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THE EXPRESSION OF UDP-GLUCURONOSYLTRANSFERASES OF THE UGT1 FAMILY IN HUMAN LIVER AND KIDNEY AND IN RESPONSE TO DRUGS

LESLEY SUTHERLAND, THOMAS EBNER and BRIAN BURCHELL*
Department of Biochemical Medicine, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY, U.K.

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Abstract—The expression of human UDP-glucuronosyltransferase (UGT) 1 gene family in the liver and kidney was examined using specific enzyme activity, antibodies and DNA probes for each of the four family members. Phenol UGT HP1 was expressed at a similar, relatively low, abundance in each liver and kidney whereas phenol UGT HP4 was more highly expressed in the kidney. Bilirubin UGTs (HP2 and HP3) were not detectable in the kidney and HP3 was the major isoform in the liver. The UGT activities towards certain specific substrates correlated well with the respective mRNA levels in the tissues. Bilirubin UGT HP3 was induced 2–3-fold in the livers from patients treated with phenytoin and phenobarbital. Storage of a human liver in University of Wisconsin solution which contains dexamethasone and insulin caused a large accumulation of all the UGT mRNAs, but these were not quantitatively translated into expressed UGT activities. The implications of these results are discussed.

The UDP-glucuronosyltransferases (UGTs† EC 2.4.1.17) are a large family of enzymes which catalyse the glucuronidation of a wide variety of endogenous and exogenous compounds. The transfer of the glucuronide moiety from the cofactor UDP-glucuronic acid (UDPGA) to the substrate renders the substrate more polar, thereby facilitating its excretion into the bile or urine [1]. Recent cloning studies using cDNA libraries constructed from human liver have resulted in the identification of a number of novel UGT isoforms from this tissue [2–5]. These clones have been broadly divided into two large subfamilies based on sequence comparisons [4, 5, 6]. The UGT2 subfamily isoforms show amino acid similarities of 60–90% between a given pair of enzymes and are most similar to the rat isoforms which have been shown to glucuronidate steroids [4, 6]. The substrate specificities of three enzymes from this subfamily have so far been examined: four isoforms have been found to glucuronidate bile acids and cathechol oestrogens [7, 8 and unpublished work]. Four members of subfamily 1 have been identified to date. Two of the members of this family, HP2 and HP3, have been found to glucuronidate bilirubin [9, 10], while HP1 and HP4 glucuronidate small planar and bulky phenols, respectively [2, 11, 12]. A particularly notable feature of subfamily 1 is revealed by DNA sequence comparisons which show that while all four isoforms share nucleotide identity within their 3′ halves, identity at the 5′ halves is between 40 and 60% for a given pair of isoforms [5, 9, 11]. This interesting cDNA structure is thought to arise as a result of alternative splicing between single exons encoding each of the 5′ (variable) regions and four downstream exons encoding the constant region [2, 5, 11, 13].

The recent expression of single UGT isoforms in mammalian tissue culture systems has allowed important advances in the study of substrate specificity [1, 11]. The construction of cells expressing stably integrated copies of these enzymes has been particularly successful as a tool for assessing glucuronidation of a wide variety of compounds [10–12, 14]. This work has revealed that while some substrates appear to be glucuronidated highly specifically, possibly by a single isoform, there also exists a degree of substrate overlap between certain isoforms [12]. This type of analysis is very useful in helping towards a rational prediction of drug metabolism as it allows compounds to be screened, in tissue culture, for their capacity to be glucuronidated by known UGT isoforms. Data of this kind will also be useful in the prediction of adverse drug reactions caused by competition between substrates. In order to have a more complete picture of what might occur *in vivo*, it would be useful to know the relative abundance of different isoforms in human tissues, and to have an understanding of what factors can influence the expression of these genes. It is well documented that many drug-metabolizing enzymes, including UGTs, are inducible in man by exogenously added substrates and it is likely that this control will reflect regulatory pathways involving natural compounds [15].

In this study, expression of UGT has been assessed by examining mRNA and protein levels, as well as measuring enzyme activities. We have examined the expression of the UGT1 gene family in human liver and kidney, both of which are important sites of

* Corresponding author. Tel. (0382) 60111 ext. 3118; FAX (0382) 644620.

† Abbreviations: 4-MU, 4 methylumbelliferone; UGT, UDP-glucuronosyltransferase; UDPGA, UDP-glucuronic acid; UW solution, University of Wisconsin solution; SSPE, sodium salts with phosphate and EDTA.

Table 1. Characteristics of patients and tissue samples

Sample	Sex	Age	Cause of surgery or death	Drug intake
K	M	68	Kidney carcinoma	Thioridazine
1	—	—	Metastatic carcinoma of bowel	Unknown
2	—	—	Transplant donor	Unknown
3	—	—	Transplant donor	Unknown
4	M	63	Metastatic carcinoma of the bowel	Unknown
5	M	3	Liver transplantation, ornithine transcarbamylase deficiency and hyperammonemia	Medication: 3 years of barbiturates
6	F	42	Ovarian metastatic adenocarcinoma	—
7	F	41	—	Repeated doses of phenytoin, ventolin

glucuronidation in man. We have used a number of different human liver samples in our analysis, including tissue from patients known to have been treated with drug-metabolizing enzyme-inducing drug therapies.

MATERIALS AND METHODS

Human tissue samples. Six human liver samples used in this study were kindly provided by Dr G. B. Odell, University of Wisconsin, Madison, U.S.A. One liver sample was taken from a patient suffering from a deficiency of ornithine transcarbamylase and two were from tissue stored in University of Wisconsin (UW) solution. A liver sample from an individual chronically treated with phenytoin was provided by Dr D. Burke, University of Aberdeen, U.K. The human kidney sample was provided by Dr J. Dick, University of Dundee, U.K. All samples were obtained with the appropriate ethical permission from each institution, directly frozen in liquid nitrogen and transported in dry ice. Further clinical details of tissue samples are described in Table 1.

Isolation and radiolabelling of UGT1 isoform-specific DNA probes. The cDNAs used to generate isoform-specific probes for subfamily UGT1A family members were cloned from human liver libraries constructed in the λ-based vectors λgt11 and λUNIZAP. The cloning of these sequences has been described in detail elsewhere [2, 4, 5, 11].

Isoform-specific probes complementary to HP1, HP2, HP3 and HP4 sequences were produced as described previously [16]. All probes are derived from sequences from the 5′ (variable) region of the HP clone. A probe which hybridizes to the constant region (and therefore recognises all HP family members) was also prepared as described [16]. The specificity of the probes used was verified by using each in turn as a probe against clones HP1–HP4, southern blotted [17] onto Hybond-N (data not shown). Probing of genomic DNA confirmed that each probe did not cross-react with any other isoform.

All probes were labelled to high specific activity ($1–2 \times 10^6$ dpm/μg) using α-^{32}PdCTP. Labelling was carried out using the "Megaprime" labelling system (Amersham, U.K.) which is a modification of the random priming procedure described by Feinberg and Vogelstein [18].

Northern blotting of RNA from tissue samples. RNA was isolated from frozen human liver and kidney tissue using a slight modification of the rapid phenol procedure described by Chirgwin *et al.* [19], in which the RNA samples were further purified by precipitation with lithium chloride [17].

The RNA samples (15 μg) were each loaded onto a 0.8% agarose gel containing 50% formamide then electrophoresed using 1 × 4-morpholinepropanesulphonic acid as running buffer. When visualization of the samples was required, ethidium bromide was included in the sample [17]. RNA samples electrophoresed in agarose as described above were transferred onto nylon membrane (Hybond-N) using the transfer and fixing conditions recommended by the manufacturer (Amersham). Northern blots were incubated at 42° in a prehybridization solution containing 5 × SSPE, 50% formamide, 5 × Denhardt's solution, 0.5% sodium dodecyl sulphate [20]. After 2 hr the solution was replaced with fresh solution to which the denatured labelled probe was added (10^6 counts/mL). Northern blots were then hybridized for 16 hr at 42°. Following hybridization, blots were washed at 42° for 45 min with a change of wash solution after every 15 min (0.1 × standard saline citrate, 0.1% sodium dodecyl sulphate). Blots were then autoradiographed at −70°, with an intensifying screen, using Hyperfilm MP film (Amersham). Quantification of blots was using a Molecular Dynamics Phosphorimager (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Construction and growth of cell lines stably expressing UGT HP enzymes. V79 (Chinese hamster fibroblast) cells which stably express high levels of

Table 2. Activities of UGT1 enzymes stably expressed in V79 cells

Substrate	UGT activity (nmol/min/mg)		
	HIugP1	HIugP3	HIugP4
1-Naphthol	3.2	0.3	0.2
4-MU	1.1	0.8	1.5
Androsterone	ND	ND	ND
Propofol	ND	ND	0.9
Bilirubin	ND	0.4	ND

ND, not detectable.

HP1, HP3 and HP4 were constructed and cultured as described elsewhere [10–12, 14].

Assay of microsomal UGT activities. Microsomes were prepared from frozen tissue samples using the procedure described by Dragacci *et al.* [21]. 1-Naphthol UGT activities were assayed as described by Otani *et al.* [22]. Bilirubin glucuronidation was assayed by the procedure of Heirwegh *et al.* [23] using 50 μM bilirubin and 5 mM UDPGA. UGT activities towards 4-methylumbelliferone (4-MU) and 2,6-diisopropylphenol were determined using the TLC assay described by Bansal and Gessner [24]. Assay mixtures contained 0.5 mM substrate and 2 mM UDPGA acid were incubated for 30 min at 37°. TLC assay were quantified using a Digital Autoradiograph (Berthold, Wildbad, F.R.G.). Microsomal enzyme activity towards each substrate was determined after optimal activation using the detergent Lubrol PX. Optimal activation curves were determined for each substrate and each microsomal preparation.

Western blotting of microsome samples. Western blotting of human liver and kidney microsome samples was carried out as described previously [11].

RESULTS

Substrate specificity of human UGT HP isoforms

We have used HP isoforms stably expressed in tissue culture (V79 cells) to try to assess the substrate specificity of individual enzymes. We have carried out an extensive analysis of the substrates which are conjugated by HP1, HP3 and HP4 [10, 12] and include in this study only a limited amount of data which is required to indicate the substrate probe activity of specific HP isoforms in human tissue samples. The results given in Table 2 show that the substrate 4-MU is a relatively non-specific substrate and can be used to give a general picture of the UGT activity of samples. The anaesthetic compound propofol (2,6-diisopropylphenol) appeared to be specifically glucuronidated by HP4, compared to the other isoforms tested, while HP1 showed relative specificity for 1-naphthol when compared to the other two isoforms tested. HP3 showed high substrate turnover for the substrate bilirubin. Previous data has shown that HP2 also catalyses the glucuronidation of bilirubin [9]. Androsterone glucuronidation was not catalysed by the cloned expressed human UGTs.

UGT activities in liver microsomes

Substrate glucuronidation for the liver and kidney samples investigated is shown in Table 3. All the liver samples showed similar levels of 4-methylumbelliferone glucuronidation, the maximum difference only being around 2-fold. This suggests that all the microsome preparations were of similar quality. The activities of all the livers towards the substrates 1-naphthol and androsterone were also very similar, with the exception of liver 7 which showed a value 2.5-fold greater than the mean activity for glucuronidation of these substrates. (4-MU glucuronidation is only 1.25 times the mean value in this sample). Liver 7 showed the highest activity towards bilirubin, while livers 1 and 6 showed very low activities. Liver sample 2 showed the highest UGT activity towards propofol, while livers 4 and 6 showed a low capacity to glucuronidate this substrate.

UGT activities in human kidney

The kidney sample examined showed high activities towards the substrates 1-naphthol and propofol. Glucuronidation of bilirubin was not detected and the activity towards 4-MU was approximately 50% of the liver average (Table 3).

Western blotting of human microsomes

The pattern obtained after Western blotting of the human liver and kidney samples revealed a good deal of variation between the samples examined

Table 3. Measurement of UGT activities in human liver and kidney microsomes

	UGT activity (nmol/min/mg) Liver							Kidney
Substrate	1	2	3	4	5	6	7	K
1-Naphthol	4.2	4.9	4.5	3.0	5.2	2.0	12.2	5.4
4-MU	25.8	28.3	26.6	14.9	32.4	16.9	31.8	15.8
Androsterone	1.4	1.4	0.5	1.4	1.5	0.6	2.7	1.5
Propofol	2.4	3.1	0.8	0.3	2.5	ND	1.4	3.5
Bilirubin	0.3	0.9	0.6	0.7	1.1	0.2	1.9	ND

ND, not detectable.

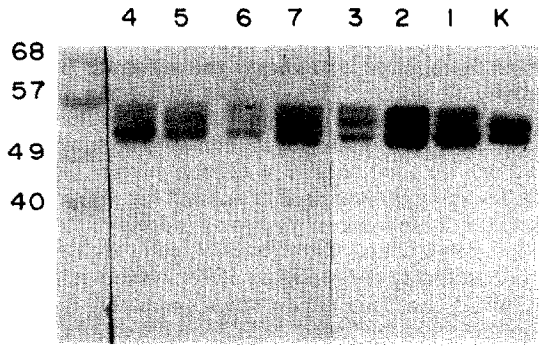

Fig. 1. Immunoblot analysis of human liver and kidney microsomes using an anti-UGT antibody. Microsomes from kidney (K) and from livers 4, 5, 6, 7, 3, 2 and 1 are loaded as indicated by the lane heading. In each lane, approximately 20 μg of protein were loaded. Immunoblotting was carried out as described in Materials and Methods using an anti-UGT antibody. Molecular mass markers (kDa) were loaded as shown.

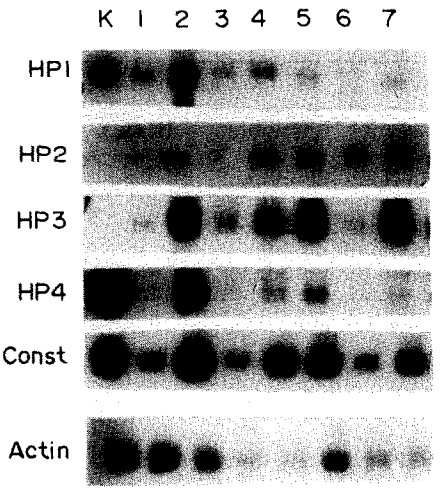

Fig. 2. Northern blot analysis of human liver and kidney RNA hybridized with specific UGT1 DNA probes. RNA from kidney (K) and from liver samples 1–7 was loaded as indicated by the lane heading. (2) Exposed to UW solution; (5) phenytoin-treated; (7) phenobarbital-treated. Each lane contains approximately 15 μg of RNA. Duplicate blots were probed with radiolabelled HP1, HP2, HP3 and HP4 isoform-specific probes as well as a general UGT1 probe (Const = constant region). The size of the UGT mRNAs observed was very similar between 2.3 and 2.7 kb. Northern blots were also hybridized with a probe against β-actin to allow normalization of the loading of RNA samples. The probe used for hybridization is as indicated at the left of each blot.

(Fig. 1). There were at least two bands not detected in the kidney which were present in the liver samples. Liver 2 showed high levels of protein while livers 4 and 6 had low amounts of UGT protein. Overall, blotting of human liver microsomes with this antibody does not provide discrete information about single isoenzymes. Therefore, UGT mRNA levels were assessed to determine relationships with the UGT activities measured.

Northern blotting of human liver RNA samples using specific probes

Isoform-specific probes which hybridized to either HP1, HP2, HP3 or HP4 were used to probe northern blots of human liver RNA samples. A probe which hybridized to the constant region of the HP cDNA family (and therefore recognises all four isoforms) was also used. Each RNA sample was hybridized with each of the five probes at least three times and the results were highly reproducible. To correct for any inaccuracies in the loading of RNA samples, the blots were also hybridized with a probe to β-actin and values normalized against the actin signal. The results of the northern blots with each of the probes described are shown in Fig. 2.

HP1-specific probe. In general the level of HP1 message in the human liver samples was relatively low. Liver 2, however, had a strikingly elevated level of this message. Expression of HP1 message in the kidney sample was at a higher level than in most liver samples, with the exception of liver sample 2.

HP4-specific probe. The levels of mRNA encoding this isoform were relatively low, and again liver sample 2 showed a greatly increased abundance. It was interesting to note that, overall, the patterns of expression of HP1 and HP4 messages appeared very similar in the liver samples tested. However, the HP4 message was most highly expressed in the kidney [greater than 3-fold higher than in most liver samples except liver 2 and correlated with the level of propofol UGT activity measured in the tissue samples ($r^2 = 0.59$) (Fig. 3b)].

HP2 and HP3 specific probes. The HP2 and HP3 mRNAs are considered together because they are known to encode isoforms which glucuronidate the same substrate (bilirubin). HP2 was expressed in human liver at a lower level than the HP3 message which in all of the livers was the more abundant species. However, the expression of both these isoforms was highly variable between individual liver samples. Three of the seven samples had very high levels of HP3 mRNA (2, 5, and 7) with liver 2 having particularly elevated levels. Livers 1 and 6 had a very low abundance of HP3 message compared to the rest of the samples. Liver sample 1 also had a low abundance of the HP2 message. It was notable that liver 2, which had very elevated levels of HP1, HP4 and HP3 messages did not have a particularly high level of HP2 message. HP2 and HP3 mRNAs were not detected in the kidney, as expected, since this tissue does not exhibit bilirubin UGT activity. The activity of bilirubin UGT expressed in the liver showed a good correlation ($r^2 = 0.73$) with the sum of the HP2 and HP3 mRNA levels to confirm the specificity of these analyses (Fig. 3c).

The effect of drug exposure on the expression of UGTs

If we are to predict the effects of administered compounds on the drug-detoxifying pathways in

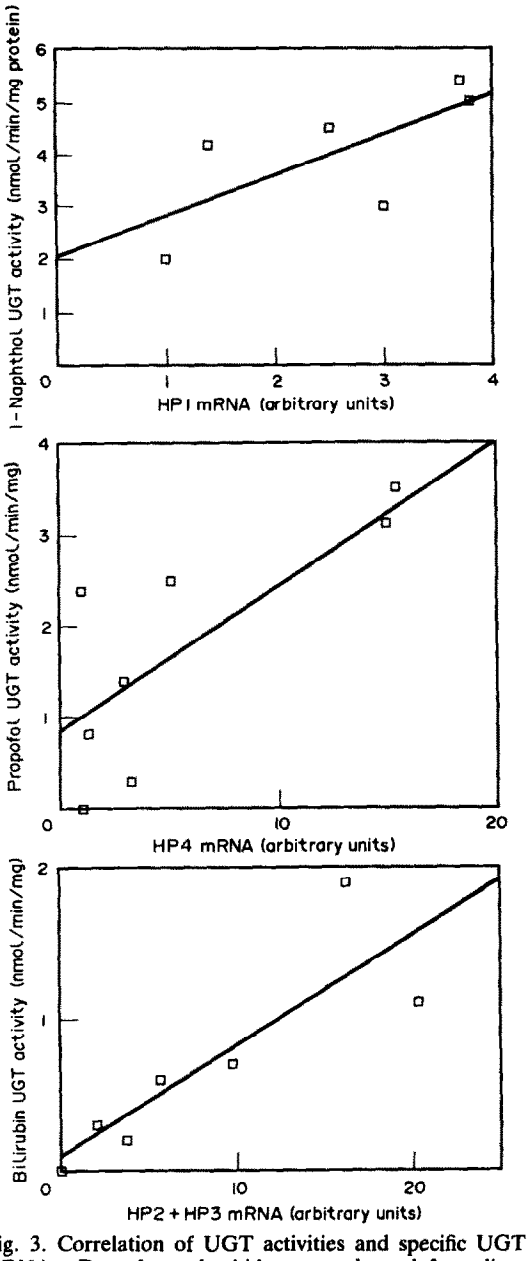

Fig. 3. Correlation of UGT activities and specific UGT mRNAs. Data from the kidney sample and from liver samples 1 and 3–7 are shown. mRNA levels were quantitated using a Molecular Dynamics Phosphorlmager and are expressed in arbitrary units. All values quoted were first normalized against an actin probe to correct for any inaccuracies in loading of RNA samples. The raw data (northern blots) are shown in Fig. 2. The activity data used to construct Fig. 3 are given in Table 2.

man, it is important that we identify and understand the mechanisms underlying the induction of drug-metabolizing enzymes. The results obtained with liver 7, from a patient known to have been treated with phenytoin, were therefore very interesting. The northern blot of this sample shows that there were approximately 3-fold increased levels of both HP2 and HP3 messages present and a corresponding increase in bilirubin UGT activity. UGT activities towards 1-naphthol and androsterone were also increased approximately 2.5-fold. Liver 5, which also showed increased HP3 mRNA levels and increased microsomal bilirubin UGT activity, was from a patient with ornithine transcarbamylase deficiency, under phenobarbital treatment for controlling convulsions due to hyperammonaemia, although the clinical details were not available to us. Interestingly, liver 2, a sample from a liver stored in UW Solution [24] and therefore exposed to dexamethasone, insulin and other drugs, had exceptionally high levels of HP1, HP3 and HP4 mRNAs. However, the large increases in UGT activity expected if all the RNA present was translated into functional protein were not observed. This effect was not observed with liver 3, which was also transplant donor tissue. Overall, these results demonstrate that exposure of human liver to various drugs caused induction of bilirubin UGT activity and mRNA.

DISCUSSION

We have analysed the expression of the UGT1 gene family in human liver and kidney. An important result, which is shown by this analysis, is that there is marked tissue-specific regulation of the members of this gene family in humans. While in most of the human liver samples both the HP1 and HP4 mRNAs are relatively rare, both these isoforms are expressed to a greater degree in the kidney sample. The differential expression is particularly marked for the HP4 mRNA. Recent work has shown that the proteins encoded by these RNAs (HP1 and HP4) are important enzymes involved in the glucuronidation of a large number of xenobiotic compounds [12]. This suggests that the kidney may be an important site for the extra-hepatic glucuronidation of xenobiotic compounds and, indeed, the kidney microsome sample studied showed high activity towards the substrates 1-naphthol, 5,6,7,8-tetrahydro-1-naphthol (data not shown) and propofol. Only one kidney was available for study, and this need not be "typical". However, whilst screening a human kidney cDNA library, using probes to the UGT1 family, we have found these isoforms to be relatively abundant. In contrast to the results obtained with the HP1 and HP4 probes, HP2 and HP3 mRNAs were not detectable in the kidney RNA and the western blot of kidney microsomes showed there to be a reduced number of UGT proteins in this tissue. Bilirubin UGT activity was also not detectable in human kidney, a finding which has also been reported previously [25]. A gene structure for the UGT1 family has been described recently in which the "common" region is encoded by four exons which are downstream of a set of exons, each encoding the variable region from one HP UGT protein [13]. Alternative splicing of one variable exon to the four common exons then produces mature HP mRNAs. It has been suggested that, because of the presence of putative TATA box sequences just upstream of each of the variable exons, a number of different primary transcripts may

be produced. Our data give us some important clues about how the choice of which variable exon is spliced to the common region is regulated. The HP1 variable exon has been mapped upstream of the HP2 and HP3 exons [13]. Although the HP4 exon was not mapped in the study of Ritter *et al.* [13], recent work from this laboratory has shown that it also maps upstream of HP2 and HP3 (D. J. Clarke and B. Burchell, unpublished data). Since there is no evidence to suggest that the common region is transcribed separately from the variable exons and joined by transsplicing of messages, a transcript containing HP1 or HP4 variable sequences must also contain the HP2 and HP3 exons. This means that, in the kidney at least, transcripts containing HP1 and HP4 exons are never, or very rarely, spliced in a way which produces HP2 or HP3 mature messages. If this splicing pattern is seen in the kidney and not in the liver, it would mean that the actual splicing reactions are regulated in a tissue-specific manner. Alternatively, it could be that this splicing pattern is also what is present in the liver and in this tissue it is increased transcription from promoter(s) upstream of HP2 and HP3 variable exons that results in increased levels of these RNAs. This tissue-specific regulation of transcription within the UGT1 gene complex could be the result of either activation of transcription in the liver or repression in the kidney. These are interesting possibilities which we are currently testing using cloned fragments of genomic DNA from upstream of the variable exons in promoter probe vectors in tissue culture.

It is clear from the data presented that the expression of UGT proteins within the liver was extremely variable. Such large differences will presumably have a significant effect on the ability of different individuals to glucuronidate compounds. This must be taken into account when trying to propose rational predictions of how drugs will be detoxified *in vivo*. In this respect, it is interesting to try to make comparisons between the variability of RNA levels and the variability of substrate glucuronidation. Surprisingly, the variations in UGT mRNA and UGT activities correlated reasonably well when substrate probes of high specificity for individual isozymes were used to match the specificity of the cDNA probes (Fig. 3). The correlation between microsomal UGT activity towards 1-naphthol and UGT HP1 mRNA was the poorest ($r^2 = 0.49$) (Fig. 3a) and this result probably reflects the value of 1-naphthol as a probe substrate, since this compound has been shown to be glucuronidated by steroid UGTs other than those listed in Table 1 [26]. The correlation between UGT activity towards propofol and UGT HP4 mRNA showed a better result ($r^2 = 0.59$). Here, the substrate probe specificity was improved (Table 1) [12]. Finally, the correlation between microsomal bilirubin UGT activity and HP2 + HP3 mRNA levels was good ($r^2 = 0.73$), again indicating the specificity of enzymes for this endogenous substrate. Interestingly, liver 2, a sample from a liver stored in UW solution and used in transplantation, did not show the large increases in enzyme activity expected if all the RNA present was translated into functional protein. This may reflect control of UGT activity post-transcriptionally, possibly in an attempt by the cell to limit the amount of UGT activity when exposed to a large induction of RNA levels by dexamethasone and insulin present in UW solution [27]. Liver 3, another donor tissue sample, did not exhibit the extensive changes observed in liver 2. However, in most untreated tissue samples there is still significant variation in the capacity of individual liver microsomes to glucuronidate certain substrates and therefore the variation is important. These results demonstrate the importance of assessing a number of samples, if possible, when working with human drug-detoxifying enzymes. Overall examination of the results revealed a good correlation in the pattern of expression of the UGT mRNAs and enzymological activity.

Treatment of patients with phenytoin, phenobarbital and possibly dexamethasone caused induction of bilirubin UGT activity and mainly HP3 mRNA. This result contrasted with the studies using monkeys, indicating that only HP2 was inducible after exposure to phenobarbital and it has been postulated that the expression of HP3 can be regarded as "high constitutive" [9]. This was thought to fit with the idea that HP3 encoded the major isoform catalysing the glucuronidation of bilirubin and therefore should be expressed at constant levels. Our data show that the expression of HP3 RNA and bilirubin UGT activity is far from constitutive, and that this isoform is inducible by phenytoin (probably as well as by other compounds). Given that drugs other than bilirubin, e.g. ethinylestradiol (T. Ebner and B. Burchell, unpublished work), are important substrates for this enzyme, variations in its expression may have serious implications.

Phenobarbital induction of gene expression has been shown to be mediated transcriptionally [15]. Since any UGT1 primary transcript containing HP2 variable sequences must also contain HP3 sequences, which lie adjacent to the common exons, it is possible that increased transcription from a promoter upstream of HP2 could be the primary effect of phenobarbital induction but that increased levels of HP3 message could also result as a consequence of splicing of HP3 exon to the common exons from this transcript. We are currently addressing this problem by examining cloned genomic DNA for a phenobarbital response element [28]. It has been reported previously that the glucuronidation of 1-naphthol was increased following treatment with phenobarbital [29]. While our results confirm this (liver 7 did indeed show elevated conjugation of this substrate) the northern blotting experiments show no evidence for induction of HP1 mRNA levels in this sample. This suggests that some other isoform, such as a steroid UGT [26] which can also catalyse the glucuronidation of 1-naphthol, might be involved in this response.

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