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ACCELERATED COMMUNICATION

Cloning and Stable Expression of a cDNA Encoding a Rat Liver UDP-Glucuronosyltransferase (UDP-Glucuronosyltransferase 1.1) That Catalyzes the Glucuronidation of Opioids and Bilirubin

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SUMMARY

A chicken anti-rat polyclonal antibody to a purified rat liver UDP-glucuronosyltransferase (UGT) with catalytic activity toward opioid substrates was used to screen a liver cDNA library prepared from phenobarbital-treated Wistar rats. A number of positive clones were obtained, and one of these clones, pM1, was further characterized. Clone pM1 was found to be a full length cDNA coding for a member of the rat *UGT1* gene family. Specifically, pM1 represents the full length homologue of the Gunn rat liver pseudo-gene product UGT1.1P and, therefore, has been designated UGT1.1r. The cDNA insert has an open reading frame of 1605 base pairs, which codes for a protein of 535 amino acids and is flanked by 2 and 632 base pairs of 5′ and 3′ noncoding sequence, respectively. The deduced amino acid sequence of pM1 contains amino acid sequences identical to the amino-terminal and internal peptides of the purified rat liver opioid UGT and to sequences reported for a rat liver bilirubin UGT [*FEBS Lett.* **299**:183–186 (1992)]. Stable expression of UGT1.1r in human embryonic kidney 293 cells showed that a protein with a subunit molecular mass (56 kDa) identical to that of the purified protein was produced. Expressed UGT1.1r protein catalyzed the glucuronidation of buprenorphine and bilirubin at high rates. Other opioids, such as nalorphine and morphine, were also substrates for the expressed UGT1.1r protein. These results show that bilirubin and opioids can be conjugated by the same rat liver UGT.

Glucuronide conjugation is an important metabolic process in which xenobiotics and endobiotics are converted to hydrophilic metabolites through the mediation of UGTs (EC 2.4.1.17). The UGTs are intrinsic membrane proteins localized in the endoplasmic reticulum and nuclear envelope of the cells of liver and other organs and are encoded by multiple genes of at least two gene families (1). The glucuronide conjugates that are formed are readily excreted by renal and hepatic mechanisms, leading to rapid removal of aglycone from the body. At this time, >30 UGT isoforms have been identified either by cloning and expression or by purification from hepatic microsomes. UGTs that are members of the *UGT1* gene complex share common second through fifth exons, with at least seven separate first exons coding for proteins with unique amino-terminal domains (2). In contrast, gene products of the *UGT2* family appear to be transcribed from unique genes (3, 4).

A major interest in our laboratory for many years has been the isolation of UGTs that catalyze the glucuronidation of opioids (e.g., morphine). The first separation of a morphine UGT from hepatic microsomes was by del Villar *et al.* (5), and the first purification of rat liver morphine (pI 7.5) UGT to homogeneity was shown by Puig and Tephly (6). Recently, Ishii *et al.* (7, 8) isolated a morphine UGT from untreated and phenobarbital-treated Wistar rats and showed that it was absent in phenobarbital-treated Gunn rats. Pritchard *et al.* (9) showed that stably expressed, rat liver UGT2B1 catalyzed the glucuronidation of carboxylic acid substrates (e.g., medium-chain saturated fatty acids, profen nonsteroidal anti-inflammatory drugs, and fibrate hypolipidemic agents) and

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ABBREVIATIONS: UGT, UDP-glucuronosyltransferase; HK293 cells, human embryonic kidney 293 cells; UDP-GlcUA, UDP-D-glucuronic acid; IgY, immunoglobulin fraction from chicken egg yolk.

phenolic and alcoholic substrates, including morphine. However, the rat UGT2B1 protein has a different amino-terminal amino acid sequence, compared with morphine UGTs purified in our laboratory and by Ishii *et al.* (8).

The current paper describes the characterization of a full length cDNA (pM1) isolated from a liver cDNA library constructed from rats treated with phenobarbital. An antibody preparation raised in chickens against the purified rat liver morphine (pI 7.5) UGT protein was used to screen the cDNA library. DNA sequence analysis of pM1 showed it to have a deduced amino acid sequence identical to that reported for UGT1.1P (clone A18) (10). In addition, amino acid sequences from proteolytic digestion fragments of purified rat liver morphine (pI 7.5) UGT, as well as from a bilirubin UGT previously reported by Clarke *et al.* (11), were present in the deduced pM1 amino acid sequence. Stable transfection of pcDNA3-M1 constructs into HK293 cells resulted in the expression of a 56-kDa protein product that was found to catalyze glucuronidation of opioids and bilirubin.

Materials and Methods

Chemicals. UDP-[U-^{14}C]GlcUA (319 mCi/mmol) was purchased from ICN Pharmaceuticals (Irvine, CA). Morphine sulfate was acquired from Merck and Co. (Rahway, NJ). Geneticin, buprenorphine hydrochloride, nalorphine hydrochloride, bilirubin, L-α-phosphatidylcholine (type XVI-E from egg yolk), dithiothreitol, and UDP-GlcUA were purchased from Sigma Chemical Co. (St. Louis, MO). Problot membranes for amino acid sequencing were obtained from Perkin Elmer-ABI (Foster City, CA). Protein assay reagents were from Bio-Rad (Richmond, CA).

Animals. Male Wistar rats (175–200 g) were obtained from Charles River Breeding Laboratories (Wilmington, MA) and injected intraperitoneally with sodium phenobarbital (80 mg/kg) once each day for 4 days. The rats were fasted for 24 hr after the last injection of phenobarbital and were then sacrificed and the livers were removed for RNA isolation and for preparation of microsomes. Only livers from rats of the phenotype exhibiting low glucuronidation activity against androsterone (12) were used. Rhode Island Red hens were used for the production of antibodies.

Amino acid sequencing. A UGT displaying activity toward opioids was isolated by chromatofocusing chromatography at pH 7.5, as described by Puig and Tephly (6). The protein was identified at 56 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a Problot membrane. Sequencing was performed with an Applied Biosystems 475 gas sequencer with an on-line 120A phenylthiohydantoin analyzer (Perkin Elmer-ABI), by the Protein Structure Facility, University of Iowa. Sequences of the internal peptides were obtained using the procedure for *in situ* digestion described by Gooderham (13), followed by transfer to Problot membranes.

Antibody production. Approximately 360 μg of the pI 7.5 UGT were purified and concentrated using an Amicon stirred cell with a Y-30 filter (Amicon, Beverly, MA). Antibodies were raised against the UGT preparation in chickens (East Acres Biologicals, Southbridge, MA). Hens received a primary immunization of 50 μg, followed by a booster injection of 30 μg every 3 weeks. After the third booster injection the presence of antibodies against pI 7.5 UGT was detected in the serum by Western blot analysis. Twelve eggs were collected from each hen, and chicken IgY was purified from the egg yolks by the polyethylene glycol precipitation method (14). Approximately 1 g of IgY/12 egg yolks was obtained. Western blot analysis showed that the IgY recognized the purified pI 7.5 UGT and a protein with a similar subunit molecular mass in rat liver microsomes (data not shown).

cDNA library construction. Total hepatic cellular RNA was isolated from phenobarbital-treated rats using the method of Chomczynski and Sacchi (15). A commercially available kit (FastTrack; Invitrogen, San Diego, CA) was used for mRNA isolation. cDNA synthesis was conducted using the ZAP cDNA synthesis kit (Stratagene, La Jolla, CA), and the double-stranded cDNA was ligated into the Uni-ZAP XR phagemid vector. cDNA library constructs were packaged using Gigapack II Gold packaging extract (Stratagene), as suggested by the supplier. Packaging efficiency was approximately 8.4×10^8 plaque-forming units/μg, with 99% of the plaques containing inserts. The cDNA library was not amplified before antibody screening.

Screening of the cDNA library. The cDNA library was plated in XL1-Blue MRF′ cells. Fusion protein expression was induced using isopropylthiogalactoside (2.5 mM), and plaque lifts were made on nitrocellulose filters. Chicken anti-rat pI 7.5 UGT was used to screen the filters (32 μg of IgY/ml at 4° in 10 mM Tris·HCl, pH 7.4, 150 mM NaCl, 0.1%, v/v, Tween-20, 5%, w/v, fat-free milk powder). The second antibody used was alkaline phosphatase-conjugated rabbit anti-chicken IgG, and positive clones were visualized with the nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate color reaction. The positive clones were then plaque purified and Bluescript plasmids were prepared according to the instructions of the manufacturer (Stratagene). Restriction enzyme analysis showed that one clone (pM1) contained a 2.4-kilobase insert. This clone was further characterized.

DNA sequence analysis. DNA sequence analysis was performed by the DNA Core Facility, University of Iowa, using an Applied Biosystems 373A automatic DNA sequencer. The pM1 insert was initially sequenced in the pBluescript plasmid using primers to the T3 and T7 promoters. Oligonucleotides were then synthesized, using a DNA synthesizer (Applied Biosystems model ABI 394), and used as primers to generate additional and overlapping DNA sequences until both strands were sequenced.

Expression of pM1 in HK293 cells. The pM1 insert was removed from pBluescript SK and ligated into the *Bam*HI/*Xho*I site of the mammalian expression vector pcDNA3 (Invitrogen). This expression vector construct is referred to as pcDNA3-M1. The HK293 cells were transfected using the calcium phosphate transfection method (16). Control cells were transfected with pcDNA3 without the insert. Cell culture media and the conditions for cell growth have been described previously (17). Forty-eight hours after transfection, the cells were split and grown in medium containing 700 μg/ml geneticin, to establish stable transfectants. Cells were harvested after 2 weeks for analysis of glucuronidation activity and immunorecognition by the chicken anti-rat pI 7.5 antibody preparation.

Glucuronidation assays. Cells expressing pcDNA3-M1 protein were resuspended in Tris-buffered saline containing 0.5 mM dithiothreitol and were disrupted by freeze-thawing three times in liquid nitrogen, followed by homogenization. Glucuronidation activity toward opioids was assayed using the method described by Puig and Tephly (6). Bilirubin glucuronidation activity was measured using the extraction method of Matern *et al.* (18). Briefly, all reactions contained 50 mM Tris·HCl, pH 7.4, 10 mM magnesium chloride, 8.5 mM saccharolactone, 100 μg/ml phosphatidylcholine, and aglycone and UDP-GlcUA at the concentrations described in Results.

Western blot analysis. Western blots of protein derived from HK293 cells transfected with pcDNA3-M1 and protein derived from HK293 cells transfected with pcDNA3 alone were analyzed using the chicken anti-rat pI 7.5 UGT preparation, as described earlier for cDNA library screening.

Results

Amino acid sequencing of the rat liver pI 7.5 UGT. A UGT displaying glucuronidation activity toward opioids was isolated by chromatofocusing chromatography at pH 7.5, as

described by Puig and Tephly (6). The amino-terminal amino acid sequence of the purified rat liver pI 7.5 UGT was found to be GKLLVIPIDGSHWLSMLG. This amino acid sequence is identical to the deduced amino-terminal amino acid sequence of a truncated cDNA, designated UGT1.1P (clone A18), that has been isolated from a Gunn rat liver cDNA library (10). In addition, the amino-terminal amino acid sequence of our purified protein agrees with that obtained recently by Ishii *et al.* (8) for a purified rat liver morphine UGT. Interestingly, the amino-terminal amino acid sequence for our purified protein and that obtained by Ishii *et al.* (8) are also identical to that obtained by Clarke *et al.* (11) for a bilirubin UGT purified from hepatic microsomes from clofibrate-treated rats. *In situ* digestion of our purified protein by *Staphylococcus aureus* V8 protease gave peptide bands of 29 kDa, 16 kDa, and 12 kDa. Amino acid sequence analysis of these peptides gave results that were identical to internal sequences of UGT1.1P (Fig. 1) and identical to proteolytic peptides reported by Clarke *et al.* (11) for a purified rat liver bilirubin UGT.

Nucleotide and deduced amino acid sequences of pM1. A cDNA library constructed from livers of rats treated with phenobarbital was screened using the antibody preparation raised in chickens against purified rat liver pI 7.5 UGT. Several cDNA clones were identified. One of these clones (pM1) was further characterized and found to be 2266 base pairs in length. This clone is a full length cDNA encoding a UGT with an open reading frame of 1605 base pairs flanked by 2 and 659 base pairs of 5′ and 3′ noncoding sequence, respectively. The nucleotide sequence is available from the GenBank database (accession number U20551).

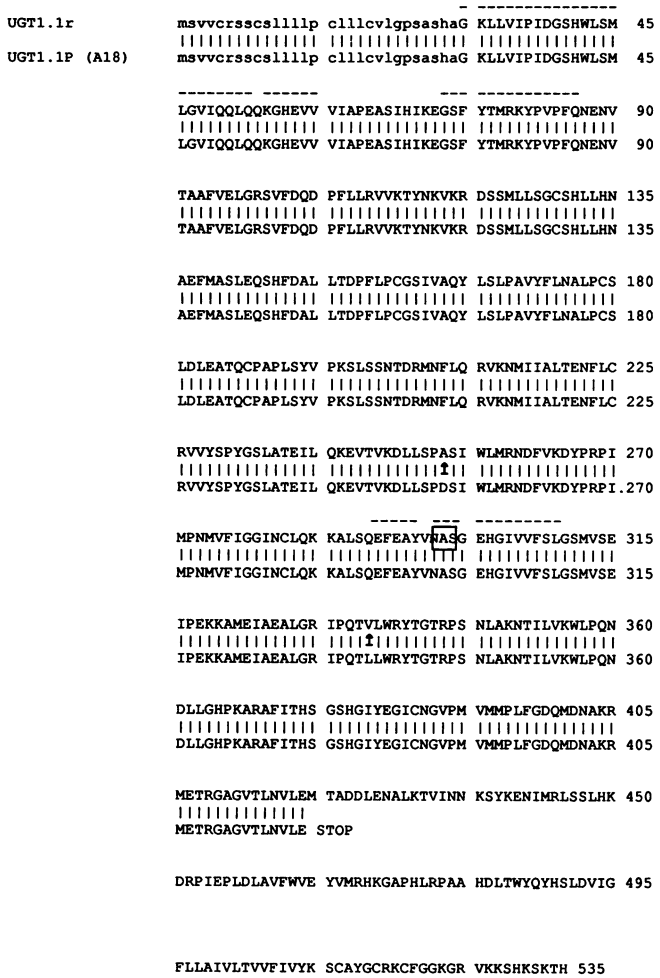

Fig. 1. Alignment of the deduced amino acid sequences of UGT1.1r and UGT1.1P (clone A18) (10). *Lowercase letters*, leader sequences. *Arrows*, amino acid differences between UGT1.1r and UGT1.1P. *Dashed lines above the sequence*, amino acid sequences of the amino terminus and proteolytic peptides for purified pI 7.5 UGT. *Box*, possible glycosylation site.

The predicted 535-amino acid sequence encoded by the pM1 insert is shown in Fig. 1, along with the deduced amino acid sequence for a truncated UGT1.1P (clone A18) isolated from Gunn rat liver (10). Iyanagi *et al.* (19) and Sato *et al.* (20) have shown that a −1 frameshift mutation in the shared exon 4 of the *UGT1* gene complex in Gunn rats results in the loss of 115 amino acids from the carboxyl end of the encoded gene products, due to the generation of a premature stop codon. This mutation is predicted to result in the production of truncated proteins for all members of the *UGT1* gene family. Indeed, Iyanagi (10) isolated three cDNAs (clones A18, A10, and B6) from a Gunn rat liver cDNA library that code for truncated proteins of the *UGT1* family. Up to the premature stop encoded by UGT1.1P, the deduced amino acid sequences of pM1 and UGT1.1P are identical except for two amino acid differences. pM1 codes for alanine at position 253 and valine at position 335, whereas UGT1.1P codes for aspartic acid and leucine, respectively. Based on these data and the generally accepted UGT nomenclature (21), as revised by Lamb *et al.* (22), we have designated pM1 as UGT1.1r.

The 245 amino acid residues at the carboxyl terminus of UGT1.1r are identical to those for UGT1.6r (23) and UGT1.4r (24), with the exception of position 335. These results suggest that UGT1.1r, UGT1.4r, and UGT1.6r share the same gene structure. Comparison of UGT1.1r and UGT1.1h (the major bilirubin UGT in humans) (25) shows that the amino acid sequences are 80% identical. There is 92% identity of amino acid sequences between UGT1.1r and Ugt1.1m (a mouse bilirubin UGT) (26). However, the amino acid sequences of UGT1.1r and UGT2B1 (27) are only 43% identical.

Expression of UGT1.1r in HK293 cells. Homogenates of HK293 cells transfected with pcDNA3 and UGT1.1r were analyzed using immunoblotting procedures with an antibody preparation raised in chickens against the purified rat liver pI 7.5 UGT. These results are shown in Fig. 2. Analysis of cell homogenates from HK293 cells transfected with UGT1.1r shows the production of a protein with a subunit molecular mass of 56 kDa. The molecular mass of this protein is identical to that of the purified rat liver pI 7.5 UGT. No protein with this subunit molecular mass was found in the HK293 cells transfected with pcDNA3 without a cDNA insert.

Table 1 shows that stably expressed UGT1.1r protein catalyzes the glucuronidation of opioids and bilirubin. Of the opioids tested, the glucuronidation rate for buprenorphine was highest and that for morphine was low. Bilirubin glucuronidation rates were also high. The apparent K_m for UDP-GlcUA, using bilirubin (0.35 mM) as substrate, was found to be 0.14 mM (Table 2). Apparent K_m values for buprenorphine and bilirubin were also determined and are shown in Table 2. We observed that concentrations of buprenorphine above 1.0 mM displayed substrate inhibition. It should also be noted that the pH optima for the glucuronidation of opioids in the

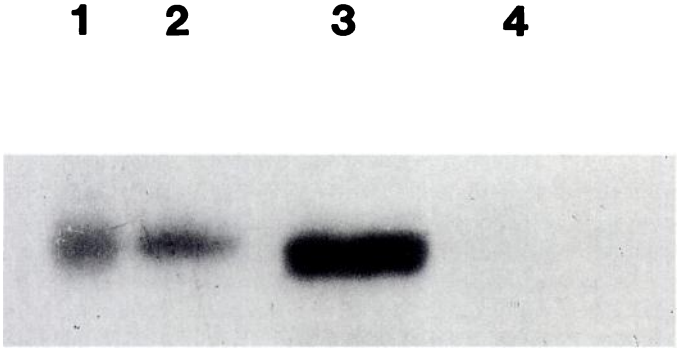

Fig. 2. Immunoblotting analysis of UGT 1.1r protein expressed in HK293 cells. Cell homogenate protein (25 μg) was separated on 10% sodium dodecyl sulfate-polyacrylamide gels together with 0.7 μg of purified pI 7.5 UGT, transferred to nitrocellulose membranes, and probed as described in Materials and Methods. *Lane 1*, glutamate dehydrogenase (56 kDa); *lane 2*, purified rat liver pI 7.5 UGT; *lane 3*, HK293 cells transfected with UGT1.1r; *lane 4*, control nontransfected HK293 cells.

TABLE 1

Glucuronidation activity of expressed UGT1.1r toward opioids and bilirubin

Enzyme assays were performed at pH 7.0 for buprenorphine, naltrexone, and morphine and at pH 7.7 for nalorphine. The glucuronidation reactions (0.1-ml final volume) were performed at 37° for 1 hr, using 1.0 mM aglycone, 2.0 mM UDP-GlcUA, and 200 μg of protein for all substrates, except morphine. Morphine glucuronidation assays were conducted for 2 hr using 0.5 mM UDP-GlcUA at higher radiochemical specific activity, to increase the sensitivity of glucuronide detection. The limit of detection for the morphine assay was 0.2 pmol/min/mg of protein. Bilirubin assays were performed at pH 7.5 using 0.35 mM bilirubin and 2 mM UDP-GlcUA. Values are the mean ± standard deviation of glucuronidation rates obtained from *n* different passages of cultured cells or the individual values obtained from two different passages of cultured cells.

Substrate	Enzymatic activity
	pmol/min/mg
Buprenorphine	114 ± 35 (n = 4)
Nalorphine	3.3, 5.0
Naltrexone	2.4, 2.5
Morphine	0.8, 0.5
Bilirubin	156 ± 13 (n = 4)

TABLE 2

Kinetics of glucuronide formation in homogenates of HK293 cells stably expressing UGT1.1r

The apparent K_m for UDP-GlcUA was determined using bilirubin (0.35 mM) as aglycone. The apparent K_m values for bilirubin and buprenorphine were determined using 2.0 mM UDP-GlcUA. Assays were conducted using the pH optimum for each substrate and using protein concentrations and reaction times yielding linear product formation. Values are the results obtained from two different passages of cultured cells or the mean ± standard deviation of results obtained from three different passages of cultured cells.

Substrate	Apparent K_m	V_{max}
	μM	*pmol/min/mg*
Buprenorphine	58 ± 7	423 ± 90
Bilirubin	16, 52	180, 177
UDP-GlcUA	143, 141	

HK293 cell homogenates varied between pH 7.0 and pH 7.7 (Table 1), pH optima that differ from those usually found for opioids in hepatic microsomes or using purified enzyme (6).

Discussion

In this study, we have isolated and characterized a cDNA (pM1, UGT1.1r), encoding a rat liver UGT, that was obtained from a cDNA library derived from livers of phenobarbital-treated rats. The cDNA was expressed in HK293 cells, which produced a protein, with a subunit molecular mass of 56 kDa, that catalyzed the glucuronidation of opioids and bilirubin. UGT1.1r is the third cDNA that has been isolated and expressed that belongs to the rat *UGT1* gene subfamily. It is the second member of this UGT subfamily to encode a protein that expresses glucuronidation activity toward bilirubin, with the other being UGT1.4r (24). Expressed UGT1.1r protein is the second rat liver UGT that has been shown to catalyze the glucuronidation of opioid substrates, with the other being UGT2B1 (9).

The existence of more than one rat liver UGT that catalyzes the glucuronidation of morphine has been suspected since the early purification studies of del Villar *et al.* (5). That group resolved two peaks of morphine glucuronidation activity by DEAE chromatography of Emulgen 911-solubilized rat liver microsomes. Ishii *et al.* (7, 8) have demonstrated the separation of two peaks of morphine glucuronidation activity using ω-(β-carboxypropionylamino)octyl-Sepharose 4B chromatography. We have recently shown, using a modification of the purification method of Puig and Tephly (6), that it is possible to resolve two proteins with morphine glucuronidation activity by using chromatofocusing chromatography.[1] One of the proteins (pI 8.3) exhibited a subunit molecular mass of 53 kDa and an amino-terminal amino acid sequence corresponding to the deduced amino-terminal amino acid sequence of UGT2B1; the second protein (pI 7.5) corresponds to UGT1.1r, the focus of this report.

The possibility that a rat liver UGT reacts with both opioids and bilirubin has been suspected for some time, based on the similarity of amino acid sequence data for purified morphine UGT and for purified rat bilirubin UGT (11). The amino-terminal and internal peptide amino acid sequences of the purified rat liver bilirubin UGT isolated by Clarke *et al.* (11) and those obtained for the purified rat pI 7.5 UGT are identical. The purified rat liver bilirubin UGT (11) was shown to be absent in Gunn rat liver microsomes. Recently, Ishii *et al.* (8) isolated a morphine UGT with the same amino-terminal amino acid sequence as we found for UGT1.1r and also showed that it was absent in Gunn rat liver microsomes.

In hepatic microsomes from untreated and phenobarbital-treated Gunn rats, Coughtrie *et al.* (28) have shown that morphine glucuronidation activity is only approximately 20% lower than that in liver microsomes from similar groups of Wistar rats. These data suggest that UGT1.1r may not be the predominant enzyme responsible for morphine glucuronidation in rat liver and that another protein (possibly UGT2B1) is the major morphine UGT in rat liver. In contrast, we have found that buprenorphine glucuronidation activity using purified UGT2B1 is lower than buprenorphine UGT activity using purified UGT1.1r, even though the specific activities for morphine in these two preparations were comparable.[2] These data suggest that the substrate reactivity for opioids differs somewhat between UGT2B1 and UGT1.1r. It is also possible that another member of the *UGT1* gene family exists that has greater reactivity for morphine glucuronidation.

[1] B. L. Coffman and T. R. Tephly. Identification and porification of two rat liver microsomal UDP-glucuronosyltransferases which glucuronidate morphine. Presented at the 7th International Glucuronidation Workshop, Pitlochry, Scotland (September 1993).

[2] B. L. Coffmann, M. D. Green and T. R. Tephly, unpublished observations.

Further studies are necessary to clarify the relative contribution of each enzyme in opioid glucuronidation in rat liver.

It is interesting to note that both UGT2B1 and UGT1.1r catalyze the glucuronidation of opioid compounds, despite having very little similarity in amino acid sequence. Lamb *et al.* (22) suggested that the hydrophobicity of the proteins may play a role in substrate specificity and that similarity in hydrophobicity does not necessarily follow similarity in amino acid sequence. Different detergents were used for solubilization and different amounts of phosphatidylcholine were used for stabilization in our studies and those reported by Clarke *et al.* (11). It is possible that the presence of the detergent and/or phospholipids used in the purification process can substantially alter the rates of glucuronidation of certain substrates and can mask the true substrate specificity.

UGT1.1 and UGT1.4, in both rats and humans, catalyze the glucuronidation of bilirubin. In human liver, UGT1.1 is the major bilirubin UGT, both because of its greater abundance (25) and because the expressed protein catalyzes bilirubin glucuronidation at higher rates than does the UGT1.4 protein (29). At the present time it is not known whether a similar situation exists in rat liver. The relative roles of UGT1.1r and UGT1.4r in bilirubin glucuronidation in rat liver remain to be determined.

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