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Cloning and Expression of Human Liver UDP-Glucuronosyltransferase cDNA, UDPGTh2

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The human liver cDNA clone UDPGTh2, encoding a liver UDP-glucuronosyltransferase (UDPGT) was isolated from a λ gt 11 cDNA library by hybridization to mouse transferase cDNA clone, UDPGTm1. UDPGTh2 encoded a 529 amino acid protein with an amino terminus membrane-insertion signal peptide and a carboxyl terminus membrane-spanning region. There were three potential asparagine-linked glycosylation sites at residues 67, 68, and 315. In order to obtain UDPGTh2 protein encoded from cloned human liver UDP-glucuronosyltransferase cDNA, the clone was inserted into the pSVL vector (pUDPGTh2) and expressed in COS 1 cells. The presence of a transferase with Mr~52,000 in transfected cells cultured in the presence of [^{35}S]methionine was shown by immunocomplexed products with goat antimouse transferase IgG and protein A-Sepharose and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. The expressed UDPGT was a glycoprotein as indicated by electrophoretic mobility shift in Mr~3,000-4,000 when expressed in the presence of tunicamycin. The extent of glycosylation was difficult to assess, although one could assume that glycosyl structures incorporated at the level of endoplasmic reticulum were always the core oligosaccharides. Thus, it is likely that at least two moieties inserted can account for the shift of Mr~3,000-4,000. This study demonstrates the cDNA and deduced amino acid sequence of human liver UDP-glucuronosyltransferase cDNA, UDPGTh2.

Key words : UDP-Glucuronosyltransferase cDNA, UDPGTh2, Tunicamycin

INTRODUCTION

Enzymatic conjugation with glucuronic acid was of major importance in the detoxification and elimination of thousands of lipophilic compounds, including carcinogens, environmental toxins, foodstuffs, and therapeutic drugs. The UDP-glucuronosyltransferase (UDPGT) enzyme system transforms many lipophilic compounds to more water-soluble products via conjugation with glucuronic acid. This conversion was responsible for enhancing the excretion of endogenous aglycons, such as steroids, bilirubin, thyroid hormones, and biogenic amines (Dutton, 1980). An aglycone must, however, contain or acquire through metabolism an appropriate substituent moiety, typically an hydroxyl or a carboxyl group, which would undergo covalent linkage to glucuronic acid.

Developmental, induction, and purification studies indicate that a family of UDPGTs with complex regulation is responsible for the glucuronidation of a wide variety of lipophilic compounds. Both developmental (Wishart, 1978) and preferential induction profiles (Bock *et al.*, 1978; Whishart, 1978) of activities provided evidence that certain substrates can be categorized into the same group based on similarities in the regulation of their glucuronidating activities. However, it has not been practically possible to exactly determine from biochemical studies how many different forms were involved in glucuronidating a particular group of substrates. Studies with purified transferases often revealed broad substrate specificities (Bock *et al.*, 1982; Mackinzie *et al.*, 1984) for chemically different aglycones and in certain cases, particular forms revealed a rather narrow substrate specificity for one or more chemically related compounds (Matern *et al.*, 1982; Kirkpatrick *et al.*, 1982; Yacoub and Tephley, 1987). Reconstitution of activity with purified forms could be influenced, however, by the detergent which remained bound to the isolated protein. Furthermore, it might be impossible to resolve by purification procedures very closely related transferase proteins. Technical difficulties associated with isolating and

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characterizing different isoforms of transferase by traditional biochemical procedures made cloning of the UDPGTs an attractive approach to determine the number of different forms, as well as to establish their regulation and expression of substrate activity. We isolated, characterized and sequenced a full-length transferase cDNA clone which encoded human liver transferase using mouse transferase cDNA UDPGTm1 as a probe.

MATERIALS AND METHODS

Materials

UDP-glucuronic acid and all aglycones tested for substrate activity were purchased from Sigma Chemical Co. (St. Louis, MO, USA). [^{14}C]UDP-glucuronic acid and [^{35}S]methionine and [^{32}P]deoxycytidine triphosphate were from Amersham (Arlington Heights, IL, USA). Restriction enzymes and other reagents used in molecular biology techniques were obtained from Bethesda Research Laboratories (Gaithersbug, MD, USA), or Boehringer Mannheim (Mannheim, Germany). The pSVL vector and the oligo-labeling kit were supplied from Pharmacia Fine Chemicals, (Uppsala, Sweden) and COS-1 cells were from the American Type Culture Collection (Rockville, MD, USA). The Bluescript plasmids and XL-1 Blue cells were product of Stratagene (La Jolla, CA, USA). The Erase-A-Base kit was purchased from Promega (Madison, WI, USA), tunicamycin was from Boehringer Mannheim (Mannheim, Germany), and Lipofectin™ was from Bethesda Research Laboratories (Gaithersbug, MD, USA). Dulbecco's modified Eagle's medium, fetal calf serum, and other cell culture reagents were obtained from GIBCO (Gaithersbug, MD, USA).

Nucleotide sequence determination of UDPGTh2

The human liver clone, UDPGTh2, which encodes a UDP-glucuronosyltransterase was isolated from a human liver λgt11 cDNA library based on its hybridization to the mouse transferase clone ^{32}P-UDPGTm1 labelled by the oligo-primed techinque using a kit, α-^{32}P-deoxycytidine triphosphate. The full-length insert was isolated and ligated into *EcoR*1-digested pSKM13$^+$ following the partial digestion of the λgt11 cDNA clone with *EcoR*1. The ligation mixture was used to transform XL-1 Blue cells. From a single colony, the plasmid was purified. A series of clones with progressively overlapping deletions was constructed by utilizing the Erase-A-Base kit and the supplier's protocol. Clones having appropriately spaced deletions were purified. Direct plasmid dideoxy sequencing was carried out as described in Mackenze *et al.* (1984). Both strands of the cDNA were completely sequenced.

Northern blot analysis of human liver mRNA with ^{32}P-Labelled-UDPGTh2

Human liver mRNA was isolated from normal biopsy material and affinity purified by chromatography through oligo(dT)-cellulose with an intermediate heat step. Poly (A$^+$) RNA (4 μg) was electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde and transferred to Zetabind™ membrane (AMF-CUNO, Meriden, CT, USA) using the manufacturer's instructions. The Northern blot was hybridized to the oligo-primed ^{32}P-labelled *EcoR*1-fragment from the most 3'end of UDPGTh2, according to Church and Gilbert (1984).

Expression of UDPGTh2 in COS1 cells

The cDNA from the pSKM13$^+$-subclone was digested with *Sma* I and *Eco* RV and then ligated into the *Sma* I-digested pSVL expression vector. After appropriate subclones were characterized for the correct or reverse orientation of the cDNA with respect to the promoter element in pSVL, plasmid preparations were purified by cesium chloride banding. The expression unit was designated pUDPGTh2. Mock-transfected cells were carried out with the cDNA inserted in the reverse direction with respect to the promoter element of pSVL. COS1 cells (80% confluent) were plated at 10^6/60×15 mm dish in Dulbecco's modified Eagle's medium with 10% fetal calf serum and grown overnight. The purified plasmid was then combined with the carrier, Lipofectin™, and added to the cell culture dishes according to the manufacturer's instructions. At the end of the 5-hour transfection period which was blocked by the addition of 10% fetal calf serum, the cells were allowed to incubate from 48 to 96 hours as indicated. Cultures received no further treatment and were harvested and stored at -70°C until assayed.

Assay for the expression and glycosylation of the UDP-glucuronosyltransferase encoded by UDPGTh2 in COS1 cells

At the end of the transfection period, cells either received no further treatment or were treated with tunicamycin (1.0 μg/ml) for various incubation periods indicated. During the final 5 hours of culture, the cells were incubated for 1 hour in methionine-free medium containing [^{35}S]methionine (100 μCi/ml) in the absence or presence of tunicamycin. The labeling period was blocked by adding excess methionine for 5 minutes. After washing the surface of the culture with phosphate-buffered saline, the cells were solubilized with 1 ml of 0.2 M potassium phosphate, pH 7.5, 0.15 M KCl, 2% sodium cholate, and 0.5% zwittergent. Insoluble materials were pelleted by centrifugation, and the solubilized cellular content was incubated with either goat control IgG or anti-mouse transferase IgG for 1

hour on ice according to the procedure of Mackenzie *et al.* (1984). An excess of protein A-Sepharose was added to the complexes and allowed to mix for at least 2 hours in the cold. The protein A-Sepharose immunocomplexes were then washed with the solubilizing buffer until all unincorporated radioactivity was removed. Control cultures (mock-transfected) and control IgG reactions were carried out as indicated in the legend. Both control and anti-mouse transferase IgG were used to immunocomplex solubilized, tritiated microsomes from untreated C57BL/6N mice. The immunocomplexes were detached and run on a single 7.5% sodium dodecyl sulfate-polyacrylamide gel and processed for autoradiography by using Autofluor according to the supplier's instructions (National Diagnostics, Manville, NJ, USA).

RESULTS AND DISCUSSION

Isolation of the human liver clone, UDPGTh2

The cDNA clone hybridized at high stringency to the mouse liver clone, UDPGTm1 (Kimura and Owens, 1987), upon screening a human liver λ gt11 cDNA library. The cDNA was subcloned and sequenced using a series of progressively overlapping deleted clones as described under Materials and Methods. Fig. 1 shows that the insert contains 1869 base pairs with an open reading frame of 1590 base flanked by 14 and 315 bases at the 5'- and 3'- untranslated regions, respectively. The clone encoded a transferase protein with 529 amino acids with the translation initiation codon indicated by nucleotide position 1. The deduc-

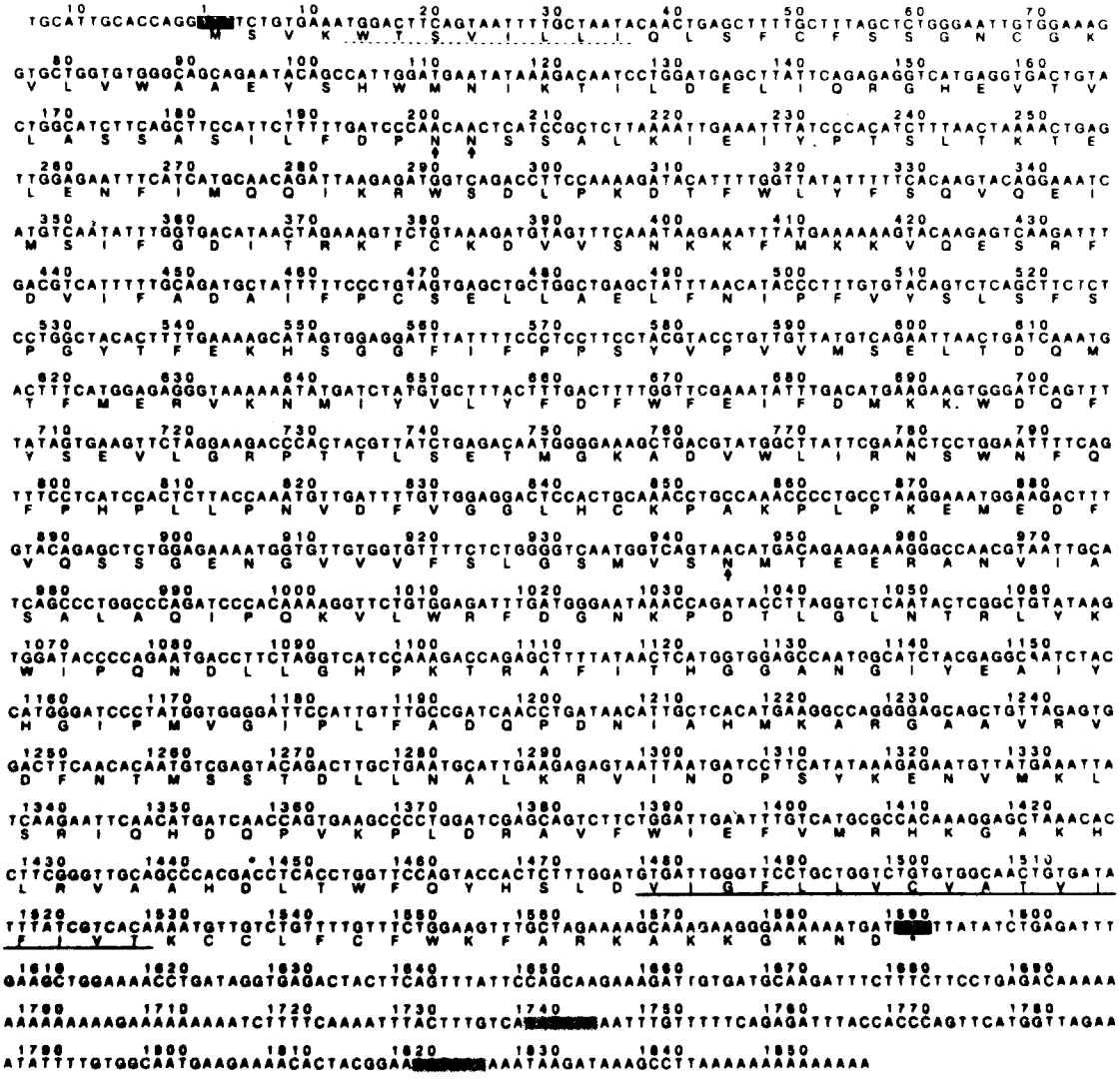

Fig. 1. Nucleotide sequence data for complete coding UDPGTh2 with deduced amino acid sequence data. The nucleotide sequence was determined according to the dideoxy method using double-stranded plasmid DNA and drawn using the PC-DNA draw program developed by Marvin Sapiro (Division of Computer Research and Technology, National Institute of Health, USA). The signal peptide sequence (residues 5-12) and membrane spanning region (residues 493-509) were underlined. The arrow (67, 68 and 315) represents consensus sequences for potential asparagine-linked glycosylation sites.

ed amino acid sequence contained a characteristic hydrophobic signal peptide from amino acids 5 through 12 for directing the protein into the endoplasmic reticulum (Burchell and Blakchaert, 1984). This sequence contained a positively charged lysine at position 4 and terminated with a possible cleavage site at the cysteine residue in position 17. The typical transmembrane hydrophobic region was located from amino acid residues 493 to 509 which was followed by a positively charged lysin (Rose *et al.*, 1980). The translation stop codon, TAG, was located after residue 529. Two consensus sequences for polyadenylation were presented in the cDNA at nucleotide 1738 and 1818 with a short segment of poly(A)$^+$ starting at nucleotide 1842. Nucleotide alignment of UDPGTh2 with other transferase clones showed overall identities: 74% to UDPGTm1 (Kimura and Owens, 1987), <40% to the human phenolic transferase clone (Harding *et al.*, 1988), 86% to HLUG25 (Jackson *et al.*, 1987), and an allelic variant of HLUG25, UDPGTh1. Fig. 2 illustrates the result of Northern blot analysis in which a 2200-base mRNA hybridized to a [^{32}P]-labeled *EcoR*I fragment from the most 3'-end of UDPGTh2. Under the same conditions used (Church and Gilbert, 1984), the full length clone cross-hybridized to a 2300-base mRNA.

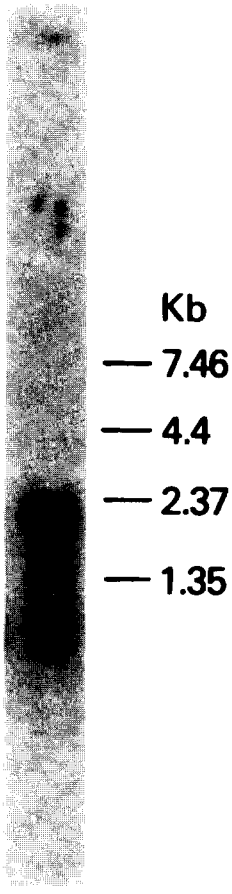

Fig. 2. Northern blot hybridization analysis of UDPGTh2. Hybridization of the ^{32}P-labeled *Eco* RI fragment (from the most 3'-end of UDPGTh2) to poly (A$^+$) (4 μg) isolated from human liver biopsy was carried out as described under Material and Methods. RNA molecular weight markers were hybridized to oligoprimed ^{32}P-labeled λ phage DNA.

Immunocomplexes of [^{32}S]methionine-labeled UDP-glucuronosyltransferase in transfected COS1 cells

In order to determine whether transferase was synthesized following transfection of COS1 cells with the expression unit, pUDPGTh2, [^{35}S]methionine-labeled and solubilized cells were immunocomplexed with goat control IgG or goat anti-mouse transferase IgG.

Transfected cells incubated for 48, 72 and 96 hours and then immunocomplexed with anti-transferase IgG showed a prominent protein with a relative molecular mass ~52,000 (Fig. 3, lanes 7, 9 and 11, respectively). With increasing incubation time these cells showed increased amounts of a second protein with a molecular mass ~49,000. The appearance of the 49,000-dalton protein with a parallel decrease in the 52,000-dalton protein between 72 and 96 hours represents, most likely, the natural turnover of the transferase in COS 1 cells. Control studies were carried out by using preimmune IgG with both 72 hours transfected cells (Fig. 3, Lane 6) and mocked transfected cells (Fig. 3, lane 3) and by exposing anti-transferase IgG to mock transfected cells (Fig. 3, lane 4). There was a trace amount of a ~51,000-dalton radioactive immunocomplexed protein with immune serum from the mock transfected cells (Fig. 3, lane 4). This result which suggested a low level of endogenous transferase protein could only be seen with exposures of cells for more than 24 hours. In addition, there appeared to be a low level of the 52,000-dalton protein with preimmune serum using transfected cells (Fig. 3, lane 6). The lack of corresponding band with preimmune serum in mock transfected cells even after longer exposure (Fig. 3, lane 3) suggested nonspecific trapping of the highly radiolabeled transferase in transfected cells. Furthermore, we considered the possibility that the expressed protein was glycosylated via the potential asparagine-linked glycosylation sites in the deduced amino acid sequence. The treatment with tunicamycin of transfected cells for 48, 72, 96 hours (Fig. 3, lane 8, 10 and 12, respectively) caused a major shift in the molecular mass of the transferase protein to about 48,000 daltons. The blockage of glycosylation by tunicamycin appeared to be incomplete as a prominent protein remained at the 52,000 dalton position. The result of a control experiment for any endogenous glycosylated protein affected by tunicamycin which reacted with anti-transferase IgG was shown in Figure 3 (lane 5). The cells were mock transfected for 96 hours. The position of the trace of

protein (Fig. 3, lane 4) which reacted with the anti-transferase IgG was not significantly affected by tunicamycin after 96 hours (Fig. 3, lane 5). Microsomes from untreated mice and then tritiated were also exposed to control IgG (Fig. 3, lane 2). The antibody reacted as previously (Mackenzie, 1984) with protein Mr~51,000~52,000. These results showed synthesis and glycosylation of a human UDPGT protein (Mr~52,000) in the COS1 cells with the peak in the specific protein level between 48 and 72 hours. The presence of three consensus sequences for asparagine-linked glycosyl moieties in the deduced amino acid sequence was consistent with the tunicamycin effect on this protein. The extent of glycosylation (number of sites occupied) was difficult to assess. If one assums that the glycosyl structures incorporated at the level of the endoplasmic reticulum would always be the core oligosaccharides (Kornfeld and Kornfeld, 1985), we could conclude that at least two moieties were inserted to account for the shift of the protein with Mr~3,000~4,000. It was reported for an expressed cDNA transferase which had one consensus sequence for glycosylation that the shift in relative molecular mass was ~2,000 (Fournel-Gigleux *et al.*, 1989). In summary, we present the deduced amino acid sequence of a human UDP-glucuronosyltransferase cDNA, UDPGTh 2, which was expressed in COS 1 cells.

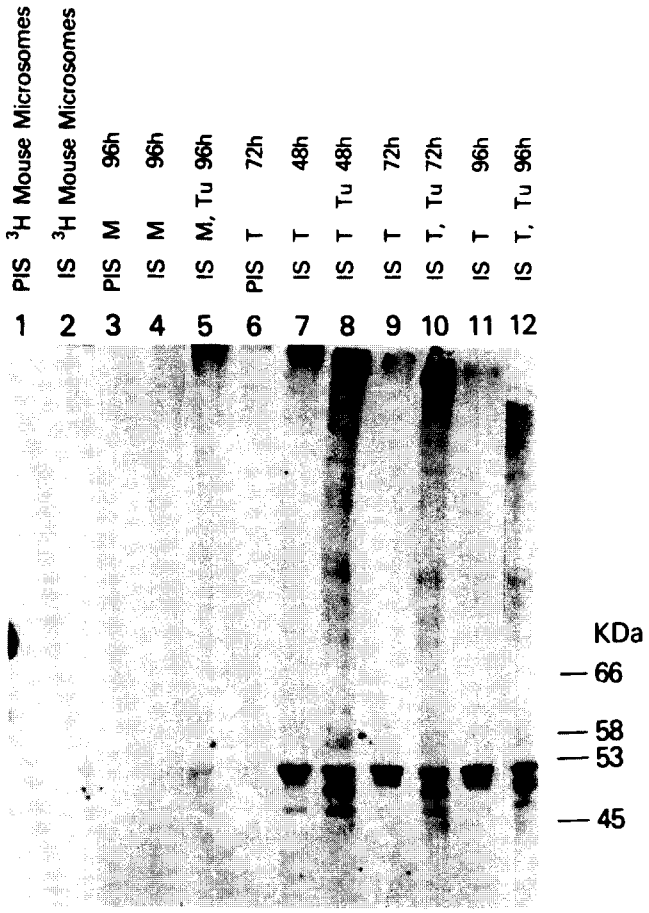

Fig. 3. Immunocomplexes of pUDPGTh2 transfected COS 1 cells following [^{35}S]methionine labeling. Cells were transfected (T) or mock-transfected (M) as described under Materials and Method. Goat preimmune IgG (PIS) or anti-mouse transferase IgG (IS) was added to mock-transfected or transfected solubilized cells after various incubation periods as shown. Both group were tunicamycin (Tu)-treated for the same time periods. Both IgG preparations were also added to tritiated mouse microsomes in lanes 1 and 2. Molecular weight markers ($\times 10^{-3}$) are albumin (66), catalase (58), glutamate dehydrogenase (53), and egg albumin (41). All samples were electrophoresed on a single gel. Autoradiography was constructed using 8 days of exposure for lane 1 and 2, 48 hours of exposure for lane 3 and 4, and 5 hours of exposure for lane 5-12.

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