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Expression and Inducibility of the Human Bilirubin UDP-Glucuronosyltransferase UGT1A1 in Liver and Cultured Primary Hepatocytes: Evidence for Both Genetic and Environmental Influences

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In Crigler-Najjar type II patients and, recently, in Crigler-Najjar type I patients treated with human hepatocyte cell therapy, phenobarbital has been used for reducing the serum bilirubin load. Its effect is attributed to induction of the enzyme required for hepatic bilirubin elimination, UDP-glucuronosyltransferase, UGT1A1. This study investigated the expression and inducibility of UGT1A1 in human donor livers and their corresponding primary hepatocyte cultures. Immunoblot analysis using a specific antibody directed against the amino terminal of the human UGT1A1 isoform showed that 5 hepatocyte donors exhibited a >50-fold difference in UGT1A1 level. UGT1A1 protein level correlated strongly with both liver microsomal bilirubin UGT activity and liver UGT1A1 mRNA level (r^2 = .82 and .72, respectively). Of the 4 patients with the lowest UGT1A1 levels, 3 were homozygotes for the UGT1A1 promoter variant sequence associated with Gilbert's syndrome, and the fourth was a heterozygote. The 3 donors with the highest levels had a history of phenytoin exposure. Hepatocytes isolated from the phenytoin-exposed donors exhibited marked declines in UGT1A1 mRNA levels during culturing. Induction studies using hepatocytes treated for 48 hours with phenobarbital (2 mmol/L), oltipraz (50 μmol/L), or 3-methylcholanthrene (2.5 μmol/L) revealed UGT1A1-inducing effects of phenobarbital, oltipraz, and, in particular, 3-methylcholanthrene. Our data suggest that both genetic and environmental factors play an important role in the marked interindividual variability in UGT1A1 expression. An understanding of these mechanisms could lead to advances in the pharmacological therapy of life-threatening unconjugated hyperbilirubinemia. (HEPATOLOGY 1999;30:476-484.)

Bilirubin is an endogenous waste product generated from the metabolism of heme.[1] At low concentrations (normal range ≤1 mg/dL), it is recognized as being beneficial as a result of its antioxidant properties. However, its accumulation in the serum to high concentrations (≥20 mg/dL) is associated with serious toxicities, including neurological toxicity (kernicterus) and renal damage.[1] Therefore, the control of bilirubin levels in the body is critical.

The major factor controlling the excretion of bilirubin is its rate of glucuronidation in liver. Glucuronidation of this substrate is catalyzed by a specific member of the UDP-glucuronosyltransferase family, UGT1A1.[2-5] The level of bilirubin glucuronidation activity (and presumably UGT1A1) in liver is determined by genetic as well as environmental factors. Such factors include exposure to drugs[6-9] and xenobiotic compounds.[9-11] The most intensively studied class of bilirubin UGT inducers has been the barbiturates, which are used clinically for lowering serum unconjugated bilirubin levels in individuals with the type II form of Crigler-Najjar syndrome.[12] More recently, phenobarbital has been shown to be effective for lowering bilirubin in a child with the type I form of Crigler-Najjar who received human hepatocyte cell therapy.[13]

The beneficial effect of the barbiturates in these cases is widely assumed to involve induction of bilirubin UGT activity, because phenobarbital increases liver microsomal bilirubin UGT activity in some animals.[8,9] However, not all species exhibit phenobarbital-inducible bilirubin UGT activity, *e.g.*, guinea pig.[14] Although bilirubin excretion is clearly increased in humans by phenobarbital, attempts to demonstrate that the effect is actually the result of elevated bilirubin UGT activity (*i.e.*, UGT1A1) in human liver has had varied outcomes.[15,16] Following the identification of UGT1A1 as the major bilirubin glucuronidating UGT,[2,3] only 2 studies have specifically addressed the inducibility of hepatic UGT1A1 in primate species using UGT1A1-specific molecular probes. No effect of phenobarbital was observed on the UGT1A1 mRNA level in liver samples from phenobarbital-exposed monkeys.[2] In contrast, liver samples from patients with a history of exposure to phenobarbital and phenytoin (a phenobarbital-like inducer) exhibited high UGT1A1 mRNA levels.[17]

Human liver–derived cells or cell lines provide direct models for studying the regulation of human UGTs. The use of partially de-differentiated hepatoma cell lines (*e.g.*, HepG2 and Hep3B) is problematic as a result of the loss of expression of endogenous UGTs, including bilirubin UGT.[18] Primary human hepatocytes, in contrast, have better-maintained expression of UGTs and other liver-specific proteins. The development of reliable methodologies for isolating and

Abbreviations: UGT, UDP-glucuronosyltransferase; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; AHR, aryl hydrocarbon receptor.

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culturing high-quality, differentiated human hepatocytes has led to their accepted use for the prediction of human drug metabolism pathways, assessment of biotransformation enzyme inducibility,[19] and, now clinically, for *in vivo* liver cell transplantation.[20,21]

Because a better understanding of the mechanisms controlling UGT1A1 expression could potentially lead to improved therapies for individuals with life-threatening hyperbilirubinemia, we initiated studies to characterize cultured primary human hepatocytes as models for studying UGT1A1 regulation. In this study, liver samples and corresponding primary hepatocytes from several human donors were examined. Differences in expression of UGT1A1 protein and mRNA in the donor liver samples and cultured hepatocytes were characterized along with an assessment of UGT1A1 inducibility by agents from 3 distinct inducing classes, phenobarbital, oltipraz, and 3-methylcholanthrene.

MATERIALS AND METHODS

Materials. The bacterial fusion protein expression vector, pQE30, and Ni-NTA resin were from Qiagen. pSVL-HugBr1 was from Ritter.[2] Restriction endonucleases were from Promega. 3-Methylcholanthrene and phenobarbital were from Sigma Chemical (St. Louis, MO). Oltipraz (4-methyl-5-pyrazinyl-1,2-dithiole-3-thione) was a generous gift from the National Cancer Institute (Bethesda, MD). Freund's complete and incomplete adjuvant was obtained from Gibco-BRL (Gaithersburg, MD). B6C3F1 mice were from the National Cancer Institute. The horseradish peroxidase–conjugated sheep anti-mouse IgG secondary antibody and the ECL Western blotting detection system were from Amersham (Arlington Heights, IL).

Development of Specific Anti-Human UGT1A1 Antiserum. A construct directing the bacterial synthesis of a 6X-Histidine-tagged UGT1A1 fusion protein was prepared using the QIAexpressionist system (Qiagen). A DNA fragment corresponding to bases 85-477 of the UGT1A1 coding region was amplified from pSVL-HugBr1 using the polymerase chain reaction technique. The 3′ end primer for polymerase chain reaction was designed to produce an in-frame TGA stop codon at the 3′ end of the amplified UGT1A1 sequence. The fragment was cloned into *Bam*HI/*Hin*dIII-cleaved and Klenow polymerase blunt-ended pQE-30. A plasmid containing the insert in the correct orientation, pQE30-h1A185-477-2, was isolated and transformed into *Escherichia coli* strain XL1-Blue (Stratagene) for fusion protein expression. The fusion protein produced is predicted to be 149 amino acids in length containing an 18-residue amino terminus corresponding to the Ni-NTA affinity purification tag sequence (MSGSHHHHHHGSPGLQEF) and a 131-residue carboxyl terminus corresponding to residues 29-159 of the UGT1A1 cDNA coding sequence.[2] The preparation of fusion proteins corresponding to the human UGT1A4 and UGT1A6 isozymes and the rat UGT1A7 isozyme were performed using a similar strategy.

The UGT1A1 fusion protein was expressed by growing pQE30-hUGT1A185-477–containing *E. coli* strain XL1-Blue (Stratagene) in 500 mL LB broth containing 100 μg/mL ampicillin at 37°C with vigorous shaking. When the A_{600} reached 0.9, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mmol/L, and the culture was grown for an additional 5 hours. Cells were harvested by centrifugation at 4,000*g* for 10 minutes. Pellets were stored at −20°C. $UGT1A1_{29\text{-}159}$ fusion protein was purified by Ni-NTA affinity chromatography using the guanidine hydrochloride/urea method (protocol 7 supplied by Qiagen). A yield of 5 mg of UGT1A1 fusion protein per 0.5 L culture was obtained. Analysis of the affinity-purified material by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) indicated >85% purity of a protein with the predicted size.

Ten female B6C3F1 mice, 8 to 10 weeks old (Taconic Farms, Germantown, NY), received an intraperitoneal injection of 0.1 mg of UGT1A1 fusion protein suspended in Freund's Complete Adjuvant (Gibco Laboratories). Three days later, the dose was repeated with the protein suspended in Freund's incomplete adjuvant (Gibco Laboratories). One week later, the dose was repeated without adjuvant. Booster shots were subsequently administered every week for 4 weeks. One week following the final injection, mice were anesthetized and blood was collected via cardiac puncture and allowed to clot at 4°C overnight. The following day, blood samples were centrifuged and the serum was collected, combined, and aliquotted in 0.2-mL aliquots for long-term storage at −80°C.

Human Liver Procurement and Hepatocyte Isolation and Culturing. Human livers used in this study were donated by the Anatomic Gift Foundation (Laurel, MD) to the Human Hepatocyte Isolation and Cryopreservation Core Facility at the Medical College of Virginia Hospital. The use of human liver and hepatocytes received from the Anatomic Gift Foundation met the approval of the Virginia Commonwealth University Medical College of Virginia Institutional Review Board. The organs had been prepared for transplantation by flushing with University of Wisconsin preservation solution and storage on wet ice until laboratory perfusion. Reasons for not using the livers included marked donor instability, poor whole-organ perfusion, or macrosteatosis greater than 40%.

Characteristics of the patients who donated livers for this study are shown in Table 1. Hepatocytes were prepared from the left lobe of each liver as described.[19,20] Briefly, cells were isolated following perfusion with collagenase-P (Boehringer Mannheim) and plated on gelatin (Sigma Chemical Co., St. Louis, MO)-coated plastic tissue culture dishes (8×10^6 cells/100-mm dish) in chemically defined medium supplemented with 5% fetal bovine serum, dexamethasone (0.1 μmol/L), insulin (5 μg/mL), transferrin (5 μg/mL), and selenium (1 ng/mL). Viabilities estimated by Trypan blue exclusion were >80%. After allowing the cells to attach to the substrate for several hours, cells were incubated for an additional 20 hours in serum-free medium supplemented as described above, before the start of induction studies. Medium was changed daily throughout the culturing period.

For the induction studies, control cultures received the vehicle

TABLE 1. Human Liver and Hepatocyte Donor Characteristics

Patient	Age/Sex/ Ethnicity*	Cause of Death	Known Inducer Exposure History	UGT1A1 Promoter Genotype†
F65	68/F/C	Stroke		5/6
F71	46/M/C	Accidental death		6/6
F73	50/F/C	Stroke		7/7
F79	58/M/C	Stroke	Dexamethasone (8 mg × 7 doses); Dilantin (100 mg × 8)	6/6
F82	49/M/C	Stroke	Dexamethasone (4 mg q 4 hr × 6 d); Dilantin (100 mg × 7)	6/6
F87	61/F/C	Stroke	Dilantin (100 mg q 8 hr × 17 d)	7/7
F89	59/M/C	Accidental death	Heavy ethanol use last 2 weeks; smoker (2ppd, 40 yr)	6/7
F90	5/M/C	Motor-vehicle accident		7/7

*F, female; M, male; C, caucasian.

†Determined using the method of Monaghan et al[27] and Buetler et al.[28] Allele 5 = $(TA)_5TAA$; 6 = $(TA)_6TAA$; and 7 = $(TA)_7TAA$.

alone (dimethylsulfoxide, 0.1% final concentration), while the test cultures received medium containing inducers dissolved in dimethylsulfoxide at concentrations of 2 mmol/L (phenobarbital), 50 μmol/L (oltipraz), or 2.5 μmol/L (3-methylcholanthrene). The inducers chosen, their concentrations, and the time of treatment (48 hours) were based on an earlier study investigating glutathione *S*-transferase mRNA regulation in primary human hepatocytes.[22] After 24 hours, the medium was replaced with fresh inducer-containing medium, and 24 hours later, the cells were harvested for analysis of protein and mRNA.

Cells were harvested for protein analysis by washing twice with 5 mL ice-cold phosphate-buffered saline and scraping the cells with a rubber policeman in 0.5 mL 0.1 mol/L potassium phosphate buffer (pH 7.5), containing 1 mmol/L ethylenediaminetetraacetic acid. After brief sonication to disperse cell clumps, the protein concentration was determined using the bicinchoninic acid protein assay kit (Pierce) and bovine serum albumin as the standard. The cells were stored at −80°C. Cells were harvested for RNA isolation by solubilization in 0.5 mL Solution D,[23] followed by storage at −80°C.

Preparation of RNA and Northern Analysis of UGT1A1, Albumin, and Cyclophilin mRNAs. Total RNA from cells and liver donor samples was isolated by the acid guanidinium method.[23] After the last precipitation, RNA was dissolved in 0.5% SDS and the concentration determined by optical density at 260 nm. Samples were stored at −80°C. Northern analyses were performed using 0.9% agarose gels containing 0.6 mol/L formaldehyde as described previously.[24] Probes for mRNA analysis were prepared by random primed synthesis of [α^{32}P]-dCTP–labeled DNA[24] using denatured DNA corresponding to the following fragments as templates: UGT1A1 (0.7-kbp *Xho*I-*Eco*RI fragment from pSVL-HugBr1); albumin (2.0-kbp *Eco*RI fragment obtained from the human albumin cDNA clone pA12 isolated in our laboratory); and cyclophilin (0.7-kbp *Bam*HI fragment from the rat cyclophilin cDNA).[24] Northern blots were analyzed using a Molecular Dynamics Phosphorimager and ImageQuant software.

Preparation of Liver Microsomes. Liver microsomes were prepared from liver donor samples frozen in liquid nitrogen and stored at −80°C as described by Kessler and Ritter.[24] After the final centrifugation step, the microsomal pellet was resuspended in 0.1 mol/L potassium phosphate (pH 7.5), 1 mmol/L ethylenediaminetetraacetic acid, and 20% glycerol. The protein concentration was determined as described above, and liver microsomes were stored at −80°C.

Western Analysis of Human Liver and Hepatocyte UGT1A1. Total hepatocellular protein or liver microsomal protein was subjected to electrophoresis through a 7.5% SDS–polyacrylamide gel using a BioRad MiniProtean II gel electrophoresis unit and the manufacturer's suggested protocols. Proteins were transferred to a nitrocellulose membrane using a Biorad Mini Trans-Blot electrophoretic transfer cell. Membranes were washed for 10 minutes in Tris-buffered saline (20 mmol/L Tris-Cl [pH 7.6], 137 mmol/L NaCl) containing 0.5% Tween 20 (vol/vol) (TBS-0.5T) and then blocked in a solution of 5% dried milk (wt/vol) dissolved in TBS containing 0.2% Tween 20 (TBS-0.2T) for 60 minutes at room temperature with gentle shaking. After 2 brief rinses followed by 2 5-minute washes in TBS-0.5T, blots were incubated in a 1:1,000 dilution of anti-UGT1A1 serum prepared in TBS-0.2T for 60 minutes at room temperature, with shaking and washed as above. Blots were then incubated with a 1:10,000 dilution of the secondary antibody (sheep anti-mouse IgG conjugated to horseradish peroxidase purchased from Amersham Life Science, Buckinghamshire, England) in TBS-0.2T for 60 minutes at room temperature, with shaking and washed as above. The signal was detected using the ECL Western blotting detection method (Amersham Life Science).

Liver Microsomal Bilirubin UGT Assay. Bilirubin UGT was determined using the method of Matern et al.,[39] modified as follows: 0.1 mol/L Tris-HCl (pH 7.6) and 0.05% Triton X-100 were substituted for triethanolamine and digitonin, respectively, and 1 mmol/L adenosine monophosphate was omitted.

Genotyping at the UGT1A1 Promoter. Genomic DNA was prepared from human liver using the method of Hogan et al.[40] Samples were genotyped for the UGT1A1 promoter polymorphism by the method of Monaghan et al.[27] using the polymerase chain reaction conditions of Beutler et al.[28] and substituting cloned *Pfu* DNA polymerase and *Pfu* polymerase buffer. This method uses sense and antisense primers C and D, respectively, to amplify a region of the UGT1A1 promoter encompassing the TATA box. Alleles corresponding to the $(TA)_5TAA$, $(TA)_6TAA$, and $(TA)_7TAA$ variants have been shown to correspond to products 96, 98, or 100 bp in length, respectively.[27,28]

Statistical Analyses. Differences in mean mRNA levels of the different treatment groups were analyzed using ANOVA with Bonferroni-Dunn post-hoc analysis. Differences were assigned statistical significance at $P \leq .05$.

RESULTS

Development of Specific Anti-Human UGT1A1 Antiserum. A polyclonal antibody directed against a fusion protein containing amino terminal residues 29-159 of the UGT1A1 enzyme was raised in mice for these studies. A schematic of the fusion protein is shown in Fig. 1A. The amino terminal region of UGTs is generally the most unique region of the protein. UGT1A1 residues 29-159 share only 36% and 39% identity to the corresponding regions of UGT1A4 and UGT1A6, respectively. A high degree of specificity would therefore be

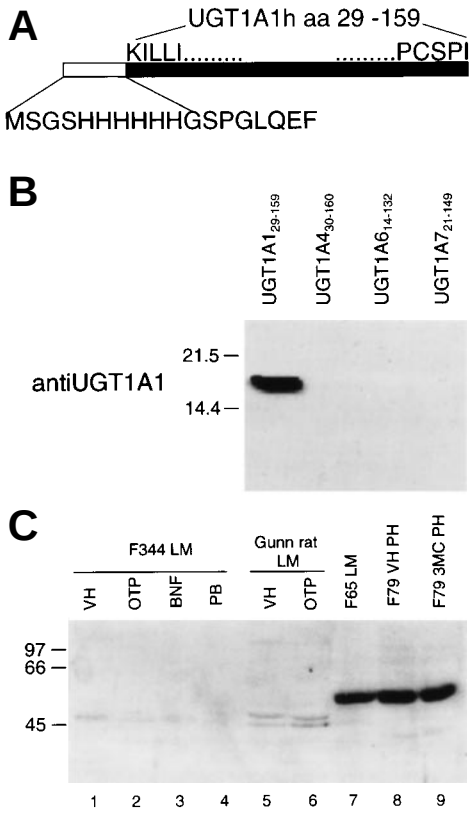

FIG. 1. Specificity of anti-human UGT1A1 antiserum. (A) Schematic of the 6XHis-tagged human UGT1A1 amino terminal fusion protein expressed in bacteria. The sequence at the amino and carboxyl termini is shown in single-letter amino acid code. (B) Immunoblot of 4 different human liver UGT amino terminal fusion proteins separated by SDS-PAGE (100 ng/lane). (C) SDS-PAGE immunoblot analysis of microsomal protein (50 μg/lane) from livers of rats treated with vehicle (VH), oltipraz (OTP), β-naphthoflavone (BNF), or phenobarbital (PB) as described,[24] from human liver donor F65, or from primary hepatocytes treated with vehicle or 3-methylcholanthrene.

expected, which was confirmed by testing for cross-reactivity toward corresponding N-terminal regions of UGT1A4, UGT1A6, and UGT1A7. Indeed, no cross-reactivity toward these other UGTs was demonstrable using an ECL-based method for visualizing antibody binding (Fig. 1B), even following extended exposure times (data not shown). An analysis of rat and human liver samples revealed a single major band in the 50-kd molecular-mass region of the human samples (Fig. 1C). The UGT detected with this antibody is slightly smaller than the band detected using a polyclonal antibody raised against UGT1A4 (data not shown). This slight difference is consistent with the predicted relative sizes of UGT1A1 (533 amino acids, 3 asparagine-linked glycosylation sites) and UGT1A4 (534 amino acids, 5 asparagine-linked glycosylation sites). Because no significant reactivity was observed with rat liver microsomal protein, it appears that the antibody does not cross-react with rat UGT1A1, despite 67% overall identity in the region used for antibody generation. A similar lack of species cross-reactivity has been reported for a polyclonal antibody directed against the amino terminal residues 12 to 131 of the human UGT1A6 isoform, which exhibits even higher homology (72% identity) with the corresponding region of the rat UGT1A6 isoform.[25]

Western and Northern Analysis of UGT1A1 in Human Liver Donor Samples. To assess variation in UGT1A1 expression in individual human liver samples and its possible causes, liver microsomes were prepared from 8 human liver/hepatocyte donors and analyzed for UGT1A1 protein using the anti-human UGT1A1 antiserum (1:1,000 dilution). A marked variation in the UGT1A1 protein level was observed (Fig. 2A). Quantitation of the data by densitometric analysis revealed >50-fold difference in the relative UGT1A1 level (Fig. 2B).

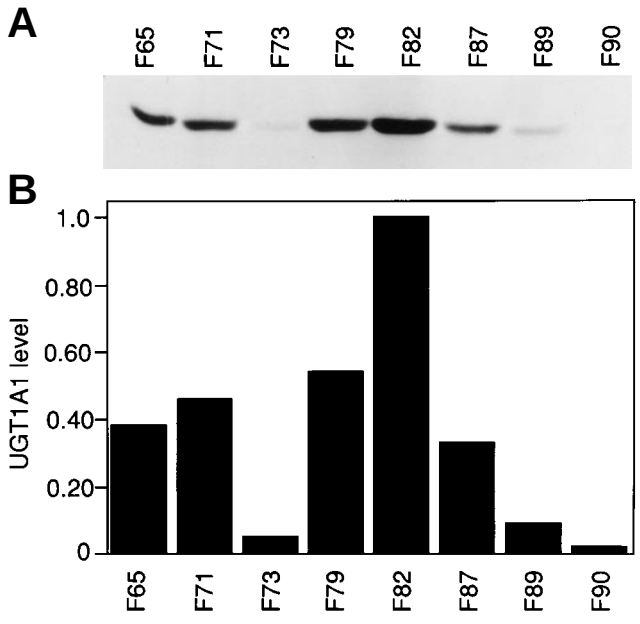

FIG. 2. Immunoblot analysis of UGT1A1 in liver microsomes from 8 donors. Liver microsomal protein (25 μg) from 8 different donors (F65-F90) was subjected to SDS-PAGE, followed by Western immunoblot analysis using anti-UGT1A1 antiserum as described in Materials and Methods. (A) Film image generated using the enhanced chemiluminescence reaction. (B) Relative levels of UGT1A1 determined by densitometric analysis of the film image.

To determine if the UGT1A1 mRNA level correlated with that of protein, RNA was extracted from donor tissue and analyzed by Northern analysis using a specific UGT1A1 probe. Large differences in the UGT1A1 mRNA level were evident (Fig. 3A). The differences were not the result of an RNA loading artifact, as demonstrated using probes for the cyclophilin or albumin mRNAs. Quantitation of the relative UGT1A1 mRNA levels by phosphorimage analysis and comparison with the UGT1A1 protein level revealed a good correlation between the UGT1A1 mRNA and protein levels ($r^2 = .72$) (Fig. 3B).

It is noteworthy that the 2 samples with the highest UGT1A1 RNA and protein levels, F79 and F82, were from individuals who received dexamethasone and phenytoin (Dilantin) during the immediate hospitalization period. A third individual in our group, donor F87, received phenytoin, and this individual also had a relatively high level of UGT1A1. These data are consistent with a previous study that reported high UGT1A1 mRNA levels in a patient treated with phenytoin. It is not clear at present whether dexamethasone contributed to the higher levels of UGT1A1 observed in donors F79 and F82, but dexamethasone has been shown to induce the UGT1A1 mRNA in livers of rats.[6] The high UGT1A1 levels in donor livers F65 and F71 suggest that additional factors besides exposure to therapeutic inducing agents can result in high UGT1A1 mRNA expression. Ethanol and possibly cigarette smoking have been speculated to be bilirubin UGT–inducing agents. However, donor F89, who had a lifetime smoking habit (2 packs/day for over 40 years) and reportedly drank heavily during the 2-week period before committing suicide, exhibited an extremely low level of UGT1A1.

UGT1A1 protein levels were also found to correlate with bilirubin UGT activity ($r^2 = .82$) (Fig. 4). The samples with the highest (F82) and lowest (F90) UGT1A1 protein levels had, respectively, the highest and lowest bilirubin UGT activities. These observations provide support for the specificity of the UGT1A1 antibody and confirm the major interindividual variability in UGT1A1/bilirubin UGT activity.

Genotypic Variation at the UGT1A1 Promoter. Because genetic variation in the TATA sequence of the UGT1A1 promoter has been associated with reduced UGT1A1 expression in individuals with Gilbert's syndrome (fasting or illness-induced unconjugated hyperbilirubinemia), it was of interest to determine whether any of the 8 individual donors exhibited the Gilbert's genotype [$(TA)_7TAA/(TA)_7TAA$ or 7/7]. The genotyping results are presented in Table 1. Four of the 8 samples were found to contain at least 1 copy of the allele associated with reduced expression, including 3 apparent homozygotes (F73, F87, and F90). Interestingly, these 4 samples exhibited the lowest levels of UGT1A1 protein (Fig. 2).

Characterization of Primary Human Hepatocytes for Alterations in Expression of UGT1A1 Protein and mRNA During Culturing. To characterize primary human hepatocyte cultures as a model for investigating mechanisms of UGT1A1 expression, we first investigated the effect of culturing the hepatocytes for 3 days on expression of UGT1A1 protein and mRNA. Hepatocytes from 5 of the donors, F79, F82, F87, F89, and F90, were studied. Figure 5A shows a comparison of the UGT1A1 protein levels in liver donor samples and the corresponding hepatocytes. Decreases in UGT1A1 level were observed in the hepatocyte cultures during the 3-day culture period. However, a strong correlation was still apparent between the

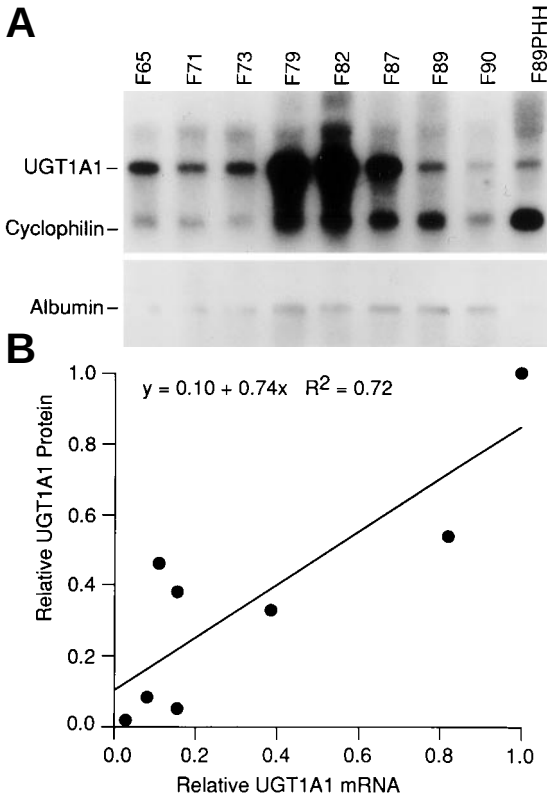

FIG. 3. Northern analysis of UGT1A1 mRNA in donor livers. Total human liver RNA from 8 donors (10 μg/lane) was subjected to agarose-formaldehyde gel electrophoresis, followed by Northern transfer and hybridization with [^{32}P]-dCTP–labeled probes for UGT1A1/cyclophilin or albumin, as described in Materials and Methods. (A) Autoradiograms of hybridized Northern blots. F89PHH represents primary human hepatocyte RNA from donor F89. (B) Correlation between liver UGT1A1 mRNA and liver microsomal UGT1A1 protein levels.

UGT1A1 protein levels in the donor livers and their corresponding hepatocytes (r^2 = .71). Hepatocytes from donor livers F79 and F82 exhibited the highest levels of UGT1A1, with F87 intermediate and F89 and F90 relatively low. These results suggested that expression of UGT1A1 is relatively well maintained during short-term culturing (up to 3 days) of hepatocytes.

In contrast to the protein data, a poor correlation existed between the mRNA levels in the donor livers and cultured hepatocytes (r^2 = .08). Figure 5B presents the comparison of RNA levels. The primary hepatocytes appeared to be differentially affected during culturing. The most affected were the F79, F82, and F87 cultures, with declines in mRNA of 90%, 94%, and 91%, respectively. In contrast, a decline of only 57% was observed for the F89 culture, and in 1 culture, F90, the UGT1A1 mRNA actually appeared to increase (70% increase). Thus, in contrast to the protein levels that were relatively stable during the 3-day culture period, expression of the mRNA was affected, but only in some of the cultures.

Induction Studies. We next investigated the inducibility of UGT1A1 protein and mRNA in the 5 donor primary hepatocyte cultures by prototypical inducing agents belonging to 3 different classes: phenobarbital, 3-methylcholanthrene, and oltipraz. At the protein level, no significant effects of any of the 3 inducing agents were apparent in cultures F79, F82, and F87 (data not shown). In cultures F89 and F90, effects of all 3 inducers were evident (Fig. 6). In culture F89, phenobarbital and oltipraz increased UGT1A1 by 46% and 67%, respectively, and in culture F90 by 31% and 125%, respectively. 3-Methylcholanthrene, in contrast, exerted an even stronger apparent effect, increasing the protein by 3.6-fold in culture F89 and 2.5-fold in culture F90.

The effects of the inducers on the UGT1A1 mRNA level were also assessed. Figure 7 shows Northern blots of the UGT1A1, cyclophilin, and albumin mRNAs in the control and inducer-treated samples from all 5 cultures (analyzed together on the same gel). Figure 8 presents the quantitative phosphorimaging data for the UGT1A1 mRNA analysis. Treatment with phenobarbital elevated the UGT1A1 mRNA in every culture, but the magnitude of this increase varied from 1.1- to 1.8-fold. The 0.45-fold mean difference in UGT1A1 mRNA level between the control (56 ± 8 expressed as mean phosphorimage units ± SE) and phenobarbital-treated groups (81 ± 16) was not statistically significant ($P <$ .43). Oltipraz treatment also produced detectable elevations in UGT1A1 mRNA in all 5 cultures. However, the most striking effects were observed with 3-methylcholanthrene treatment. The mean UGT1A1 mRNA level of the 3-methylcholanthrene treatment group was 205 ± 33 phosphorimage units, for an average fold increase of 3.6 (statistically significant, $P \leq .001$). This effect was not the result of differences in RNA loading between samples as demonstrated using probes for either albumin or cyclophilin mRNA.

DISCUSSION

This study describes a strong correlation between the levels of UGT1A1 protein and RNA and microsomal bilirubin UGT activity in 8 randomly selected human liver donor samples. A surprisingly high degree of variability was observed for each of these parameters, including more than a 50-fold variation in UGT1A1 protein level. The finding that 4 of the 8 donor

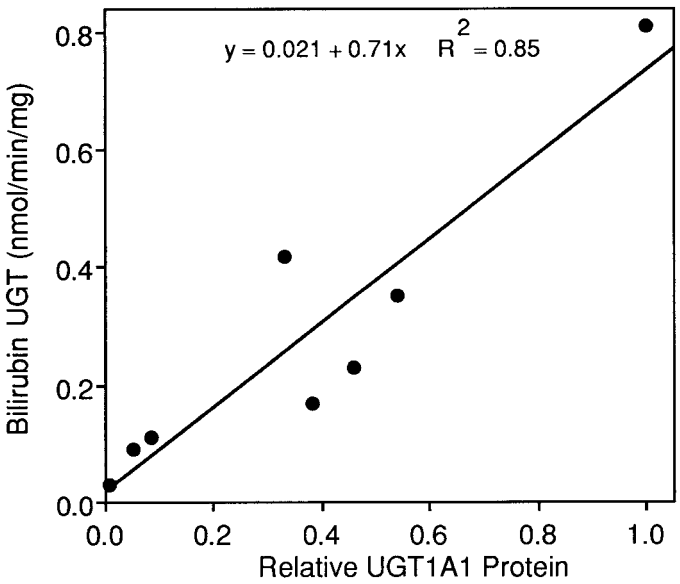

FIG. 4. Relationship between bilirubin UGT activity and UGT1A1 protein levels in donor liver samples. Bilirubin UGT activities of liver microsomes from human liver donors were determined as described in Materials and Methods and plotted versus the liver microsomal UGT1A1 protein level determined by immunoblot analysis. The best-fit line determined by least-squares linear regression analysis is shown.

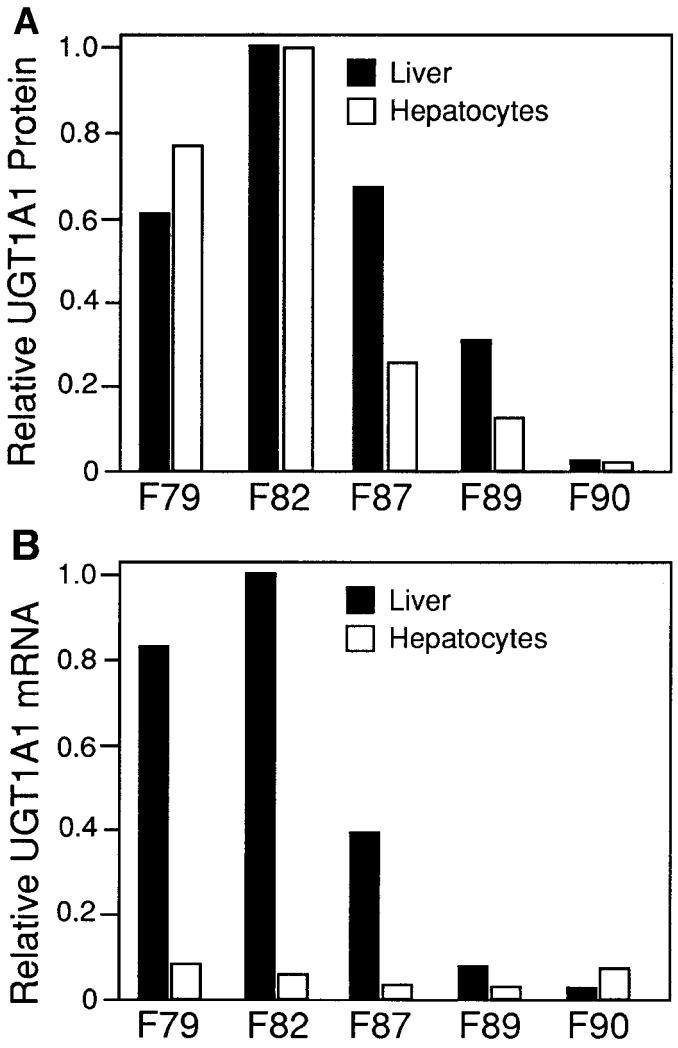

FIG. 5. Relationship between UGT1A1 protein and mRNA levels in donor liver and 3-day primary hepatocyte cultures. (A) UGT1A1 protein analysis. Samples of homogenized liver (15 μg/lane) and control primary hepatocytes (15 μg total cell lysate/lane) were subjected to SDS-PAGE immunoblot analysis in parallel as described above. Data are presented in relative units (with the F82 liver sample arbitrarily assigned a value of 1.0). (B) UGT1A1 mRNA analysis. Total cellular RNA prepared from either donor tissue or corresponding primary human hepatocytes cultured for 3 days was analyzed by Northern hybridization with the UGT1A1 mRNA probe as described above. Data were quantitated by phosphorimage analysis. The results are expressed in relative units (with the F82 liver sample assigned a relative value of 1.0).

samples had the UGT1A1 promoter sequence associated with decreased UGT1A1 expression (*i.e.*, the Gilbert's defect), including 3 homozygotes (F73, F87, and F90), supports an important role for genetic factors as 1 cause of the high UGT1A1 expression variability.

Environmental influences such as exposure to alcohol and drugs are also believed to exert an influence on UGT1A1 expression,[26] and at least 2 lines of evidence in this study support this conclusion. The first is based on the observation that introducing hepatocytes from the donors into culture, *i.e.*, a common environment, had an equalizing influence on UGT1A1 mRNA expression in the samples (Fig. 5). Before culturing, a 50-fold difference was observed in UGT1A1 mRNA, whereas after 3 days in culture, only a 2.6-fold difference was observed between the samples. The equalization appeared to be mainly the result of large declines in expression in the samples with the highest starting levels (F79, F82, and F87), with little or no decrease in the 2 samples with lower levels. One interpretation of these data is that the large decreases in RNA level observed in cultures F79, F82, and F87 resulted from the loss of an induction stimulus that was present *in vivo* in these patients, *e.g.*, phenytoin exposure. Each of these 3 samples was from individuals with a documented history of phenytoin exposure, and phenytoin has previously been associated with elevated UGT1A1 levels.[17]

The second line of evidence is from our UGT1A1 induction study using primary human hepatocytes as a cellular induction model. The value of this model, compared with human hepatoma cell lines that do not express bilirubin UGT activity,[18] is demonstrated by the detectable expression of the UGT1A1 mRNA and protein in all 5 of the cultures studied in this report. However, the data also underscore a significant limitation of this model. Some of the cultured primary hepatocytes, in particular those having the highest starting UGT1A1, exhibited a marked decrease in UGT1A1 mRNA. Yet, after 3 days in culture, parallel changes in UGT1A1 protein were not evident, suggesting that UGT1A1 protein had not reached the new "steady-state" level established by the lower level of UGT1A1 mRNA expression.

Of particular interest in this study was whether expression of the UGT1A1 mRNA could be increased in the cultured hepatocytes by phenobarbital treatment. Induction of bilirubin UGT is widely accepted as the mechanism by which phenobarbital therapy lowers serum bilirubin levels in individuals with unconjugated hyperbilirubinemia.[12,15,16,29] Fur-

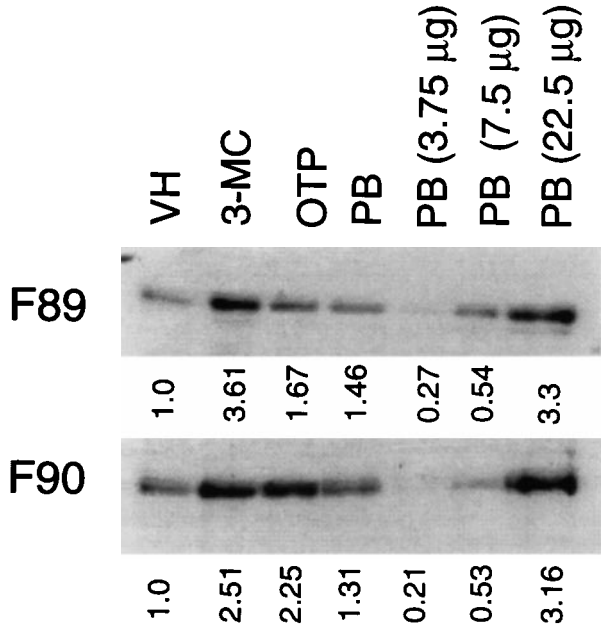

FIG. 6. Immunoblot analysis of UGT1A1 protein in control and inducer-treated primary human hepatocyte cultures. Total cell lysate (15 μg/lane) from donor primary hepatocytes F89 and F90 treated for 48 hours with either vehicle (VH, DMSO), 3-methylcholanthrene (3-MC, 2.5 μmol/L), oltipraz (OTP, 50 μmol/L), or phenobarbital (PB, 2 mmol/L) were analyzed for UGT1A1 protein as described above. To assess the linearity of ECL, differing amounts of cell lysate from phenobarbital-treated cells (3.75, 7.5, or 22.5 μg) were included. The relative UGT1A1 protein levels are indicated under each lane.

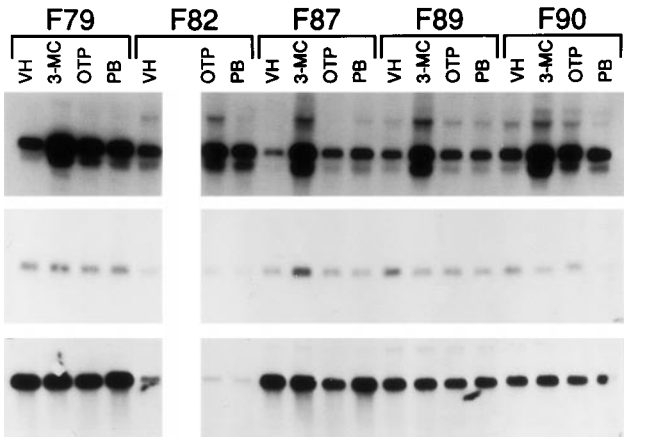

FIG. 7. Inducibility of the UGT1A1 mRNA in cultured primary human hepatocytes by phenobarbital, oltipraz, and 3-methylcholanthrene. Human hepatocytes from the 5 human liver donors (F79-F90) were prepared as described in Materials and Methods and then incubated for 48 hours in the absence or presence of inducing agents. VH, 0.1% dimethylsulfoxide; MC, 3-methylcholanthrene (2.5 μmol/L); OT, oltipraz (50 μmol/L); PB, phenobarbital (2 mmol/L). RNA was isolated and analyzed for UGT1A1, cyclophilin, or albumin message as described in Materials and Methods. Blots were exposed to film to generate the autoradiographs shown.

thermore, Sutherland et al.[17] reported markedly higher UGT1A1 mRNA levels in 2 patients exposed to phenobarbital or the phenobarbital-like inducer, phenytoin, and in this study, the 3 donor samples with the highest levels of UGT1A1 protein were from individuals with a documented history of phenytoin therapy at the time of death. Yet our study revealed surprisingly weak hepatocyte UGT1A1-inducing effects of phenobarbital. The weak inducing effect of phenobarbital on human hepatocyte UGT1A1 expression may be the result of limitations of the primary hepatocyte induction model. Isolated primary hepatocytes undergo progressive loss of differentiated functions during continued culturing. The latter includes liver-specific factors necessary for the phenobarbital induction mechanism. However, human hepatocytes cultured as described in this report have been noted for their significantly greater stability of hepatic functions and metabolizing enzyme activities compared with rodent hepatocytes.[19] Moreover, it should also be noted that the mRNA for a second isoform in the human UGT1 family, UGT1A4, was found to be more markedly increased by phenobarbital treatment of the F79, F82, F87, F89, and F90 hepatocyte cultures (Auyeung et al., manuscript in preparation). Thus, our data indicate that the hepatocytes in this study remained responsive to phenobarbital and that UGT1A1 is relatively less responsive to phenobarbital than other UGT genes. In this regard, it is interesting that the hepatocyte induction data agree qualitatively with the effects of phenobarbital described in monkeys. Hepatic UGT1A4 mRNA was phenobarbital-responsive, whereas UGT1A1 mRNA was not as affected.[2]

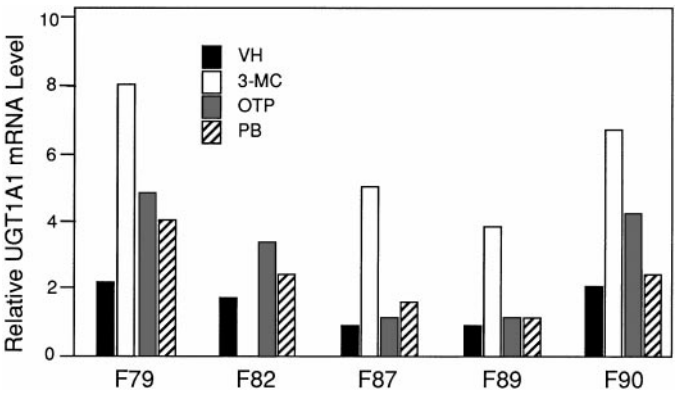

FIG. 8. Relative mRNA levels in control and drug-treated primary human hepatocytes. The Northern blot hybridized with UGT1A1 probe was analyzed by phosphorimaging. The results are presented in relative phosphorimage units.

Another novel finding of our study is that the human UGT1A1 mRNA is responsive to 3-methylcholanthrene. Previous studies have reported elevations in bilirubin UGT activity in hamsters and mice[9] exposed to 3-methylcholanthrene. However, species and strain differences in responsiveness have also been reported,[9,11] and it has remained unclear, until now, whether the human UGT1A1 counterpart is responsive to polyaromatic hydrocarbons. The effect on UGT1A1 mRNA presumably involves transcriptional activation mediated through the aryl hydrocarbon receptor (AHR), a conclusion based on genetic evidence from inbred strains of mice carrying functional or defective alleles of the AHR.[9] Induction through this mechanism involves ligand-induced formation of a complex between AHR and AHR nuclear translocator protein that then binds to and activates a DNA regulatory sequence known as the xenobiotic response element. Xenobiotic response elements have not yet been reported in the upstream region of the human *UGT1A1*.

Interestingly, bilirubin itself has been proposed as an AHR agonist. Treatment of mouse hepatoma cells with bilirubin (100 μmol/L) resulted in a 15-fold elevation in 7-ethoxyresorufin *O*-deethylase activity,[30] a marker for AHR activation. Although it is not clear if the mechanism is operative when serum bilirubin is in the normal range (≤1.5 mg/dL or 25 μmol/L), it has been shown that CYP1A1 mRNA and protein levels are elevated in young jaundiced Gunn rats,[31] which exhibit high unconjugated bilirubin levels as a result of an inactive UGT1A complex. CYP1A1 has been proposed to contribute to bilirubin elimination when the UGT excretory mechanism is impaired. Together, these data indicate that bilirubin might regulate its own elimination through AHR-dependent control of 2 different pathways.

Our data also suggest that individuals who smoke cigarettes (a source of aryl hydrocarbons) may exhibit reduced steady-state bilirubin levels as a result of UGT1A1 induction. Indeed, 2 different population surveys each have reported an inverse association between cigarette smoking and serum bilirubin levels.[32,33] It is intriguing to speculate about the possible health significance of this effect. Bilirubin, a potent antioxidant, has been shown to protect low-density lipoprotein from oxidative damage.[34] Oxidation of low-density lipoprotein is considered the initial event in the formation of foam cells and, ultimately, atherogenesis. In support of the protective role of bilirubin in heart disease, recent studies

have reported evidence of an inverse association between serum bilirubin level and the risk of the early familial form of coronary heart disease.[35]

Finally, it is interesting to note that the effects of the Gilbert's genotype on UGT1A1 expression level appear to be variable in the cultured hepatocytes. UGT1A1 mRNA levels appear to be lower in hepatocytes from donors F87 and F89. However, 1 of the Gilbert's homozygotes (F90) exhibited levels that were comparable with the control (6/6) cultures (F79 and F82). This may reflect the fact that many factors besides those involved in the initiation of RNA synthesis (the event predicted to be affected by the Gilbert's sequence variation) act to determine steady-state UGT1A1 mRNA levels, and that their relative importance varies as a result of *in vitro* culturing conditions. A second possibility is that the effect of the TATA box sequence variation requires strong transcriptional pressure to be manifest. However, arguing against this possibility is the observation that 3-methylcholanthrene–induced UGT1A1 mRNA levels are comparable in the F79 and F90 cultures.

In summary, this study supports an important influence of both genetic and environmental factors as determinants of the marked interindividual variability observed in liver UGT1A1 expression. An understanding of the environmental factors may be exploitable for the clinical relief of hyperbilirubinemia in individuals with limiting UGT1A1 activity. This category of patients now includes Crigler-Najjar type I patients treated with hepatocyte cell therapy.[13] Oltipraz is currently being tested in clinical trials as a cancer chemoprotective agent.[36] While 3-methylcholanthrene obviously has no potential therapeutic utility, less hazardous agents are available that induce hepatic metabolizing enzymes by the same mechanism.[37,38] Furthermore, the risk of adverse effects associated with the long-term use of these agents may be outweighed by the risk of developing life-threatening kernicteric encephalopathy.

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