Disruption of the Ugt1 Locus in Mice Resembles Human Crigler-Najjar Type I Disease*

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Nghia Nguyen†, Jessica A. Bonzo‡, Shujuan Chen†, Sarah Chouinard§, Michael J. Kelner‡, Gary Hardiman¶, Alain Bélanger§, and Robert H. Tukey†‡¶

From the †Laboratory of Environmental Toxicology, Departments of Chemistry & Biochemistry and Pharmacology, University of California, San Diego, La Jolla, California 92093, the §Department of Pathology, University of California, San Diego, La Jolla, California 92093, the ‡Biomedical Genomics Microarray Facility, Department of Medicine, School of Medicine, University of California, San Diego, La Jolla, California 92093, and the ¶Oncology and Molecular Endocrinology Research Center, Centre Hospitalier Universitaire de Québec, Québec G1V 4G2, Canada

The 9 UDP-glucuronosyltransferases (UGTs) encoded by the UGT1 locus in humans are key enzymes in the metabolism of most drugs as well as endogenous substances such as bile acids, fatty acids, steroids, hormones, neurotransmitters, and bilirubin. Severe unconjugated hyperbilirubinemia in humans that suffer from Crigler-Najjar type I disease results from lesions in the UGT1A1 gene and is often fatal. To examine the physiological importance of the Ugt1 locus in mice, this locus was rendered non-functional by interrupting exon 4 to create Ugt1−/− mice. Because UGT1A1 in humans is responsible for 100% of the conjugated bilirubin, it followed that newborn Ugt1−/− mice developed serum levels of unconjugated bilirubin that were 40–60 times higher than Ugt1+/− or wild-type mice. The result of extreme unconjugated bilirubin in Ugt1−/− mice, comparable to the induced levels noted in patients with Crigler-Najjar type I disease, is fatal in neonatal Ugt1−/− mice within 2 weeks following birth. The extreme jaundice is present as a phenotype in skin color after 8 h. Neonatal Ugt1−/− mice exhibit no detectable UGT1A-specific RNA, which corresponds to a complete absence of UGT1A proteins in liver microsomes. Conserved glucuronidation activity attributed to the Ugt1 locus can be defined in Ugt1−/− mice, because UGT2-dependent glucuronidation activity is unaffected. Remarkably, the loss of UGT1A functionality in liver results in significant alterations in cellular metabolism as investigated through changes in gene expression. Thus, the loss of UGT1A function in Ugt1−/− mice leads to a metabolic syndrome that can serve as a model to further investigate the toxicities associated with unconjugated bilirubin and the impact of this disease in humans.

Jaundice is frequently observed and stems from a variety of hepatic and non-hepatic conditions such as infection, biliary obstruction, hemolysis, liver disease, and genetic diseases that influence hepatic metabolism and transport. It is symptomatic clinically by the accumulation of indirect or unconjugated bilirubin (UCB), which accumulates resulting in hyperbilirubinemia. The most benign genetic disease leading to hyperbilirubinemia is Gilbert syndrome, which is a common inheritable condition resulting in transient levels of UCB (1). In humans, bilirubin is conjugated with glucuronic acid, which is catalyzed solely by UDP-glucuronosyltransferase (UGT) 1A1 (2). In Gilbert syndrome, hyperbilirubinemia is tied to a TA insertion in the promoter region of the UGT1A1 gene (3, 4), characterized genetically as UGT1A1*28, and is felt to lead to reduced transcriptional activation of the gene and lowering of hepatic UGT1A1 levels. More detailed analysis of genetic variants associated with UGT1A1*28 indicate that Gilbert syndrome is part of a haplotype of four UGT1A1 variants spanning at least three UGT1A genes (5). The more serious of the genetic defects associated with UGT1A1 is Crigler-Najjar type 1 (CNI) disease (6), an autosomal recessive syndrome where mutations in the coding region render the protein completely non-functional. The accumulation of UCB within focal brain regions leads to neural dysfunction followed by cell death and permanent disability (7, 8). If untreated by liver transplantation (9, 10), the complete inactivation of UGT1A1 in humans is fatal (11), and lethality is directly attributed to toxic concentrations of UCB.

The UGT1A1 gene is part of the UGT1 locus (12), which encodes nine functional UGT1A proteins (13), together playing a crucial role in the metabolism of drugs, xenobiotics, environmental toxicants, as well as endogenous substances such as fatty acids, bile acids, hormones, steroids, neurotransmitters, and bilirubin (14). The organization of the UGT1 locus on chromosome 2 is encoded by >220 kDa of DNA, with four conserved exons located at the 3′-end and flanked consecutively by an array of nine cassette exons that encode the N-terminal portion of each UGT1A protein. Thus, the UGT1 locus encodes nine functional UGT1A proteins, each composed of a unique N-terminal region encoded by one of the 5′-flanking exons while...
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sharing an identical C-terminal 245 amino acids encoded by exons 2–5. Each UGT1A gene is regulated independently from each other based upon tissue-specific control (15–17), which is considered to be the biochemical basis of organ-specific glucuronidation activity. Development of transgenic mice that express the entire human UGT1 locus (18) confirms that the human UGT1A genes can be regulated in a tissue-specific fashion similar to that observed in human tissues. In addition, the human UGT1A genes are regulated by the xenobiotic receptors pregnane X receptor (18), the peroxisome proliferator-activated receptor α (19), the liver X receptor (20), and the Ah receptor (18). Each of these receptors, including the activation of Nrf2 by an antioxidant response (21), target the UGT1A1 gene for regulation and potentially contribute to the steady-state balance of important endogenous substrates for glucuronidation such as bilirubin.

Although jaundice and hyperbilirubinemia can lead to drug toxicities associated with the impairment of functional UGT1A1 (22) as well as encephalopathy or irreversible brain damage (23), animal models that resemble CN1 deficiencies have not been used to examine the biochemical and physiological conditions associated with defects in either the murine Ugt1a1 gene or the Ugt1 locus. Although the Gunn rat model exhibits a genetic defect in the Ugt1 locus (24, 25) and develops hyperbilirubinemia (26, 27), the animals are healthy based upon their ability to reproduce. To assess the functional role of the Ugt1 locus in mice, we have created a mouse model in which the coding region of exon 4 has been interrupted. Results are presented demonstrating complete interruption of the Ugt1 locus in Ugt1<sup>−/−</sup> mice leading to levels of UCB that equate to comparable increases observed in patients with CN1 disease. The absence of functional UGT1A protein predisposes neonatal mice to fatal consequences associated with non-hemolytic unconjugated hyperbilirubinemia.

**EXPERIMENTAL PROCEDURES**

**Generation of the Ugt1<sup>−/−</sup> Mice**—A 14-kb Ugt1a1 λ-genomic DNA clone from a 129/SvJ genomic DNA library (Stratagene, 946313) was isolated by screening the library with a cDNA fragment to exons 2–5 of the mouse Ugt1a6 cDNA (28). This Ugt1a1-λ DNA fragment contained exon 1 through exon 5 with additional 3′ intronic sequence. From this λ DNA, we cloned two EcoRI fragments (see Fig. 1) that spanned 9.4 kb into pBluescript II KS (Stratagene) and generated the targeting vector by interrupting amino acids Thr-413/Leu-414 of exon 4 with the G418 (neo)-resistant gene. The vector by interrupting amino acids Thr-413/Leu-414 of exon 4 was engineered as previously described (18) with 10 μg of protein loaded into each well. Following electrophoresis at 200 V, the resolved proteins were transferred onto nitrocellulose membrane (Immobilon-Millipore), and the membrane was blocked with 5% nonfat dry milk in Tris-buffered saline solution (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20) for several hours. The membranes were then incubated with primary human UGT1A (29) or UGT2B7 (30) antibodies overnight at 4 °C, followed by incubation with horseradish peroxidase-conjugated secondary antibody. Each membrane was washed again, and the conjugated horseradish peroxidase was detected using the ECL plus Western blotting detection system (Amersham Biosciences), and the proteins were detected using a MolecularImager® ChemiDoc™ XRS system (Bio-Rad).

**UDP-glucuronosyltransferase Activity Determination**—Liver microsomes were prepared from 5-day-old neonatal mice. Liver from three animals of each genetic strain (WT, Ugt1<sup>+/−</sup>, or Ugt1<sup>−/−</sup>) were combined and homogenized at 4 °C in 0.5 ml of phosphate-buffered saline (pH 7.4) using a motorized glass Teflon homogenizer. The tissue homogenate was completed at 4 ml with microsome buffer (2.62 mM KH<sub>2</sub>PO<sub>4</sub>, 1.38 mM K<sub>H</sub>PO<sub>4</sub>, 2% glycerol, and 0.5 mM dithiothreitol) and was first centrifuged at 12,000 × g for 20 min at 4 °C, and this resulting supernatant was centrifuged at 105,000 × g for 60 min at 4 °C. The pellet was suspended in microsome buffer, and the protein concentration was determined by the Bradford method. Glucuronidation assays were performed for 1 h in triplicate with 50 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 8.5 mM saccharolactone (except for serotonin, MPA, imipramine, bilirubin, and T4), 10 μg/ml phosphatidylcholine (except for T4), 1 mM UDP-glucuronic acid (Sigma), 2.5 μg/ml pepstatin (except for T4⁰), 0.5 μg/ml leupeptin (except for MPA and T4), 10 μg of microsomal protein, and 10 μM of the substrate (bilirubin, testosterone, 2-OH-estrone, 4-nitrophenol, 4-methylumbilferone, and 1-naphthol) and 200 μM of the substrate (serotonin, MPA, imipramine and T4) in a total volume of 100 μl. Assays were stopped with 100 μl of methanol (1-naphthol, 4-nitrophenol, 4-MU, testosterone, 2-OH-estrone, and bilirubin (with 0.02% butylated hydroxytoluene)) or methanol with 0.02 M HCl (MPA) or acetonitrile with 0.02% butylated hydroxytoluene (serotonin) or acetonitrile with 0.2% formic acid (imipramine).
or acetonitrile with 6% formic acid and 0.02% butylated hydroxytoluene (T4) prior to centrifugation at 10,000 \( \times g \) for 10 min at 4 °C, and supernatants were collected for high-performance liquid chromatography analyses.

**Mass Spectrometry Analysis of Enzymatic Assays**—Each sample (50 µl) was diluted 1:1 with water, vortexed, and then transferred into a conical vial for injection into the mass spectrometer. The high-performance liquid chromatography system consisted of a mass spectrometer (model API 3200, PerkinElmer Life Sciences/MDS Sciex, Thornhill, Ontario, Canada) and was used to detect glucuronide conjugates formed. It was operated in the multiple reactions monitoring mode and equipped with an electrospray ionization interface in negative or positive ion mode and a high-performance liquid chromatography pump plus autosampler model 1200 series (Agilent, Montreal, Canada). For testosterone-glucuronide analysis, chromatographic separation was achieved with a 10 mm × 4.6 mm, 4-µm particle size Synergy Hydro-RP column at a flow rate of 1.0 ml/min (Phenomenex, Torrance, CA). The mobile phase A was water plus 1 mM ammonium formate, B was methanol plus 1 mM ammonium formate, and C was tetrahydrofuran. Separation was achieved using an isocratic condition of 48% A, 48% B, and 4% C in 7 min at a flow rate of 0.9 ml/min. Afterward, the column was flushed 1 min with 4% A, 92% B, and 4% C and re-equilibrated to initial conditions over 3 min. For 1-naphthol-glucuronide, separation was achieved using a linear gradient of 85% A, 5% B, and 10% C in 2 min at a flow rate of 0.9 ml/min. After 2 min, the column was washed with 5% A, 5% B, and 90% C. Afterward, the column was re-equilibrated to initial conditions over 3 min. For 2-hydroxyestrone-glucuronides, samples were analyzed with a 4- to 6-mm inner diameter × 50-mm Luna Phenyl-Hexyl column (3-µm particle size, Phenomenex) using the mobile phase (solvent A, water; solvent B, methanol plus 0.1% ammonium hydroxide; and C, methanol). Separation was achieved using a linear gradient of 85% A, 5% B, and 10% B to 70% A, 5% B, and 20% C in 2 min at a flow rate of 0.9 ml/min. After 2 min, the column was washed with 5% A, 5% B, and 90% C. Afterward, the column was re-equilibrated to initial conditions over 3 min. For 4-hydroxyestrone-glucuronides, samples were analyzed using an isocratic separation of 50% A and 50% B (3.5 min). For serotonin-glucuronide, 60% A and 40% B (2 min) was used with the same chromatographic system. For MPA-glucuronide and MPA acetylglucuronide, samples were analyzed with a 4.6-mm inner diameter × 100-mm Gemini C18 column (5-µm particle size, Phenomenex). The mobile phases were solvent A, water plus 3 mM ammonium formate plus 0.5% acetic acid and B methanol plus 3 mM ammonium formate plus 0.5% acetic acid. Separation was achieved using a linear gradient of 65% to 85% B in 3 min at a flow rate of 0.9 ml/min. After 3 min, the column was washed with 85% B. Afterward, the column was re-equilibrated to initial conditions over 3 min. For bilirubin monoglucuronide and diglucuronide, samples were analyzed with a 4.6-mm inner diameter × 100-mm Luna C18 column (3-µm particle size, Phenomenex). The mobile phase A was water plus 1 mM ammonium formate; B was methanol plus 1 mM ammonium formate. Separation was achieved using a linear gradient of 70% B to 95% B in 2.5 min at a flow rate of 0.9 ml/min. After 0.5 min, the column was re-equilibrated to initial conditions over 3 min. For thyroxine (T4)-glucuronide, samples were analyzed with a 4.6-mm inner diameter × 100-mm Synergy RP-Hydro column (4-µm particle size, Phenomenex). The mobile phase A was water plus 0.1% formic acid, and B was acetonitrile plus 0.1% formic acid. Separation was achieved using an isocratic separation of 70% A and 30% B (3.5 min).

**Detection of Ugt1a RNAs by Reverse Transcription-PCR and Northern Blot**—Total RNA for Northern blot and reverse transcription-PCR was prepared using the RNasey RNA purification kit from Qiagen. For reverse transcription-PCR analysis, 2 µg of total RNA was used for generation of cDNA following the Omniscript RT kit protocol (Qiagen) with oligo(dT) primers in a 20-µl reaction. Following cDNA synthesis, 2 µl of the reaction was used for PCR amplification using 2 units of Choice TaqDNA polymerase (Denville Scientific). For detection of mouse UGT1A1 RNA, the forward primer was designed to a highly specific region of exon 1a1 (5′-GGTGGAACTTG-GACGGACTG-3′). For detection of the common UGT1A transcripts the forward primer was designed toward exon 2 (5′-GCCCTATGTCAGCCCTCTTG-3′). The reverse primer annealing in exon 5 (5′-GGCGCATGATGTTCCTTCTTG-3′) was used for both UGT1A1 and UGT1A1 amplification. For detection of UGT1A6, the forward primer was designed to anneal to an identical region in exons 1a6a and 1a6b (5′-CAGGATGCGCTTGCTCTTCCC-3′). The reverse primer for UGT1A6 was directed toward exon 5 (5′-TGTTCTTGGCAA-GATTGCGAT-3′). Expression of actin was used as a loading control. For detection of UGT1A1, UGT1A, and actin RNA expression, the polymerase was activated for 2 min at 95 °C followed by 23 cycles of 95 °C 30 s, 58 °C 30 s, 72 °C 45 s, and a final 72 °C extension for 7 min. The PCR for detection of UGT1A6 RNA expression was performed under the same conditions except the annealing temperature was 55 °C. Eight microliters of the PCR product was resolved on a 1.5% agarose gel and imaged using a Bio-Rad ChemiDoc XRS.

For Northern blot analysis, 10 µg of RNA from each liver was separated in 1% formaldehyde-agarose gels with the resolved RNA transferred to GeneScreen membrane (PerkinElmer Life Sciences) by capillary diffusion. The membrane was pre-hybridized at 42 °C for several hours in a solution containing 5 × SSC, 5 × Denhardt’s solution, 1% SDS, 100 µg/ml single-stranded DNA, and 50% formamide. A 1.2-kb DNA probe that spans a portion of exon 5 was amplified from the targeting vector by PCR (5′-AAAGGAAACCTCCAGAGACC-3′ and 5′-GCTACAGGGAGAATCATGCG-3′), purified, and labeled using [α-32P]ATP and a Rediprime II Random Prime Labeling System (Amersham Biosciences), followed by hybridization to the membrane overnight at 42 °C. The membrane was then washed with 0.5% SDS and 2 × SSC several times and exposed to phosphorimaging screens (Molecular Dynamics).
followed by detection of signal using a Molecular Dynamics Storm 840 optical scanner.

Microarray Analysis—Total RNA was isolated as described above from three 5-day-old WT and three Ugt1/H11002/H11002 livers. Biotinylated cRNA was prepared using the Illumina RNA amplification kit according to the manufacturer’s instructions (Ambion, Inc, Austin, TX). Messenger RNA was converted to cDNA and then amplified and labeled by T7 DNA polymerase. The Mouse 6 Sentrix Expression BeadChip was used (Illumina). Following hybridization and washing, the arrays were scanned on an Illumina BeadArray Reader. The signals were computed with weighted averages of pixel intensities, and local background was subtracted. Sequence-type signal was calculated by averaging corresponding bead signals from the three liver samples with outliers removed (median absolute deviation). Simultaneous normalization of multiple microarrays was done using the “mloess” method (31). Array data have been deposited in the EBI Array Express Database (accession number is pending).

RESULTS

Hyperbilirubinemia Associated with Mutagenesis of the Ugt1 Locus—The mouse Ugt1 locus encodes nine functional Ugt1a genes (32) resulting in the translation of UGTs that contain identical C termini encoded by conserved exons 2–5. Elimination of the mouse UGT1A activity from the germ line was accomplished by interrupting exon 4 at a BstEII restriction enzyme site and inserting the neomycin gene followed by recombination of the targeting vector (Fig. 1) into ES cells. Heterozygous Ugt1/H11001/H11002 mice generated from one of the clones was bred, and littermates that were Ugt1/H11002/H11002 were identified by Southern blot analysis from tail DNA (Fig. 1). Using BglII to digest DNA, the WT band is identified as a 4.8-kb fragment that is extended to 6.0 kb following insertion of the neo gene. Both 4.8- and 6.0-kb fragments are identified in Ugt1/H11001/H11002 mice, whereas the WT band is not present in Ugt1/H11002/H11002 mice, demonstrating that the knock-out allele is heritable when Ugt1/H11002 mice serve as the breeders. Within 8 h following birth, the visible appearance of jaundice in Ugt1/H11002 mice is evident by the orange skin color of the neonates (Fig. 2).
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FIGURE 4. Detection of Ugt1a RNAs. A, reverse transcription-PCR analysis using liver RNA to detect UGT1A1 and UGT1A1A RNA in 5-day-old Ugt1−/−, Ugt1+/−, and WT mice. Mouse actin RNA was detected and used to monitor Ugt1a abundance. B, Northern blot analysis. The 3′ DNA probe shown in Fig. 1 was used to determine the level of total UGT1A mRNA expressed in liver of WT, Ugt1+/−, and Ugt1−/− mice.

TABLE 1
Serum bilirubin concentrations

| Genotype | Bilirubin | Bilirubin-G | Total |
|----------|-----------|-------------|-------|
| WT, +/−  | 0.13 ± 0.16 | 0.46 ± 0.23 | 0.59 ± 0.30 |
| −/−      | 7.82 ± 1.25 | 0.80 ± 0.25 | 8.62 ± 1.14 |
| (−/−/WT, +/−) (fold increase) | 59.68 | 1.73 | 14.53 |
| p value  | <0.0001 | 0.005 | <0.0001 |

Longevity studies show that all of the Ugt1−/− neonatal mice die within the first 2 weeks following birth (Fig. 2), demonstrating that interruption of the Ugt1 locus generates a lethal mutation.

In humans, UGT1A1 is solely responsible for generating the mono- and digluconic metabolites of bilirubin (2). Bilirubin digluconic is required for elimination from the liver into the biliary system and is secreted into the intestine. Genetic defects in CN1 patients (33) result in UCB levels usually exceeding 20 mg/dl (350 μmol/liter), which correlate to bilirubin levels of 1.2 mg/dl. In littermates from heterozygous Ugt1+/− offspring that were sacrificed at 5 days and from which serum was collected for blood chemistry analysis, UCB levels from Ugt1−/− mice were 50- to 60-fold more concentrated than in WT or Ugt1+/− mice (Table 1). The normal levels of UCB in 5-day-old mice ranged from 0.1 to 0.3 mg/dl in comparison to 7–10 mg/dl in Ugt1+/− mice. The rise in total bilirubin results from the accumulation of UCB. There were no differences in total or indirect bilirubin levels between WT and Ugt1−/− mice. Markers of liver injury such as serum alanine aminotransferase and aspartate aminotransferase were normal in Ugt1−/− mice along with several other serum analyte measurements (see “Experimental Procedures”) indicating there is no obstruction of biliary flow or kidney damage (data not shown).

The Ugt1 Locus Is Non-functional in Ugt1−/− Mice—Using livers from Ugt1−/−, Ugt1+/−, and WT mice, microsomes were prepared from 5-day-old neonatal mice for immunoblot analysis using an anti-human UGT1A antibody that was prepared against the conserved carboxyl region of human UGT1A1 and shown to recognize only UGT1A proteins (29). Western blot analysis demonstrated that the anti-human UGT1A antibody reacted with liver microsomal proteins prepared from Ugt1+/−, WT, and adult mice, confirming that the antibody identifies mouse UGT1A proteins (Fig. 3). However, there were no detectable UGT1A proteins in liver microsomal preparations from Ugt1−/− mice. When an immunoblot was performed using a human anti-UGT2B7 antibody, cross-reactive mouse UGT2 proteins were detected in microsomal preparations from Ugt1−/−, Ugt1+/−, and WT mice. The family of UGT2 proteins was not impacted by mutation of the Ugt1 locus. Further analysis of murine UGT1A RNA expression defined the complete lack of UGT1A isoform-specific RNA such as UGT1A1 and UGT1A6 (Fig. 4A). In murine liver, UGT1A1 and UGT1A6 gene products are abundant (34) and detected in 5-day-old Ugt1−/− and WT mice as well as in adult liver. In addition, Northern blot analysis using a probe to exon 5 of the Ugt1 locus confirmed that the interruption of the Ugt1 locus leads to the complete absence of expressed UGT1A-specific RNA (Fig. 4B). Analysis of UGT1A protein and RNA expression in Ugt1−/− mice also indicates that heterozygosity at the Ugt1 locus can be identified by approximately a 50% reduction in expressed RNA and protein when compared with WT mice at the same age.

The complete absence of Ugt1a proteins in Ugt1−/− mice was further investigated by determination of liver UGT activity with microsomes and selective endogenous and exogenous substrates (Fig. 5). In human liver, UGT1A1, UGT1A3, UGT1A4, UGT1A6, and UGT1A9 are the only UGT1A isoforms expressed (15). In murine liver, UGT1A1, UGT1A5, UGT1A6a, UGT1A6b, and UGT1A9 are the predominant UGT1A isoforms that are expressed (34). As expected, conjugation activity using bilirubin as a substrate was not detected in Ugt1−/− mice, whereas it was comparable in WT and Ugt1+/− mice. T4 is also specifically conjugated by human UGT1A proteins (35), and, as expected, we observed a 50% reduction in T4 glucuronidation activity in Ugt1−/− mice and the complete absence of activity in Ugt1+/− mice. The glucuronidation profile for T3 (data not shown) resembles that of T4. However, total serum T3 and T4 levels were no different in serum from 5-day-old Ugt1−/−, Ugt1+/−, or WT mice. By contrast, testosterone, which is a specific substrate for UGT2B proteins in several species (36–38), displays a rate of glucuronidation by liver microsomes that is similar in Ugt1−/− and WT mice and
slightly increased in \textit{Ugt1} \textsuperscript{+/−} mice. Liver glucuronidation activity for 2-hydroxyestrone at position 2 was reduced by 25% in microsomes from \textit{Ugt1} \textsuperscript{+/−} mice further reduced by 75% in \textit{Ugt1} \textsuperscript{−/−} mice, linking this activity to UGT1A activity. However, glucuronidation of the same substrate at position 3 was not affected in \textit{Ugt1} \textsuperscript{+/−} mice and reduced by only 19% in \textit{Ugt1} \textsuperscript{−/−} mice, indicating that the glucuronidation at this position was mainly due to UGT2B proteins, confirming previous findings for conjugation of this substrate in humans (39). Serotonin, a substrate that has been shown to be glucuronidated by human UGT1A6 (40) and proposed as a highly selective \textit{in vitro} probe for UGT1A6 (41), was also assayed, but, surprisingly, the absence of UGT1A proteins diminished the activity by only 20%. This result suggests that serotonin glucuronidation may not be critically linked to murine UGT1A6-directed metabolism, because it is efficiently metabolized by the family of UGT2 proteins in mice.

Of the five exogenous substrates investigated, MPA, imipramine, 4-nitrophenol, 1-naphthol, and 4-MU, all of which are known to be substrates for human UGT1A proteins (14), showed a decrease in glucuronidation activity with liver microsomes from \textit{Ugt1} \textsuperscript{−/−} mice, followed by a highly significant reduction in the \textit{Ugt1} \textsuperscript{−/−} mice. MPA, the active metabolite of the immunosuppressant mycophenolate mofetil, is metabolized as an MPA-phenylglucuronide by several UGT1A proteins (42–46) and as an MPA-acetylglucuronide by UGT2B4 (46) and UGT2B7 (45). MPA-phenolglucuronide is reduced by almost 50% in liver from \textit{Ugt1} \textsuperscript{−/−} mice and is further decreased to near undetectable levels in the \textit{Ugt1} \textsuperscript{−/−} mice, demonstrating that in liver MPA is a specific UGT1A substrate. Interestingly, the production of MPA-acetylglucuronide was reduced by 43% in liver from \textit{Ugt1} \textsuperscript{−/−} mice, indicating that both UGT2 and UGT1A proteins are involved in the formation of that metabolite. Although small phenols have been historically categorized as UGT1A substrates, our data indicate that glucuronidation of these agents with microsomes from \textit{Ugt1} \textsuperscript{−/−} mice confirms they serve as efficient substrates for the other members of the UGT2 family of proteins.

The Impact of UGT1A Loss on Cellular Metabolism—In an effort to understand the impact of UGT1A deficiency and UCBl-directed toxicity, a microarray experiment was designed to compare gene expression patterns in liver between 5-day-old WT and \textit{Ugt1} \textsuperscript{−/−} mice. These experiments were conducted using Lumina microarray chips that encompassed the most current assembly of the mouse genome. When the averages of RNA expression values from three WT livers were compared with those from \textit{Ugt1} \textsuperscript{−/−} livers, increases in \textit{Ugt1} \textsuperscript{−/−}/WT RNA expression profiles indicated that >350 genes were being expressed 1.5-fold or greater in \textit{Ugt1} \textsuperscript{−/−} mice, with >75 being expressed at 2.5-fold and greater. The loss of UGT1A function was concordant with reduced gene expression in \textit{Ugt1} \textsuperscript{−/−} mice, with >300 genes being underexpressed >1.5-fold. When sta-
statistically significant alterations in gene profiling were categorized into functional groups, it could be seen that aspects of normal cellular metabolism and function were potentially affected in Ugt1−/− mice (Table 2). For example, genes linked to cell cycle dependence are significantly repressed, whereas genes linked to the inhibition of cellular kinases are induced (Fig. 6). Other important pathways such as those linked to fatty acid and pyrimidine metabolism as well as steroid metabolism show a significant degree of change in Ugt1−/− liver. These combined changes in gene expression in developing hepatocytes would be anticipated to have a significant impact on the early phases of liver development, and potentially contribute to the overall toxicity associated with the mutation.

Examination of gene expression profiles linked to xenobiotic metabolism shows a number of impressive regulated gene expression patterns (Fig. 7). We have confirmed similar patterns of expression for CYP2B9, heme oxygenase (HO-1), cholesterol 7α-hydroxylase (CYP7A1), and CYP4A10 RNA by real-time PCR (data not shown). Induction of HO-1, which catabolizes heme to bilirubin, occurs under situations of oxidative stress and is felt to be an adaptive response to injury and disease (47, 48). However, with increasing concentrations of serum bilirubin resulting from Ugt1a1 deficiency, the induction of HO-1 would add to the already heightened bilirubin accumulation. Cholesterol is metabolized to either steroids or bile acids in mammals. CYP7A1 is liver-specific and converts cholesterol into 7α-hydroxycholesterol producing primarily cholic acid (49). In the intestine, bile acids play an important role in facilitating the uptake of fats, sterols, and fat-soluble vitamins (49). The only other genes that are repressed to the same degree as Cyp7a1 (−3.0) are the deficient Ugt1a1 genes. In Cyp7a1−/− mice, the mutation leads eventually to liver failure and postnatal lethality (50). We might predict from this result that the Ugt1−/− mice are also deficient in bile acid synthesis, which might contribute to a lack of nutrient and vitamin uptake through their diet (49). It is also interesting to note that several of the genes that encode some of the major regulatory factors of xenobiotic metabolism, such as PXR (51) and CAR (52), are also induced in Ugt1−/− mice, potentially linking their regulation to the induced levels of several of the Phase I genes.

**DISCUSSION**

An animal model has been developed by introducing a genetic lesion into the common exon 4 of the Ugt1 locus that displays physiological conditions similar to what is observed in human CN1 patients. Neonatal Ugt1−/− mice accumulate severe levels of UCB that results in death within 2 weeks following birth. The interruption of the reading frame in exon 4 leads to a complete absence of UGT1A RNA and protein, similar to what is observed in a CN1 patients. The accumulation of UCB resulting from non-hemolytic hyperbilirubinemia is attributed to the presence of the UGT1A1*28 polymorphism, which is linked to Gilbert syndrome, or sense mutations in any of the five UGT1A1 exons that results in either CN1 or CN2 disease (1). Criger-Najjar type 2 is less severe resulting from less dramatic missense mutations or heterozygous expression of mutant and normal alleles and is treatable by phototherapy or inducing the UGT1A1 gene with phenobarbital therapy (53). More severe lesions are associated with CN1, an inborn error of metabolism disease that results in stop mutations or frameshifts and renders either the UGT1A1 gene or the entire UGT1 locus non-functional. Interruption of exon 4 in Ugt1−/− mice inactivates the entire murine Ugt1 locus resulting in neonatal death, thus resembling the toxicity patterns seen in CN1 patients.

The most pronounced phenotype in the Ugt1−/− mice is observable neonatal jaundice and serum UCB levels that range from 40- to 60-fold normal neonatal indirect bilirubin values. Indirect bilirubin values in humans that are 20- to 40-fold those measured in normal individuals (14) have been linked to brain damage following the accumulation of bilirubin in the basal ganglia and brainstem nuclei. Experiments have not been performed at this time to examine the impact of the hyperbilirubinemia on CNS toxicity in Ugt1−/− mice. The role of UGT1A1 as the sole enzyme involved in bilirubin glucuronidation in humans has been definitively established in patients with CN1 through linkage studies of mutations only in the unique exon of the UGT1A1 gene (2). Although it is unclear if mouse UGT1A1 serves as the only enzyme tied to bilirubin glucuronidation, interruption of the Ugt1 locus through lesions in one of the conserved exons disrupts all of the Ugt1a1 genes followed by the early development of hyperbilirubinemia. There is no detectable bilirubin glucuronidation activity in liver microsomes from Ugt1−/− mice. Over 77 genetic lesions in exons 1 through 5 of the human UGT1A1 gene have been reported and tied to either CN1 or CN2 (54). Although there exists a wide spectrum of UGT1A1 mutations that are associated with the onset of hyperbilirubinemia, little information exists on the biochemical outcome of the UGT1A1 mutations that lead to bilirubin induced toxicities.

In post-mortem evaluation of patients with hyperbilirubinemia, necrosis as well as neuronal degeneration is evident in regions of bilirubin deposition (55). Studies using cultured neurons and astrocytes have provided some evidence for the induction of apoptosis after elevated bilirubin exposure. Low concentrations of unconjugated bilirubin (<5 μM) that more accurately reflect in vivo exposures demonstrate poly(ADP-ribose) polymerase cleavage, DNA fragmentation, cytochrome c release, and mitochondrial membrane permeabilization (56–

**TABLE 2**

List of functional pathways that are altered significantly in Ugt1−/− liver compared to WT liver

| Pathway description                        | p value     |
|--------------------------------------------|-------------|
| Cell cycle                                 | 0.504 E-18  |
| Pentose and glucurionate interconversions  | 0.340 E-14  |
| Porphyrin metabolism                       | 0.472 E-13  |
| Starch and sucrose metabolism              | 0.134 E-12  |
| Androgen and estrogen metabolism           | 0.337 E-12  |
| Metabolism of xenobiotics                  | 0.337 E-11  |
| Fatty acid metabolism                      | 0.810 E-09  |
| Pyrimidine metabolism                      | 0.336 E-07  |

In ranking genes of interest we utilized a method similar to that described by Cole et al. (71) for their software package Focus, and we calculated p values for groups of genes. For pathway analysis, the groups of genes are defined by the individual pathways. The pathways included are all those listed in the Kyoto Encyclopedia of Genes and Genomes and present in the commercial BioCarta Database (BioCarta, San Diego, CA). As the p values increase, the pathways become less and less significant. Based upon the ordering, the cell cycle pathways are the most significant. Shown are only those pathways with a p value of <10−5, because p values greater than this substantially increase the possibility of false positives.
FIGURE 6. Microarray analysis of cell cycle genes. Total RNA from 5-day-old neonatal WT and Ugt1−/− mice were used in microarray analysis. Using the Illumina Mouse 6 Sentrix Expression BeadChip, the expression of each gene in either WT of Ugt1−/− mice was analyzed in triplicate using three independent liver RNA samples. Shown is a heat map for gene clusters that are linked to the cell cycle.
Bilirubin also activates the mitogen-activated protein kinases p38, Jun N-terminal kinase 1/2, and extracellular signal-regulated kinase 1/2 as well as NF-κB adding to the complexity of cellular signaling pathways involved in neuronal and astrocyte cell death (59–61). The activation of the inflammatory and signaling pathways is directly associated with activation of apoptosis, but the initiating stimulus remains elusive. Thus, Ugt1−/− mice may serve as an excellent model to examine the physiological and biochemical implications of severe hyperbilirubinemia as well as those consequences associated with selective cellular signaling events.

Previous communications have indicated that the homozygous j/j Gunn rat model mimics the clinical symptoms seen in CN1, because the single base pair mutation in exon 4 of the rat UGT1 locus leads to a premature stop codon, and the rats develop spontaneous hyperbilirubinemia (24). Total serum bilirubin levels in Gunn rats are slightly lower (7.15 mg/dl) than those detected in Ugt1−/− mice (8–10 mg/dl). Gunn rats have served as an excellent animal model to examine the impact of elevated UCB on spontaneous hydroencephalitis as well as the potential of gene therapy for long term correction of hyperbilirubinemia (62). But there are several significant differences in the phenotype between the Gunn rat and Ugt1−/− mice. The most dramatic is reflected in the immediate health of the animals, with the accumulation of UCB in Gunn rats having no impact on neonatal morbidity or the ability of the rats to reproduce. In contrast, the genotype associated with interruption of exon 4 in Ugt1−/− mice predisposes newborns to the toxic effects associated with elevated UCB leading to death within 11 days. Although we observed a 2 mg/dl difference in total serum bilirubin from that in Gunn rats, this difference may be sufficient in Ugt1−/− mice to initiate central nervous system toxicity and lethal encephalopathy in neonates. It has also been determined that blood T4 levels in Gunn rats are normal up to 5–10 days after birth but increase by 50% at 15–20 days following birth (63). In agreement with this observation, liver glucuronidation activity toward T4 in Gunn rats is impaired ~50% compared with that observed in Wistar rats (64).

### FIGURE 7.
**Microarray analysis of genes involved in xenobiotic metabolism.** Listed are heat maps of genes categorized as Phase I, Phase II, and transcriptional activation. The cutoff for inclusion are those genes that show a response >1.5 or <−1.5.

58). Bilirubin also activates the mitogen-activated protein kinases p38, Jun N-terminal kinase 1/2, and extracellular signal-regulated kinase 1/2 as well as NF-κB adding to the complexity of cellular signaling pathways involved in neuronal and astrocyte cell death (59–61). The activation of the inflammatory and following birth (63). In agreement with this observation, liver glucuronidation activity toward T4 in Gunn rats is impaired ~50% compared with that observed in Wistar rats (64). However, liver T4 glucuronidation capacity is completely absent in Ugt1−/− mice, which might contribute to elevated levels of cir-
increases in CYP1A2 protein in activated levels of UCB in serum, there is no detectable CYP1A1 or CYP1A proteins. However, at 5 days following birth and at early development, and a host of genes tied to the control of cell cycling are repressed. Other significant changes were noted with fatty acid and steroid metabolism. Impressively, these later changes in gene expression profiles were attributed in part to genes linked to xenobiotic metabolism, such as the Ugt1a genes, because they have been tied to estrogen metabolism and the Cyp4a genes, which play a role in arachidonic acid metabolism (70). Although several of the cytochrome P450 genes such as Cyp2b9 are impressively induced, the repression of Cyp7a1 expression may contribute to poor uptake of ingested fats and fat-soluble vitamins. It is interesting to note that neonatal Ugt1<sup>−/−</sup> mice appear to be malnourished about 1-day before they die as evident by weight differences when compared with their WT and Ugt1<sup>−/−</sup> littermates.

The entire human UGT1 locus has recently been shown to be regulated in transgenic Tg-UGT1 mice in a tissue-specific pattern that closely resembles expression profiles that have been documented in human tissues (18). For example, a significant abundance of the UGT1A proteins accumulates in the small and large intestines of Tg-UGT1 mice (18, 19), similar to what has been observed in human tissue. However, there is little information on how these genes are regulated in other important tissues associated with glucuronidation such as those linked to hormonal and steroid control. Having available a humanized mouse model would help to define the role of human glucuronidation as it pertains to inducibility, tissue specificity, and its role in homeostatic control of hormones and steroids. With the ability to cross-breed Ugt1<sup>−/−</sup> mice with Tg-UGT1 mice, future experiments can be designed with Tg-UGT1/Ugt1<sup>−/−</sup> mice to examine the role of human glucuronidation as it pertains to regulation by tissue, inducibility by xenobiotics, and its role in drug metabolism and toxicity.

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