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cDNA CLONING AND EXPRESSION OF TWO NEW MEMBERS OF THE HUMAN LIVER UDP-GLUCURONOSYLTRANSFERASE 2B SUBFAMILY

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SUMMARY: Two new UDP-glucuronosyltransferase cDNAs, designated UGT2B10 and UGT2B11, encoding 528 amino acid proteins were isolated from a human liver cDNA library. The deduced amino acid sequences of UGTs 2B10 and 2B11 share > 76% sequence similarity with other known human liver UGT2B subfamily isoforms and <48% sequence similarity with UGT1 family proteins. COS-7 cells transfected with UGT 2B10 and 2B11 synthesized proteins with respective molecular masses of 49kDa and 51kDa. UGT2B11 expressed in COS-7 cells glucuronidated a number of polyhydroxylated estrogens (estriol, 4-hydroxyestrone and 2-hydroxyestriol) and xenobiotics (4-methylumbelliferone, 1-naphthol, 4-nitrophenol, 2-aminophenol, 4-hydroxybiphenyl and menthol). Despite the screening of more than forty potential substrates, glucuronidation activity was not observed for expressed UGT2B10.

Conjugation with glucuronic acid is an important metabolic pathway for drugs (and their phase I metabolites), environmental chemicals, and a range of endogenous compounds, including bile acids, bilirubin and steroid hormones. Glucuronidation reactions are catalysed by the enzyme UDP-glucuronosyltransferase (UGT). It is now apparent that the versatility of the glucuronidation process is due to the fact that UGT exists as a gene superfamily, with the individual UGT isoforms tending to exhibit distinct but overlapping substrate specifities (1-4). The total number of UGT gene products is unknown, but at least twelve rat UGT cDNAs have been isolated to date (2-5). Microsomal kinetic and inhibitor studies, enzyme purification and the cloning of UGT cDNAs have similarly demonstrated UGT multiplicity in humans (6-16).

Following comparison of known cDNA sequences, UGT genes have been separated into two families (designated UGT1 and UGT2) on the basis of evolutionary

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divergence (5). Human UGTs classified in gene family 1 include UGT1*1 (9), UGT1*4 (9), UGT 1*02 (10) and UGT1*6(11). UGT1*1 and 1*4 both glucuronidate bilirubin whereas UGT1*02 and 1*6 apparently both preferentially conjugate xenobiotic phenols. Human UGT gene family 2 currently comprises three members, all of which have been classified in the B subfamily; UGT2B4 (12, 13), UGT2B7 (13-15) and UGT2B8 (16). The human 2B subfamily UGTs appear to preferentially glucuronidate hydroxy-metabolites of steroid hormones and/or bile acids. However, UGT2B7 has also been shown to conjugate a range of drugs possessing an aliphatic carboxylic acid function (15) while UGT2B8 additionally glucuronidates certain xenobiotic phenols and 1-naphthylamine (16).

In this communication we report the cDNA cloning and expression of two human hepatic UGTs. Both of the newly characterised enzymes belong to the UGT2B subfamily and have been designated UGT2B10 and UGT2B11(5).

MATERIALS AND METHODS

Chemicals: The sources of all chemicals and reagents, including the UGT substrates listed in Results, are given in reference 15.

Isolation of UGT cDNAs: A cDNA library in the EcoRI site of lambda gt11, prepared from the mRNA of a single human liver (15), was screened by plaque hybridization with a rat liver cDNA, UGT2B1 (17), as probe. Briefly, the probe was labelled by the random primed labelling technique using ^{35}S-dATP and hybridizations were performed in 1.5 x SSPE, 1% SDS and 0.5% skim milk powder at 65°C. The inserts of positive plaques were digested with EcoRI and the size of their cDNA inserts determined by electrophoresis in 1.5% agarose gels. cDNAs greater than 1.6kb were partially sequenced and compared to the sequences of previously isolated human liver UGT cDNAs to identify those with full coding regions. Two of the cDNAs, subsequently designated UGT2B10 and UGT2B11, were chosen for further analysis.

DNA sequencing: UGTs 2B10 and 2B11 were amplified by the polymerase chain reaction (PCR) using the oligonucleotide primers 5'-GGCTCTAGAGACTAGTCC-TGGAGCCCGT-3' and 5'-GACTCTAGACCAACTAGTAATGGT-3'. The primers were complementary to the 5' and 3' regions of lambda gt11 that flanked the cDNA insert and additionally contained SpeI recognition sites. Inclusion of the SpeI recognition sites was necessary since each of the cDNAs contained internal EcoRI sites. The UGT 2B10 and 2B11 cDNAs were subcloned into the SpeI site of the Bluescript II SK+ plasmid (Stratagene, La Jolla, CA) for sequencing. Sequences were assembled from nested deletions generated by the Erase-a-Base system (Promega, Madison, WI) and the dideoxy protocol, as previously described (18). Nucleotide sequences were determined fully for both DNA strands. One region of UGT2B10 and three regions of UGT2B11 sequences along the reverse strand could not be obtained from the nested deletions. Sequences of these regions of the reverse strand were therefore determined using synthetic oligonucleotides as primers.

cDNA expression in COS-7 cells: The EcoRI site of the mammalian expression vector p91023(B) was modified by the addition of linkers to create an SpeI site. PCR

amplified UGT 2B10 and 2B11 were digested with SpeI and subcloned into the modified vector in the correct and reverse orientations with respect to the adenoviral transcription signals. Vectors containing inserts were transfected into COS-7 cells using DEAE-dextran and subsequent chloroquine treatment (18). Cells transfected with plasmid containing cDNA inserts in the reverse orientation were used as negative controls. Transfected cells were harvested after 48 hr. Nascent UGT in an aliquot of these cells was labelled with ^{35}S-L-methionine and analyzed on SDS-PAGE gels as previously described (15,20). The remaining cells were homogenised in 50mMTris-HCl (pH 7.4) and stored at -70°C until used in the substrate specificity studies.

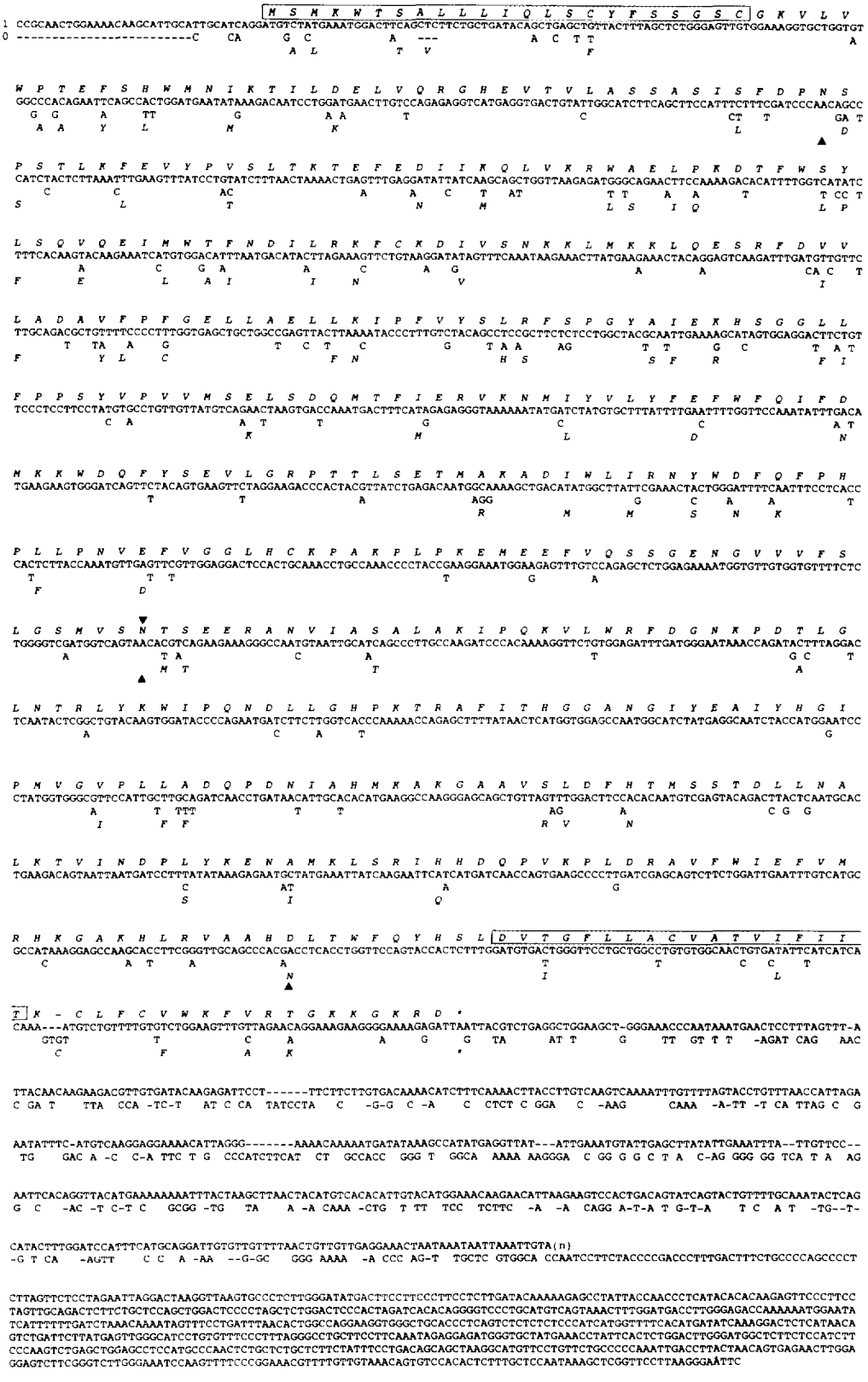
Enzyme activities:
The glucuronidating activity of human liver microsomes and transfected or mock transfected COS cells towards all substrates except imipramine was assayed using a modification (15) of the radiometric thin-layer chromatographic assay of Bansal and Gessner (21). The presence of glucuronides was confirmed by treatment with β-glucuronidase (1000 units, pH 4.6). After autoradiography, the glucuronide-containing regions of the plates were removed and radioactivity was quantitated by liquid scintillation counting. The imipramine glucuronidation activity of human liver microsomes and COS cells was assessed using the method of Coughtrie and Sharp (22).

RESULTS

Nucleotide and deduced amino acid sequences of UGTs 2B10 and 2B11: Nucleotide sequences for the UGT 2B10 and 2B11 cDNAs are shown in Fig.1. The open reading frame, from the first ATG triplet to the TAG stop codon, is 1584 nucleotides for both cDNAs. The UGT2B10 open reading frame is flanked by 10- and 1205-base pairs (bp) of 5'- and 3'- non-coding sequence, while the UGT2B11 open reading frame is flanked by 35- and 472-bp of 5' and 3'-non-coding sequence. A poly (A) addition consensus sequence occurs 13bp upstream from the poly (A) tract of UGT2B11. Although the UGT2B10 cDNA does not contain a poly (A) tract, a potential poly (A) addition signal occurs 22bp from the 3'- terminus.

The UGT 2B10 and 2B11 cDNAs encode predicted UGT proteins of 528 amino acids (Fig. 1). Both deduced amino acid sequences contain a characteristic hydrophobic membrane-insertion signal peptide at the amino terminus and a typical carboxyl terminus membrane-spanning hydrophobic region. There are potential

Figure 1. Nucleotide and deduced amino acid sequences of UGT 2B10 and 2B11 cDNAs.
The complete nucleotide sequence of UGT2B11 cDNA is given and its deduced amino acid sequence shown above in italics. The nucleotides and deduced amino acids of the UGT2B10 cDNA which differ from those of the UGT2B11 cDNA are shown below the UGT2B11 sequence data. Nucleotide and amino acid sequences (in italics) are numbered from their first residues. Predicted N-glycosylation sites and poly(A) addition signals are denoted by solid triangles and underlined regions, respectively. The probable signal peptide and transmembrane regions are boxed.



asparagine-linked (Asn-X-Ser/Thr) glycosylation sites at residues 66, 314 and 481 of UGT2B10 and at residue 315 of UGT2B11. The deduced amino acid sequences of UGT2B10 and UGT2B11 share > 76% sequence similarity with other known human liver UGT2B subfamily proteins and <48% sequence similarity with human liver UGT1 family proteins.

Expression of the UGT 2B10 and 2B11 cDNAs in transfected COS-7 cells: The synthesis of UGT protein by COS-7 cells transfected with UGT 2B10 or 2B11 cDNA was confirmed by immunoadsorption with a UGT-specific polyclonal antibody (24). The SDS-PAGE determined molecular masses of the predominant proteins resulting from UGT 2B10 and 2B11 expression were 49 and 51 kDa, respectively (Fig 2.). The origin of the additional less abundant polypeptides is unknown, although it is possible that the lower molecular weight bands represent unglycosylated protein.

Catalytic activity: Nine of the forty three xenobiotics and steroids screened for activity were glucuronidated by cDNA-expressed UGT2B11 (Fig 3). In order of activity, these were 4-methylumbelliferone, 4-nitrophenol, 1-naphthol, 4-hydroxyesterone, 4-

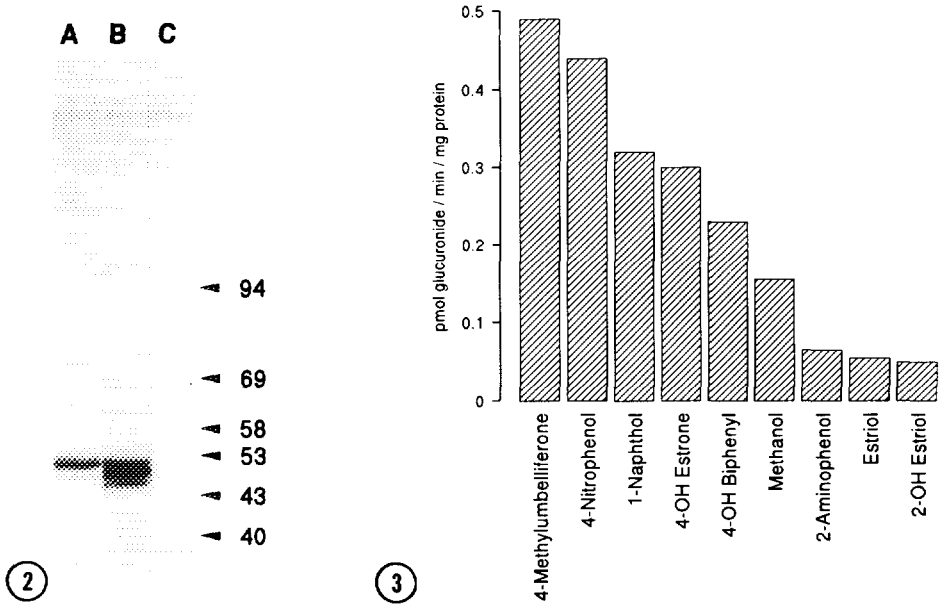


Figure 2. Expression of UGT 2B10 and 2B11 by COS-7 cells. Expressed proteins were labelled 48hr post-transfection with ^{35}S-methionine for 1 hr. After solubization, the cell lysate was immunoadsorbed with anti-mouse UGT IgG and analysed by SDS-PAGE and autoradiography. Lane A, UGT 2B11; Lane B, UGT 2B10; Lane C, COS-7 cells transfected with UGT2B11 cDNA in the reverse orientation with respect to the adenoviral transcription signal.

Figure 3. Glucuronidation of steroids and xenobiotics by expressed UGT 2B11. *Results represent the mean of two determinations with the same batch of transfected* cells.

hydroxybiphenyl, menthol, 2-aminophenol, estriol and 2-hydroxyestriol. Although glucuronidated by human liver microsomes under the assay conditions employed, the following compounds were not metabolised by expressed UGT2B11; androsterone, R,S-benoxaprofen, chloramphenicol, clofibric acid, diflunisal, digitoxigenin monodigitoxoside, ethinylestradiol, R,S-fenoprofen, harmol, hyodeoxycholic acid, R-, S- and R,S-ibuprofen, imipramine, indomethacin, R- and S- and R,S- ketoprofen, lorazepam, mexiletine, morphine, R- and S-naproxen, oxazepam, paracetamol, phenolphthalein, propranolol, salicylamide, salicylic acid, temazepam, testosterone, R,S-tiaprofenic acid, valproic acid and zomepirac. Expressed UGT2B10 exhibited no activity towards any of the substrates tested with UGT2B11.

DISCUSSION

It has been demonstrated here that UGT2B11 has the capacity to glucuronidate estriol and the catechol estrogens 4-hydroxyestrone and 2-hydroxyestriol, but lacks activity towards hyodeoxycholic acid. All previously characterised human UGT2B isoforms (viz. 2B4, 2B7 and 2B8) have been shown to catalyse the glucuronidation of estriol (13-16). Moreover, UGT2B4 and UGT2B7 both glucuronidate 4-hydroxyestrone and hyodeoxycholic acid (12-15), while UGT2B7 additionally conjugates other catechol estrogens (including 2-hydroxyestriol) (14,15). Thus, at least with respect to estrogen derivates, UGT2B11 substrate specificity parallels that of UGT2B7. Rates of conjugation of these compounds were, however, 30- to 90-fold lower for UGT2B11 than those reported for UGT2B7 (15).

Apart from metabolising hydroxysteroids, it has been demonstrated that UGTs 2B7 and 2B8 may also glucuronidate xenobiotics. In particular, UGT2B7 glucuronidates a range of drugs containing an aliphatic carboxylic acid function and a limited number of xenobiotics containing an aliphatic alcohol or phenolic group (15). UGT2B8 is additionally known to have the capacity to conjugate the phenols 4-methylumbelliferone and 4-nitrophenol (16). Like these two isoforms, UGT2B11 metabolised a limited number of xenobiotic phenols including 2-aminophenol, 4-methylumbelliferone, 1-naphthol, 4-hydroxybiphenyl and 4-nitrophenol. Again, however, rates of conjugation were low, both in comparison to UGT2B7 and the phenol-glucuronidating forms of the UGT1 family (10, 11, 14, 15). The only non-phenolic xenobiotic glucuronidated by UGT2B11 was the alicylic alcohol menthol.

From a comparison of the substrate profiles of UGT2B4, UGT2B7, UGT2B8 and UGT2B11 it is apparent that there is a degree of overlap in substrate specificity. In particular, all have the capacity to glucuronidate estriol, and probably certain

catechol estrogens and xenobiotic phenols. As indicated earlier, however, there are marked inter-enzyme differences in catalytic activity towards these and other substrates. Xenobiotic carboxylic acids, for example, appear to be glucuronidated only by UGT 2B7 (15). Of additional interest is the fact that marked differences in substrate specificity may occur for closely related members of the UGT2B subfamily. Hyodeoxycholic acid is among the substrates most efficiently glucuronidated by UGT2B4 but it is not metabolised by UGT2B11, despite the fact that these isoforms share almost 96% sequence similarity.

Given the relatively low activity of UGT2B11, particularly in comparison to UGT2B7, it is likely that the precise physiological role of this enzyme relates to the metabolism of yet to be identified compounds. Similarly, the lack of activity of UGT2B10 towards the more than forty compounds screened means the functional role of this isoform similarly remains unknown. It is conceivable that the UGT2B10 cDNA isolated here could contain a mutation in a region critical for catalytic activity. However, partial sequence analysis of PCR products encoding UGT2B10 in three separate human livers has not provided evidence in support of this.

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