- and PH-treated animals at all time points, whereas easily measured signals were obtained with all of the other cDNAs. Because of the inconsistent result with the Pi-class GST cDNA it was not examined further. Each blot with the other GST cDNA probes was performed in duplicate. Figure 1 shows a representative blot. As previously reported [23,25] the mRNA levels for albumin changed little following PH. In contrast, the transcript levels of Ya and Yc in animals having undergone PH compared with control animals were lower at 12 and 24 h. Transcript levels of Yb₁ were reduced at 12 and 24 h also, whereas levels of Yb₂ were little affected by PH. Variations in loading, as shown by the weak signals obtained with the 12 h and 72 h sham-treated animals with the Yc cDNA probe on a single blot (Figure 1), complicate the interpretation of any changes. Semi-quantitative measurements of transcript levels can be made if variations in loading can be controlled by use of an internal standard such as albumin. We therefore probed each blot with the cDNA for albumin as well as with a GST cDNA. Gels were then scanned as described in the Materials and methods section and the area obtained for the GST mRNA normalized by using the area obtained with the albumin cDNA from the same membrane. Table 1 shows the normalized average areas from two separate blots obtained with each GST cDNA relative to the sham-treated animals. The mRNAs for GST Ya and Yc were reduced maximally at 12 h, being 13% and 42% of sham-operated controls respectively. The mRNA levels of Ya and Yc then increased and had recovered by 48 h. Different results were obtained with the Mu-class GST. The mRNA for the GST Yb₁ subunit was maximally depressed at 24 h (22% of sham level) whereas the level of Yb₂ was never much less than the sham level and usually was greater (Table 1).

Protein levels

To determine if changes in mRNA levels correlated with changes in GST protein levels, Western blots were performed on cytosol from sham- and PH-treated animals using an anti-Ya antiserum. The antiserum cross-reacts with other subunits in the Alpha class but does not cross-react with subunits in other classes. Alpha-class protein levels 6, 12, 24, 48 and 72 h after PH were 97%, 12%, 26%, 62% and 67% of levels in sham-treated controls respectively (Figure 2).

Enzymic activity

Activity was measured using chlorodinitrobenzene, which serves as a substrate for most forms of GST, and cumene hydroperoxide, which is largely an Alpha-class substrate [3]. Both activities decreased, with a somewhat greater fall in activity measured with chlorodinitrobenzene as compared with cumene hydroperoxide (Table 2). Maximum depression occurred between 12 h and 24 h and recovery was complete by 48 h. The fall in activity as measured with cumene hydroperoxide may not have been as great as when measured with chlorodinitrobenzene because the former is also a substrate for glutathione peroxidase [32].

Level of transcriptional activity

Nuclear run-off assays were performed 6 h and 24 h after surgery. Transcriptional activities at 24 h in PH-treated animals were equal to, or greater than, activities in sham-operated controls (Figure 3 and Table 3). At 6 h, levels of transcription were

Figure 2 Effect of PH on Alpha-class protein levels

The Western blot was performed as described in the Materials and methods section using anti-Ya antiserum. Abbreviations: PH, PH-treated; S, sham-operated.

Table 2 Effect of PH on enzymic activity of GSTs

Enzymic activities were determined in pooled cytosols from three to five animals with both CDNB (chlorodinitrobenzene) and CuOOH (cumene hydroperoxide) as described in the Materials and methods section. Each assay was performed in triplicate and is expressed as mean $\pm$ S.D.

Operation	Time after surgery (h)	Enzymic activity (μ mol/min per mg of protein)	
		CDNB	CuOOH
PH	6	1.24 $\pm$ 0.05	0.39 $\pm$ 0.07*
Sham	6	1.44 $\pm$ 0.12	0.67 $\pm$ 0.05
PH	12	0.50 $\pm$ 0.04*	0.23 $\pm$ 0.02*
Sham	12	0.87 $\pm$ 0.16	0.39 $\pm$ 0.09
PH	24	0.76 $\pm$ 0.03*	0.42 $\pm$ 0.01*
Sham	24	2.07 $\pm$ 0.24	0.75 $\pm$ 0.04
PH	48	1.28 $\pm$ 0.23	0.72 $\pm$ 0.09
Sham	48	1.74 $\pm$ 0.19	0.84 $\pm$ 0.07
PH	72	1.14 $\pm$ 0.28	0.52 $\pm$ 0.03
Sham	72	1.15 $\pm$ 0.16	0.56 $\pm$ 0.05

* Enzymic activities were significantly ($P < 0.05$) less in PH-treated as compared with sham-operated animals at these time points. Significance was determined using Student's *t*-test for two means.

Figure 3 Effect of PH on rates of transcription of GSTs and albumin

Nuclear run-off assays were performed as described in the Materials and methods section. pBR322 served as a negative control.

Table 3 Effect of PH on transcriptional activity

Each assay was performed twice and scanned as described in the Materials and methods section.

cDNA	Time after surgery (h) ...	Nuclear run-off (% of sham-operated control)	
		6	24
Ya		78	116
Yc		60	128
Yb ₁		76	107
Yb ₂		43	115
Albumin		89	90

somewhat lower for Yc and Yb₂ subunits in animals having undergone PH whereas they were similar to levels in sham-operated controls for the other subunits (Table 3). No change in transcription rates for albumin was noted at either time point.

GSH levels

The effect of PH on GSH levels is shown in Table 4. At 6 h GSH levels were reduced by 49 % in PH-treated animals as compared with sham-operated animals. By 12 h the levels were similar in both groups and then levels steadily increased in the animals having undergone PH, reaching a maximum at 48 h, which exceeded that in sham-operated controls by 2-fold. The difference persisted up to 72 h.

DISCUSSION

The GSTs play an important role in preventing hepatic damage from a variety of injurious agents. In addition, they metabolize potential carcinogens to inactive compounds [3–6]. Hepatic regeneration is a sequelae of hepatic injury and may play a critical role in promoting carcinogenesis [33]. Therefore, defining the effect of hepatic injury and regeneration on the GSTs is important for understanding how the regenerating liver deals with exposure to toxic compounds. In the current study, PH had significant effects on GST mRNA levels, protein levels and enzymic activity. The effects were more pronounced on the Alpha-class GSTs (Ya and Yc) but changes in the Yb₁ subunit of the Mu class were observed also. Decreases in GST mRNA levels were associated with decreases in protein levels (Alpha class) and GST activity. The decreases in activity were modest, but may be of a sufficient degree to make the liver following PH susceptible to injury by toxic compounds during this early phase of regeneration. Hepatic levels of GSH also fell during the first 6 h after PH, which would contribute to the increased susceptibility to hepatic injury by toxic electrophiles.

The maximum decreases in mRNA and protein levels were observed 12–24 h after PH. This is the pre-replicate phase of hepatic regeneration, with the first round of cell division occurring about 36 h after PH [24]. Return of GST levels to normal occurred at the time the cells began to divide. Levels of GSH also increased during this time period and actually exceeded the levels in sham-operated animals by 2-fold at 24, 36 and 48 h after surgery. The cause of the increase in GSH concentration was not investigated, but it could reflect either increased production or decreased excretion by the liver [34]. Therefore, in contrast with the low levels of GST and GSH seen early after PH, 36 h after PH the regenerating liver has fully recovered its ability to deal with injury by GST-metabolized toxins.

Table 4 Effect of PH on liver GSH levels

Assays were performed on pooled homogenates from three to five animals as described in the Materials and methods section. Three separate determinations were made on each pooled homogenate. *P* indicates level of significance. Abbreviation: N.S., not significant.

Operation	Time after surgery (h)	GSH content		<i>P</i>	<i>P</i>
		($\mu\text{mol/g}$ of tissue)	(nmol/mg of protein)		
PH	6	0.94 ± 0.15	16.5 ± 2.6	< 0.0001	< 0.05
Sham	6	1.91 ± 0.11	20.0 ± 1.04		
PH	12	1.20 ± 0.12	17.32 ± 1.78	N.S.	N.S.
Sham	12	1.31 ± 0.15	16.0 ± 1.81		
PH	24	3.10 ± 0.16	33.13 ± 1.89	< 0.0001	< 0.0001
Sham	24	1.26 ± 0.12	16.42 ± 1.56		
PH	48	3.26 ± 0.38	37.93 ± 4.48	< 0.0001	< 0.0001
Sham	48	1.79 ± 0.23	19.02 ± 2.47		
PH	72	2.23 ± 0.27	34.62 ± 4.17	< 0.0001	< 0.0001
Sham	72	0.90 ± 0.11	15.45 ± 1.95		

Regulation of expression of GST in previous studies has appeared to be largely at the level of transcription. Increased expression following exposure to drugs such as phenobarbital, 3-methylcholanthrene and electrophilic compounds is due to an increase in transcription [12]. Changes in expression of GST during culture of hepatocytes are also thought to be at the transcriptional level [17]. In the current study changes in mRNA levels for GST appear to reflect transcriptional and post-transcriptional events. We base this conclusion on the observations that transcriptional activity, although decreased at 6 h for some subunits, correlated poorly with levels of mRNA. For example, transcriptional activity for the Ya subunit after PH differed little from that of sham-operated animals at 6 h and yet levels of mRNA for that subunit had begun to fall. Transcriptional activity was reduced for the Yb₂ subunit and yet levels of Yb₂ mRNA were equal to, or greater than, the control. Transcriptional activity for all four of the subunits had returned to baseline values 24 h after surgery and yet the levels of mRNA for three of the four subunits were still well below the levels present in the sham-operated animals. Thus, though PH causes a decrease in rates of transcription for some subunits, mRNA stability also appears to contribute to the changes seen after PH. What accounts for the changes in GST mRNA stability after PH is unknown but similar mixed effects of regeneration on the mRNA of other proteins have been reported [22,23]. It is interesting that the Yb₂ mRNA appeared to have increased stability after PH. Similarly, during culture hepatocytes lose Yb₃ mRNA, whereas the level of mRNA for Yb₂ changes very little [15]. These findings suggest that there may be undefined differences in the mRNA structure of Yb₂ compared with that of other GST mRNAs, which affect its stability after PH [35,36].

In the fetal liver GST π is the predominant isoenzyme. Soon after birth levels of GST π decrease and Alpha- and Mu-class isoenzymes become the predominant forms of GST [37]. Hepatocytes in culture appear to undergo a reverse process where levels of Alpha- and Mu-class forms decrease whereas π increases [15]. Hepatic regeneration also may be associated with a change from an adult toward a fetal phenotype [22]. In the current study, however, despite the decrease in the levels of the

adult forms of GST there was no apparent increase in expression of GST π . Although we could not consistently detect GST π in either sham- or PH-treated animals, in another study GST π mRNA was detectable in normal and regenerating liver [25]. However, in the latter study levels of GST π mRNA decreased following PH, which contrasts with the finding in cultured hepatocytes. Therefore, regenerating hepatocytes do not appear to assume a fetal GST phenotype. GST π is present in hepatic lipocytes and bile-duct epithelial cells [38,39]. Our inability to obtain consistent signals most likely reflects the small amount of RNA in the extracts from these cells relative to that from hepatocytes.

In conclusion, expression of GSTs is regulated both transcriptionally and post-transcriptionally after PH. There appear to be significant differences between the Alpha and Mu classes as to the effects of PH on expression, which is consistent with previous observations that the expression of GST subunits are regulated differently [4,12,17]. Lastly, the changes in GST expression observed with hepatocytes in culture do not parallel the changes observed during hepatic regeneration.

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