Cloning and Characterization of cDNAs Encoding Mouse Ugt1.6 and Rabbit UGT1.6: Differential Induction by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin[†,‡]

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ABSTRACT: In this report, cDNAs for mouse liver Ugt1.6 and rabbit liver UGT1.6 have been cloned and characterized. The predicted amino acid sequence of mouse Ugt1.6 is 93% and 78% similar to the rat and human UGT1.6 sequences, respectively, while the rabbit UGT1.6 is 79% and 83% similar to the rat and human UGT1.6 sequences, respectively. To examine the substrate specificities of the proteins encoded by the mouse Ugt1.6 and rabbit UGT1.6 cDNAs, the recombinants were expressed in monkey kidney COS-1 cells. Transfection of the mouse and rabbit recombinants allowed for the expression of the UGT1.6 proteins as determined by immunoprecipitation of newly synthesized protein. The expressed UGTs conjugated small planar phenolic molecules such as 4-nitrophenol, 1-naphthol, and 4-methylumbelliferone. While the bulky phenol 4-hydroxybiphenyl was not a substrate for the enzymes, 2-hydroxybiphenyl was an excellent substrate. Androgens and estrogens were not conjugated by either mouse Ugt1.6 or rabbit UGT1.6. In rodents, UGT1.6 mRNA is expressed constitutively and induced when the animals are treated with the Ah receptor ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Using wild-type mouse hepatoma cells and the Ah receptor deficient class II cells, it was demonstrated that induction of mouse Ugt1.6 was dependent upon a functional Ah receptor complex. However, when New Zealand white rabbits were treated with TCDD and liver mRNA was examined by Northern blot analysis, it was shown that TCDD had no effect on the induction of UGT1.6 mRNA. These results indicate that the Ah receptor is involved in the induction of Ugt1.6 mRNA by TCDD in rodents, but there exist species differences related to the induction and expression of the *UGT1*6* gene by TCDD.

UDP-glucuronosyltransferases (UGTs)[1,2] are a superfamily of catabolic enzymes involved in the glucuronidation of endogenous compounds such as steroid hormones, bilirubin, and bile acids as well as thousands of exogenous compounds that include drugs and xenobiotics. These enzymes are located in the endoplasmic reticulum and are believed to be positioned on the lumenal surface of the membrane (Iyanagi et al., 1986). The role of UGT enzymes in drug metabolism and detoxification occurs through the transfer of the D-glucuronic acid from the cosubstrate UDP-glucuronic acid to aglycons, forming β-D-glucopyranosiduronic acids. The addition of glucuronic acid can be to hydroxyl (alcoholic and phenolic), carboxyl, thiol, amino (primary, secondary, and tertiary), or carbonyl groups. The generation of glucuronides increases the compounds' water solubility and facilitates their excretion from the cell. Results of UGT enzyme purification and the more recent cloning of multiple UGT cDNAs demonstrate that multiple UGT isozymes function as the terminal catabolic process in the elimination of endogenous agents or those substances that are consumed as drugs or through our diet (Dutton, 1980; Burchell & Coughtrie, 1989).

The human UGT1 locus encodes seven UGTs (Ritter et al., 1992). Flanking exons 2–5 in a linear array are seven unique exon 1 sequences, which are individually spliced to exons 2–5 to produce the different RNA transcripts. The flanking exon sequences resemble cassette exons (Smith et al., 1989) and can be included or excluded depending upon which transcript is synthesized. For example, the RNA that encodes human UGT1.6 is a product of transcribed sequences from the sixth unique exon (located in front of five other first unique exons), in addition to the conserved sequences from exons 2–5. The cDNAs encoding the human (Harding et al., 1988) and the rat UGT1.6 (Jackson et al., 1988; Iyanagi et al., 1986) have been characterized and the expressed proteins (Harding et al., 1988; Jackson et al., 1988) have been shown to encode UGT activity toward small planar phenols such as 4-nitrophenol. The predicted amino acid sequences of the rat and human UGT1.6 are 78% similar. The majority of the divergence between the rat and human UGT1.6 occurs in the first 285 amino acids, which is encoded by the first exon. The high degree of UGT1.6 amino acid similarity between evolutionarily divergent species such as rat and human would indicate that the UGT1 locus is most likely conserved in other species such as rabbits.

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[‡] The DNA sequences reported in this paper have been submitted to Genbank under Accession Numbers U09930 (mouse Ugt1.6) and U09030 (rabbit UGT1.6).

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[1] Abbreviations: UGT, UDP-glucuronosyltransferase; 3MC, 3-methylcholanthrene; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; UGT1.6, liver 4-nitrophenol UDP-glucuronosyltransferase; RIPA buffer, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM phenylmethanesulfonyl fluoride, and 50 mM Tris-HCl, pH 7.5.

[2] The nomenclature used for the designation of the UGTs follows a modified format to that already outlined (Burchell et al., 1991). When discussing UGT1 proteins or cDNAs, we have elected to drop the 0* preceding the designation of the first unique exon location. For example, we are classifying UGT1*06 and UGT1*6 as UGT1.6. The asterisk notation, which was originally used to designate those genes and gene products that arose from alternative splicing, is replaced by a period. In the previous nomenclature, the 0 was used to indicate that the exact location of the first exons flanking the common exons of the UGT1 locus were unknown. However, we feel that the similarity in alignment of UGT1 proteins from different species is adequate proof that those proteins are indeed orthologs of the human UGT1 transcripts. Since the asterisk was used to designate the assignment of an exon position in the *UGT1* locus, it is appropriate to continue the use of this designator in discussions of the *UGT1* gene. For example, the gene encoding UGT1.6 will be designated *UGT1*6*. Designation of the murine UGTs is indicated in lower case (i.e., Ugt1.6).

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One of the first UGT enzymes purified from rats and rabbits catalyzed the glucuronidation of 4-nitrophenol (Burchell, 1978; Gorski & Kasper, 1977; Tukey & Tephly, 1981). This enzyme, now termed UGT1.6, displayed UGT activity toward planar phenols such as 4-nitrophenol, 1-naphthol, and 4-methylumbelliferone. The 4-nitrophenol UGT attracted additional attention since it was shown to be induced in rodents by chemical carcinogens such as polycyclic aromatic hydrocarbons (PAHs), in addition to tumor promoters such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Owens, 1977). Since PAHs and TCDD are ligands for the Ah receptor, it was felt that induction of UGT1.6 in rodents was dependent upon a functional Ah receptor. Induction studies in rats have clearly shown that PAHs induce the UGT1.6 mRNA (Iyanagi et al., 1986) and that this process occurs as a result of transcriptional activation (ElAwady et al., 1990). However, the 4-nitrophenol UGT activity appears to be differentially induced between species such as rat and rabbit, with no induction of 4-nitrophenol UGT activity elicited in rabbits following treatment with TCDD (Hook et al., 1975). This would indicate that the regulation of 4-nitrophenol UGT in rabbits may not involve coordination with the Ah receptor.

To examine the role of the Ah receptor and the expression of UGT1.6 in mice and rabbits, the UGT1.6 cDNA in these species has been cloned and characterized. The cDNAs were then used to examine the levels of expression of the UGT1.6 mRNA in rabbits as well as in rodent and mouse hepatoma cells that are deficient in a functional Ah receptor complex.

MATERIALS AND METHODS

Materials. 3-Methylcholanthrene (3MC), phenylmethanesulfonyl fluoride, formalin-fixed *Staphylococcus aureus* cells, tunicamycin, and agarose were purchased from Sigma Chemical Company (St. Louis, MO). TCDD was purchased from Chemsyn Science Laboratories (Lenexa, KA). Polynucleotide kinase was purchased from Promega Corp. (Madison, WI). Restriction endonucleases, T4 DNA polymerase, and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). Nick translation kits and [α-^{32}P]dCTP (3000 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). Oligo(dT)–cellulose was purchased from Boehringer-Mannheim (La Jolla, CA). Reverse transcriptase was purchased from Life Sciences (St. Petersburg, FL). The eukaryotic pSVL expression vector was purchased from Pharmacia (Piscataway, NJ). Genamp DNA amplification kits were purchased from Perkin-Elmer Cetus (Irvine, CA). Glass-backed linear K TLC plates with preabsorbant strips were purchased from Whatman (Hillsbro, OR).

Isolation of RNA and Northern Blot Analysis. Mice were given 250 mg/kg 3MC in corn oil as a single intraperitoneal injection or 100 μg/kg TCDD in DMSO as a single intraperitoneal injection and sacrificed 18–24 h later by cervical dislocation. Rats received 100 μg/kg TCDD in DMSO by a single intraperitoneal injection and were sacrificed 18–24 h later by CO_2 asphyxiation. Rabbits received 10 μg/kg TCDD in DMSO by a single intraperitoneal injection and were sacrificed by phenobarbital injection into an ear vein 48 h following treatment with TCDD. Total RNA was isolated from 2–5 g of liver tissue by the guanidine hydrochloride procedure (Cox, 1968), and poly(A)+ RNA was purified by passing the RNA over a column of oligo(dT)–cellulose (Aviv & Leder, 1972).

Ruber H4IIE rat hepatoma cells (American Type Tissue Culture), mouse hepatoma (Hepa 1c1c7), and class II (BP$^-$) hepatoma cells (gift from Dr. James Whitlock, Stanford University), cultured as described (Postlind et al., 1993), were treated with TCDD at a final concentration of 10 nM for 24 h. Total RNA was isolated from four 150-mm plates by the guanidine hydrochloride procedure (Glisin et al., 1973), followed by centrifugation through cesium gradients (Glisin et al., 1973). Five micrograms of poly(A)+ RNA or 10 μg of total RNA was electrophoresed on a 1% denaturing agarose gel containing formaldehyde and the RNA was transferred to nitrocellulose filters (Sambrook et al., 1989). The RNA-bound filters were annealed with labeled cDNA probes (10^8 cpm/μg) generated by nick translation of the purified cDNA inserts.

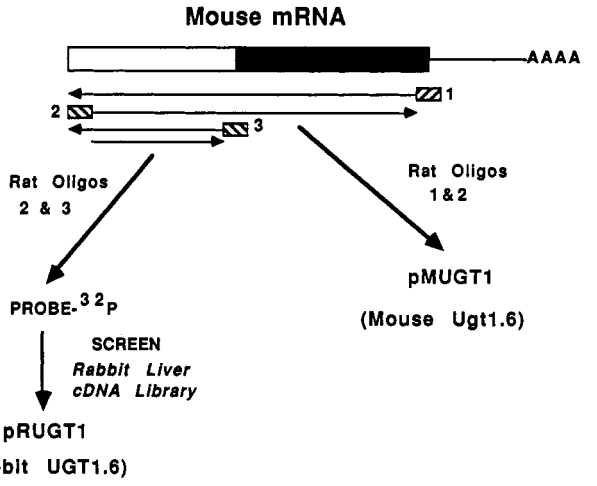

FIGURE 1: Cloning mouse Ugt1.6 and rabbit UGT1.6 cDNA. The scheme for cloning mouse Ugt1.6 and rabbit UGT1.6 is shown. All oligonucleotides were made to match the sequence from the rat UGT1.6 cDNA (Iyanagi et al., 1986). Oligonucleotide 1 was generated to match the sequence from +1610 to +1591 (antisense). Oligonucleotide 2 was generated to match the sequence from −2 to +18 (sense). Oligonucleotide 3 was generated to match the sequence from +470 to +451 (antisense). To generate the mouse Ugt1.6 cDNA, oligonucleotide 1 was used in a reverse transcriptase reaction with poly(A)+ RNA isolated from 3-MC-treated mouse liver. Oligonucleotides 1 and 2 were then used in a PCR reaction with a diluted aliquot (1:100) of the reverse transcriptase reaction. A 1.6-kilobase-pair cDNA fragment was subcloned into a pBluescript (KS) vector for sequencing. The rabbit UGT1.6 was cloned by using oligonucleotides 2 and 3 in a PCR reaction with a diluted aliquot (1:100) of the reverse transcriptase reaction to generate a 450-base-pair fragment. This 450-base-pair fragment was cloned and then used as a Ugt1.6-specific probe to screen a rabbit liver λZAP library.

Cloning of Mouse and Rabbit UGT1.6 cDNAs. An outline of the cloning strategy is shown in Figure 1. Oligonucleotides 5′-GGATGGCTTGCCTTCTTCCT-3′ (sense) corresponding to bases −2 to +18, 5′-TCATGTTATTCCTGTACTCT-3′ (antisense) corresponding to bases +470 to +451, and 5′-CCTTCACTTCCTGCCACTTC-3′ (antisense) corresponding to bases +1610 to +1591 of the rat UGT1.6 cDNA (Iyanagi et al., 1986) were synthesized using an Applied Biosystems 380B DNA synthesizer. In 0.25 M KCl, 10 mM Tris, pH 7.5, and 1 mM EDTA, the 3′-antisense oligonucleotide (bases +1610 to +1591) was mixed with 5 μg of liver mRNA isolated from C57BL/6 mice that had been treated with 3MC. This mixture was heated to 70 °C for 2 min and then cooled to 42 °C over 15 min. To this mixture was added 0.75 mM each of the dNTPs, 3.75 μg of actinomycin D, and 40 units of RNasin in 98 μL containing 75 mM Tris, pH 7.5, 12 mM $MgCl_2$, 15 mM DTT, and the complementary single-strand DNA generated following the addition of 10 units of AMV reverse transcriptase. The reaction was allowed to proceed at 37 °C for 1 h, and 1 μL of the mixture was diluted 1:100 in H_2O. From this dilution, 10 μL was added to two separate

solutions containing 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 0.2 mM each dCTP, dATP, dGTP, and dTTP, and 2.5 units of *Taq* polymerase. To one reaction was added 1 μM sense (bases −2 to +18) oligonucleotide and 1 μM antisense oligonucleotide (bases +470 to +451) in a total volume of 100 μL. With a Perkin-Elmer Cetus DNA thermal cycler, the samples were denatured at 94 °C for 1 min, annealed at 37 °C for 1 min, and extended at 72 °C for 3 min. Primer extension was repeated for 30 cycles with the duration of the extension segment at 72 °C increased by 3 s/cycle. This 450-base-pair fragment was subcloned into the *Eco*RV site of pBluescript KS+. To a second reaction was added 1 μM sense oligonucleotide and 1 μM antisense (bases +1610 to +1591) oligonucleotide. This 1.6-kilobase-pair PCR fragment was subcloned into the *Sma*I site of pBluescript KS+ and identified as pMUGT1. Both cDNAs were characterized by DNA sequence analysis (Vieira & Messing, 1982).

To clone the rabbit UGT1.6 cDNA, a cDNA library was constructed from NZW adult rabbit liver mRNA in λZAP, as described previously (Tukey et al., 1993). The 450-base-pair mouse Ugt1.6 cDNA was labeled by nick translation and the probe was used to screen the cDNA library. From approximately 50 000 colonies, two clones were purified. The largest recombinant, pRUGT1, contained a 2.1-kilobase-pair cDNA insert, and this plasmid was further characterized by DNA sequence analysis (Vieira & Messing, 1982).

Expression of Mouse and Rabbit cDNAs in COS-1 Cells. The mouse Ugt1.6 cDNA was removed from pMGT1 following *Sma*I/*Hin*dIII digestion, and the purified insert was blunt-ended using the large fragment of DNA polymerase I (Klenow). The insert was then subcloned into the *Sma*I site of the eukaryotic expression vector pSVL, and this vector is referred to as pSVLM1. The rabbit cDNA was removed from pRUGT1 by digestion of the plasmid with *Xho*I/*Xba*I, and the purified insert was subcloned into the *Xho*I/*Xba*I site of pSVL. The rabbit expression vector is referred to as pSVLR1.

DNA transfection experiments were conducted as outlined (Zuber et al., 1986). COS-1 cells were grown to 50% confluency in Dulbecco's modified Eagle's medium (DMEM) in either 35-mm or 150-mm diameter plates, supplemented with 5% fetal calf serum and 5% Nu-serum. After the cells were washed twice with PBS, they were transfected by incubation with DMEM, 5 μg/mL plasmid DNA, 0.25 mg/mL DEAE-dextran (MW, 500 000), and 20 mM Hepes, pH 7.5. The transfection medium was aspirated and cells were incubated for 5 h in medium containing 100 μM chloroquine. After the cells were washed twice with PBS, they were further incubated for 48 h in culture medium.

Tunicamycin Treatment, ^{35}S-Labeling, and Immunoprecipitation. Approximately 24 h after transfection, cells from a single 35-mm tissue culture dish were supplemented with fresh medium that contained either 1 μg/mL tunicamycin (T+) or no tunicamycin (T−). Forty-eight hours after transfection (24 h with tunicamycin), the cells were washed three times with Hank's balanced salt solution and incubated for 4 h in cysteine- and methionine-free modified eagle's medium supplemented with 100 μCi/mL [^{35}S]methionine (ICN). During the labeling period the medium of the tunicamycin-treated cells (T+) was again supplemented with the drug (2 μg/mL). The cells were then washed twice in PBS and lysed on ice in 600 μL of RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM phenylmethanesulfonyl fluoride, and 50 mM Tris-HCl, pH 7.5). Cellular DNA was broken by shearing the solution through a 25-gauge needle, and cellular debris was removed by centrifugation in an Eppendorf microfuge.

Immunoprecipitation of labeled proteins was carried out as described (Straub et al., 1993). The cleared supernatant was incubated for 4 h at 4 °C with 22 μg of the IgG fraction of an antiserum raised against purified rabbit 4-nitrophenol UGT (Tukey & Tephly, 1981; Green et al., 1988). After a 20-min incubation of the lysates with 80 μL of a 10% suspension of formalin-fixed *S. aureus* cells in RIPA buffer, unbound material was removed by washing the cells four times in RIPA buffer and once in 50 mM Tris-HCl, pH 7.5. The *S. aureus* cells were suspended in 100 μL of loading buffer (63 mM Tris-HCl, pH 6.8, containing 2% SDS, 5% glycerol, 5% β-mercaptoethanol, and 0.02% bromophenol blue), and the radiolabeled antigens were released by boiling the sample for 5 min followed by removal of the cells by centrifugation in an Eppendorf microfuge. The immunoprecipitated proteins were analyzed by SDS–polyacrylamide gel electrophoresis on an 8% polyacrylamide gel. The gels were stained with Coomassie blue to visualize protein standards, and the gel was treated with 1M sodium salicylate for 30 min before drying. Radiolabeled bands were detected by fluorography at −70 °C.

Transferase Assays. From five 150-mm tissue culture plates transfected with plasmid, the cell pellet was homogenized in 2 mL of 0.1 M potassium phosphate buffer, pH 7.4. UGT activities were determined using a modified procedure from Bansal and Gessner (1980). Reactions were performed in 50 mM Tris buffer, pH 7.6, containing 10 mM $MgCl_2$, 1 mM UDPGA, 0.04 μCi of ^{14}C-UDPGA, 0.1 mM substrate, and 100 μg of COS-1 cell extract protein. When 2-aminophenol was assayed, 1 mM substrate was used. The final volume was 100 μL. Reactions were incubated at 37 °C for 1 h and the reactants extracted with 200 μL of 100% ethanol. The protein was removed by centrifugation at 12000*g* for 5 min in an Eppendorf centrifuge. The supernatant was dried and resuspended in 30 μL of 100% methanol and spotted on a Whatman glass-backed linear K TLC plate. Chromatography was performed in a mixture of 1-butanol/acetone/acetic acid/water (35:35:10:20). The TLC plate was then dried and exposed to X-ray film. The spots containing the glucuronides were removed and quantitated by placing the appropriate portion of the TLC cellulose in 10 mL of Ecolite scintillation cocktail and counting in a Beckman scintillation counter. The substrates selected were 1-naphthol, 4-nitrophenol, 4-methylumbelliferone, 4-hydroxybiphenyl, 2-hydroxybiphenyl, estrone, testosterone, and 2-aminophenol. The assays were performed with concentrations of protein and incubation times that were linear for product (glucuronide) formation. Under these conditions, the expressed UGT1.6 proteins were capable of forming approximately 150 pmol of glucuronide/h.

RESULTS

Characterization of the Rabbit and Mouse cDNAs. When all of the UGT amino acid sequences are aligned, the 3′ regions of the proteins are highly related, while the 5′ regions show considerable divergence (Burchell et al., 1991). However, the rat and human 4-nitrophenol UGT1.6 proteins have evolved at similar rates and are 80% related in amino acid sequence. For this reason, we rationalized that the 5′ region of the rat mRNA UGT1.6 mRNA would be related in sequence to mouse and rabbit mRNA that may encode proteins with similar substrate specificities. Since the mouse and rabbit 4-nitrophenol UGT1.6 RNAs have not been cloned, experiments were initiated to characterize these gene products. Using liver mRNA isolated from 3MC-treated C57BL/6 mice, oligo-

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UGT1.6l   - MACLLSAAQRASAGVLFVALWGTVLGDRLLVVPQDGSHWLSMQDIVEALG -50
UGT1.6m   - MACLLPAAQTLPAGFLFLVLWASVLGDKLLVVPQDGSHWLSMKEIVEHLS -50

UGT1.6l   - ARGHEIVVLVPEVNLLLRESRFYTRRIYPVPFDQEEQSYRYRTFGEKHFT -100
UGT1.6m   - ERGHDIMVLVPEVNLLLGESKYYRRKIFSVTYSLEELQTRFRTFGNNHFL -100

UGT1.6l   - DRSWLSGPQTEYRNNMVVIDMYFINCQSLLRHGDTLDFLRAGKFDALFTD -150
UGT1.6m   - PGASLMGPLREYRNNMIVVDMFFSNCQSLLKDSATLSFLRENKFDALFTD -150

UGT1.6l   - PALPCGVILAEYLGLPSVYLFRGFPCSLEHGFGGSPNPVSYIPRCYTKFS -200
UGT1.6m   - PAMPCGVILAEYLNLPSVYLFRGFPCSLEHMLGQSPSPVSYVPRFYTKFS -200

UGT1.6l   - DQMSFPQRVVNFLVNLLEVPLFYCLYSKYEDLAVELLKREVDLPTLFQKD -250
UGT1.6m   - DHMTFPQRLANFIVNILENYLYYCLYSKYEIIASDLLKRDVSLPSL-HQN -249

UGT1.6l   - PVWLLRYDFVFEYPRPVMPNMVLIGGINCKKPDVLSQEFEAYVNASGEHG -300
UGT1.6m   - SLWLLRYDFVFEYPRPVMPNMIFLGGINCKKKGKLTQEFEAYVNASGEHG -299

UGT1.6l   - IVVFSLGSMVSEIPEKKAMEIADALGKIPQTVLWRYTGSRPSNLAKNTYL -350
UGT1.6m   - IVVFSLGSMVSEIPEKKAMEIAEALGRIPQTVLWRYTGTRPSNLAKNTIL -349

UGT1.6l   - VKWLPQNVLLGHPKTRAFITHSGSHGIYEGICNGVPMVMLPLFGDQMDNA -400
UGT1.6m   - VKWLPQNDLLGHPKTRAFITHSGSHGIYEGICNGVPMVMMPLFGDQMDNA -399

UGT1.6l   - KRIETRGAGVTLNVLEMTSDDLANALKTVINDKSYKENIMRLSSLHKDRP -450
UGT1.6m   - KRMETRGAGVTLNVLEMTADDLENALKTVINNKSYKENIMRLSSLHKDRP -449

UGT1.6l   - VEPLDLAVFWVEFVMRHKGAAP-RPAAHDLTWYQYHSLDVIGFLLAIVLT -499
UGT1.6m   - IEPLDLAVFWVEYVMRKKGAPHLRPAAHDLTWYQYHSLDVIGFLLAIVLT -499

UGT1.6l   - VAFVTFKCCAFAWGKCFGKKGRVKKAHKSKVH -531
UGT1.6m   - VVFIVFKCCAYGCRKCFGGKGRVKKSHKSKTH -531
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FIGURE 2: Alignment of rabbit and mouse UGT1.6. Based upon the position of the largest open reading frame, an alignment of the predicted amino acid sequences derived from the rabbit and mouse cDNAs is shown. The sequences are designated UGT1.6l, which was derived from the rabbit cDNA clone, and Ugt1.6m, which was derived from the mouse clone. Solid lines indicate perfect homology of amino acids. Alignments were performed using the PALIGN program in PCGENE (Intelligenetics Inc.).

nucleotides encoding the rat UGT1.6 mRNA were used to generate by PCR analysis a 1650-base-pair fragment that spanned the entire coding region (Figure 1). In addition, a 450-base-pair fragment designed to encode part of exon 1 was generated and used as a probe to screen a rabbit liver λZAP cDNA library (Figure 1). From these studies, a mouse recombinant, pMGT1, generated from PCR analysis, and a rabbit liver cDNA, pRUGT1, were characterized by DNA sequence analysis.

Clone pRUGT1 contains an open reading frame of 1592 bases that encodes a predicted protein of 531 amino acids. The cDNA contains 95 base pairs of 5′ untranslated and 370 base pairs of 3′ untranslated DNA but does not contain a consensus polyadenylation signal or a poly(A) tract, indicating that the cDNA represents a truncated cDNA transcript. The recombinant mouse cDNA, generated by PCR analysis, is 1592 base pairs in length and encodes a predicted protein of 531 amino acids. Both DNA sequences can be obtained from GenBank.[3]

When the predicted amino acid sequences encoded by the mouse and rabbit UGT cDNAs were aligned, they displayed 78% identity (Figure 2). The mouse Ugt displayed 93% and 78% identity with the rat (Iyanagi et al., 1986) and human (Harding et al., 1988) UGT1.6 transferases, respectively, while the protein encoded by the rabbit UGT displayed 79% and 83% identity with the rat and human UGT1.6 transferases, respectively.

In humans, it is thought that generation of the individual UGT1 RNAs occurs by an RNA splicing mechanism of the

[3] The DNA sequence of the mouse Ugt1.6 (accession number U09930) and rabbit UGT1.6 (accession number U09030) can be obtained from Genbank.

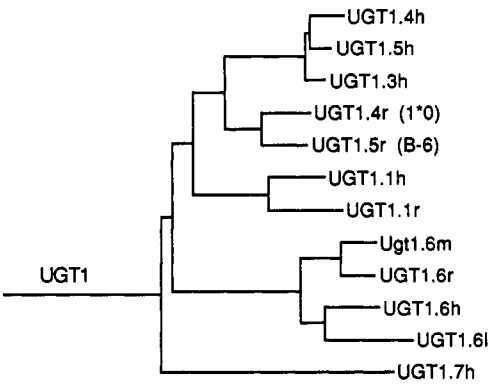


FIGURE 3: Phylogenetic tree showing relationships between the predicted amino acid sequences of the UGT1 proteins. The tree was generated by progressive alignment (Doolittle, 1990). Each sequence is followed by a lower case letter designating the species, i.e., h, human; r, rat; m, mouse; and l, legamorph. Shown in parentheses are the trivial names assigned by the different laboratories. Shown are human UGT1.3 and UGT1.5, which are predicted from the gene sequences (Ritter et al., 1992); rat UGT1.4 (Sato et al., 1990); rat UGT1.5, which is predicted from clone B-6 (Iyanagi, 1991); human UGT1.1 and UGT1.4 (Ritter et al., 1991); rat UGT1.1, predicted from clone A-18 (Iyanagi, 1991); rat UGT1.6 (Iyanagi et al., 1986); human UGT1.6 (Harding et al., 1988); and human UGT1.7 (Wooster et al., 1991). The rabbit UGT1.6, designated as UGT1.6l, and mouse Ugt1.6 sequences are from Figure 2.

seven individually regulated 5′ nested first exon sequences with four shared exons (Ritter et al., 1992). Exon 1 of the human UGT1.6 encodes the first 285 amino acids. Within this region, the proteins encoded by the mouse and rabbit UGTs are 69% identical. The mouse Ugt is 69% related to the human UGT1.6, while the rabbit UGT is 75% similar to the human UGT1.6. Over this same region the proteins encoded by the mouse pMGT1 and rabbit pRUGT1 cDNAs are 88% and 69% identical, respectively, to the rat UGT1.6 (Iyanagi et al., 1986). When the mouse and rabbit sequences were aligned with the UGT1 sequences from different species, the proteins encoded by pMGT1 and pRUGT1 cluster with the other UGT1.6 sequences (Figure 3). This result allows us to predict that the proteins encoded by pMGT1 and pRUGT1 can be classified as the mouse Ugt1.6 and rabbit UGT1.6.

Expression of the Mouse and Rabbit cDNAs. Additional confirmation that the rabbit and mouse recombinants encode the UGT1.6 proteins can be derived from expression of the recombinant proteins. Purified preparations of rabbit and rat UGT1.6 (Falany & Tephly, 1983; Gorski & Kasper, 1977; Tukey & Tephly, 1981), as well as recombinant rat and human UGT1.6 (Harding et al., 1988; Jackson et al., 1988), catalyze the glucuronidation of small planar phenols such as 4-nitrophenol, 1-naphthol, and 4-methylumbelliferone. Bulky phenols such as 4-hydroxybiphenyl and steroids are poor substrates for these enzymes. To examine the substrate specificities of the mouse Ugt1.6 and rabbit UGT1.6, the cDNAs were expressed in COS-1 cells.

Transfection of the expression vectors pSVLM1 and pSVLR1 into COS-1 cells resulted in the synthesis of glycosylated proteins of 55 and 54 kDa, respectively (Figure 4). Since both proteins contain potential N-linked glycosylation sites (rabbit, Asn^{294}; mouse, Asn^{293} and Asn^{431}), transfected cells were treated with tunicamycin to inhibit asparagine-linked glycosylation. Both proteins exhibited a reduced molecular weight, indicating that N-linked glycosylation occurs as a posttranslational event. Together, these results demonstrate that recombinant rabbit UGT1.6 and mouse Ugt1.6 can be efficiently expressed in COS-1 cells.

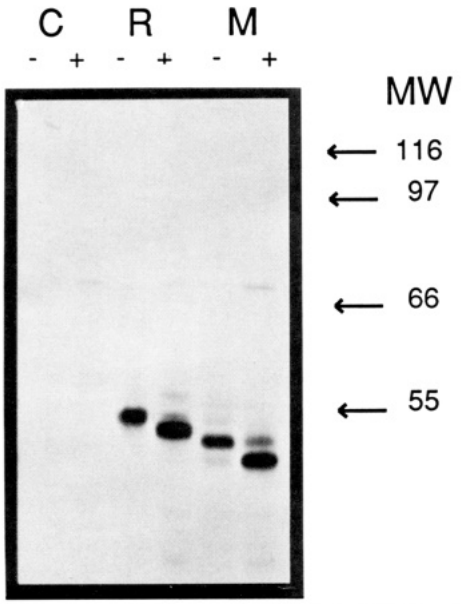


FIGURE 4: Immunoprecipitations of radiolabeled COS-1 cell extracts. COS-1 cells were transfected with expression vectors pSVLR1 (R) and pSVLM1 (M). Control (C) cells were transfected with the pSVL plasmid. As outlined in Materials and Methods, one plate from each transfection was treated (+) with tunicamycin. Forty-eight hours after transfection, cells were labeled for 4 h with 100 μCi/mL [^{35}S]-methionine/cysteine. After the cells were lysed and incubated with antibody, the antigen/antibody complexes were precipitated with formalin-fixed *S. aureus* cells and subjected to SDS–PAGE on an 8% acrylamide gel. The gel was stained with Coomassie blue, impregnated with 1 M sodium salicylic acid as a fluorographic enhancer, dried, and exposed at –70 °C overnight.

Whole cell extracts were prepared from cells transfected with pSVLM1 or pSVLR1 as well as COS-1 cells mock-transfected with calf thymus DNA. UGT assays were performed using planar and bulky phenols as well as steroids as substrates. The mouse Ugt1.6 and rabbit UGT1.6 catalyzed the glucuronidation of 1-naphthol, 4-nitrophenol, 4-methylumbelliferone, and 2-aminophenol (Figure 5). COS-1 cells mock-transfected with calf thymus DNA showed no activity toward these substrates. No glucuronides were observed when the steroids estrone and testosterone (not shown) were used as substrates.

A characteristic of the human UGT1.6 is the inability to glucuronidate bulky phenols such as 4-hydroxybiphenyl (Jackson et al., 1988; Ebner & Burchell, 1993). The expressed rabbit UGT1.6 and mouse Ugt1.6 are no exception, since neither protein generated 4-hydroxybiphenyl glucuronide. However, 2-hydroxybiphenyl, which is a bulky phenol with the same molecular mass as 4-hydroxybiphenyl but with different steric properties, serves as a very efficient substrate for glucuronidation by both the expressed rabbit UGT1.6 and mouse Ugt1.6 (Figure 6). The rate of 2-hydroxybiphenyl glucuronidation was 80% the value achieved when 4-nitrophenol was used as substrate.

Expression of Mouse mRNA in BP Cells. The treatment of rats with TCDD or 3MC induces 4-nitrophenol UGT activity in liver, in addition to induction of the UGT1.6 mRNA (Iyanagi et al., 1986). Studies examining the induction of 4-methylumbelliferone glucuronidation by 3MC and TCDD in Ah receptor-responsive and -deficient mice has suggested that induction of the 4-methylumbelliferone UGT is regulated by the Ah receptor (Owens, 1977). Once the mouse Ugt1.6 mRNA, which encodes a protein that catalyzes 4-methylumbelliferone glucuronidation, was cloned, studies were undertaken to examine the role of the Ah receptor in the induction of Ugt1.6 mRNA.

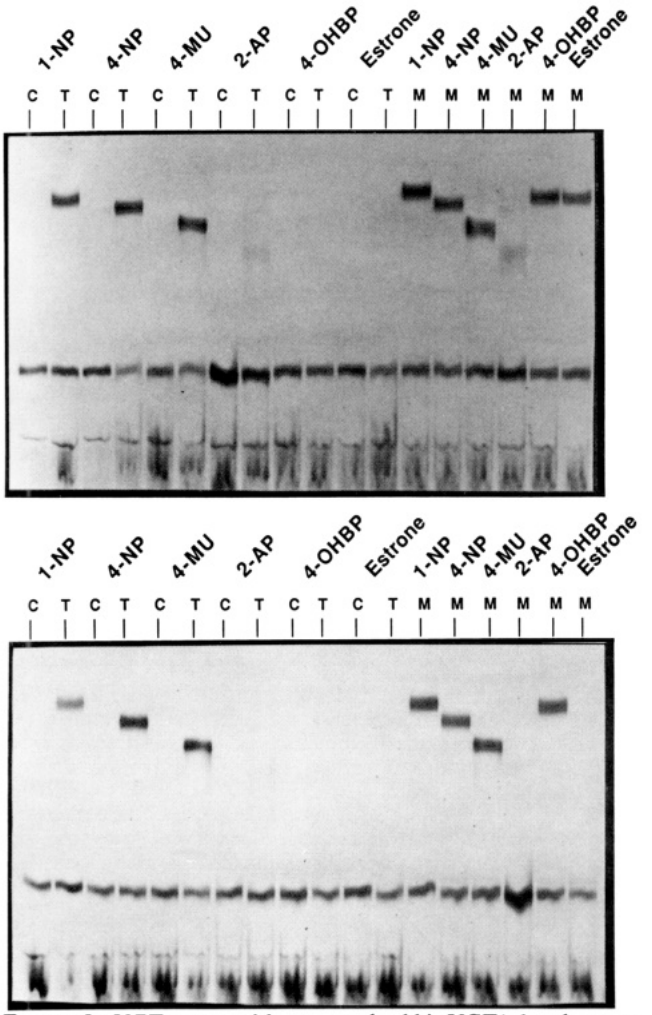
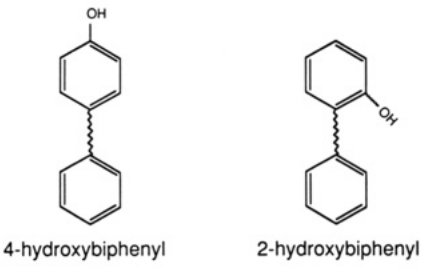


FIGURE 5: UGT assays with expressed rabbit UGT1.6 and mouse Ugt1.6 cDNAs. The rabbit pSVLR1 (top panel) and mouse pSVLM1 (bottom panel) plasmids were transfected into COS-1 cells as outlined in Materials and Methods. Total cell homogenates were prepared and UGT assays were performed as described (Bansal & Gessner, 1980). Assays were performed using 100 μg of COS-1 cell extracts from cells that were mock-transfected with calf thymus DNA (C) or transfected with pSVLR1 or PSVLM1 (T) or with 10 μg of rabbit and mouse liver microsomes (M). Substrates shown are 1-naphthol (1-NP), 4-nitrophenol (4-NP), 4-methylumbelliferone (4-MU), 2-aminophenol (2-AP), 4-hydroxybiphenyl (4-OHBP), and estrone. TLC plates were exposed for 7 days at –70 °C.

Using the region of the Ugt1.6 cDNA that encodes only exon 1 as a probe, it was demonstrated that UGT1.6 mRNA in Reuber H4IIE rat hepatoma cells and mouse hepatoma cells was induced following exposure of these cells with 10 nM TCDD (Figure 7). The 5-fold induction of UGT1.6 mRNA in the Reuber H4IIE cells by TCDD was comparable to the levels of induced 4-methylumbelliferone UGT activity (Malik et al., 1979). Transcriptional induction of the *Cyp1a*-1 gene by TCDD and the accumulation of Cyp1a-1 mRNA, a phenomenon reported to involve ligand-dependent activation of the Ah receptor (Okino et al., 1992), was also observed. When the class II variant BP$^-$ cells, which are unable to translocate the Ah receptor complex to the nucleus (Miller et al., 1983; Israel & Whitlock, 1984; Jones et al., 1985), are treated with TCDD, there was no induction of Ugt1.6 mRNA. The induction of mouse Ugt1.6 and Cyp1a-1 mRNA in wild-type cells and the lack of Ugt1.6 mRNA induction in BP$^-$ cells indicates involvement of the Ah receptor, since there was also no induction of the Ah receptor-regulated Cyp1a-1 mRNA in the BP$^-$ cells.



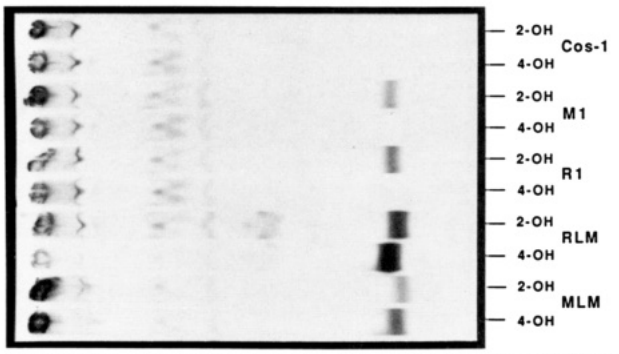


FIGURE 6: Glucuronidation of 2- and 4-hydroxybiphenyl. The rabbit pSVLR1 (R1) and mouse pSVLM1 (M1) cDNAs were transfected into COS-1 cells as outlined, and total cell homogenates were used to assay for glucuronide formation using 2- and 4-hydroxybiphenyl as substrates. Cells were mock-transfected (Cos-1) with calf thymus DNA. As controls, rabbit and mouse liver microsomes, designated as RLM and MLM, were also included. For the cell extracts, 100 μg of protein was used, while 10 μg of microsomal protein was employed.

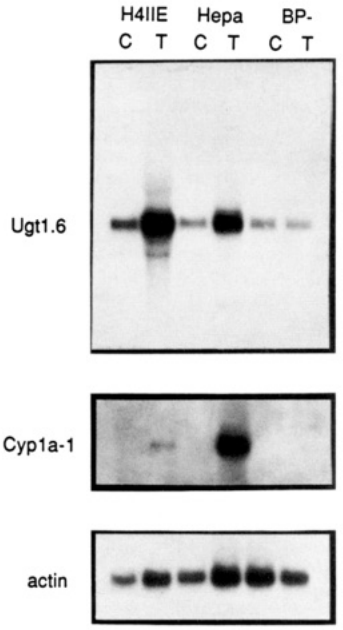


FIGURE 7: Induction of UGT1.6 mRNA by TCDD in vitro. Total RNA was isolated from rat H4IIE and mouse Hepa and BP-hepatoma cell lines, and 10-μg portions were subjected to electrophoresis in a 1% denaturing agarose gel. The RNA was transferred to nitrocellulose filters and probed with a ^{32}P-labeled cDNA probe corresponding to the first 754 base pairs of the mouse Ugt1.6 clone. The filters were then reprobed with a cDNA probe specific for mouse Cyp1a-1 (Okino et al., 1992).

Expression of UGT1.6 mRNA in Rats, Mice, and Rabbits. When rodents and rabbits were treated with TCDD, significant variation in the relative accumulation of UGT1.6 mRNA was detected between the different species. Northern blot analysis using the 5′ divergent region of the mouse cDNA demonstrated that a 24-h treatment with TCDD induced rat liver UGT1.6 mRNA by over 20-fold (Figure 8). High levels of induced UGT1.6 mRNA have also been observed when rats were treated with 3MC (ElAwady et al., 1990). However, the actions of TCDD on the induction of mouse live Ugt1.6 mRNA were not as dramatic. While the response was very reproducible, TCDD elicited only a 2–3-fold induction of mouse Ugt1.6 mRNA. With both rats and mice, the actions of TCDD induced significant increases in CYP1A1 mRNA.

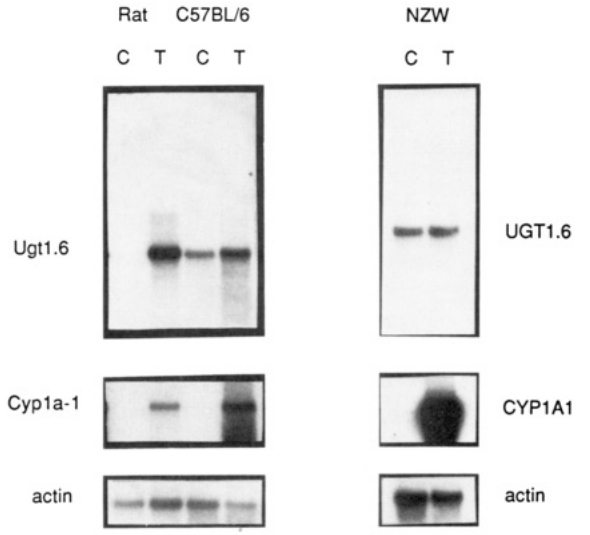


FIGURE 8: Induction of UGT1.6 mRNA by TCDD in vivo. Poly-(A)+ RNA was isolated from adult Sprague-Dawley rats, C57BL/6 mice, and NZW rabbits and 5-μg portions were subjected to electrophoresis on a 1% denaturing agarose gel. The RNA was transferred to nitrocellulose filters and probed with a ^{32}P-labeled cDNA probe. Rodent RNA was probed with the first 754 base pairs of the mouse Ugt1.6 cDNA, while the rabbit RNA was probed with the first 798 base pairs of the rabbit UGT1.6 cDNA. The filters were then reprobed with a cDNA specific for mouse Cyp1a1 or rabbit CYP1A1 (Okino et al., 1993, 1985). Actin levels were determined for loading efficiency. RNA from untreated animals is indicated with a C, while TCDD-treated animals are indicated with a T.

When adult rabbits were treated with 10 μg/kg TCDD, a large increase in the levels of CYP1A1 mRNA resulted (Figure 8), similar to what has been previously observed (Strom et al., 1992). This Northern blot was stripped and reprobed with the 5′ portion of the rabbit UGT1.6 cDNA (Okino et al., 1985). Unlike the results we observed in rodents, where TCDD clearly induced UGT1.6 mRNA, TCDD did not induce UGT1.6 mRNA in adult rabbits.

DISCUSSION

Rabbit UGT1.6 and mouse Ugt1.6, when expressed in COS-1 cells, display substrate activities toward planar phenolic compounds such as 4-nitrophenol and 1-naphthol as well as 4-methylumbelliferone, similar to that observed for the rat and human UGT1.6 (Jackson et al., 1988; Harding et al., 1988). The predicted amino acid sequence of the rabbit UGT is most related to the human (81%), rat (78%), and mouse (78%) phenolic UGT1.6, while the mouse Ugt1.6 is closely related to the rat (93%) and human (78%) UGT1.6. When all of the UGT1 proteins are examined for evolutionary similarity by the method of progressive alignment (Doolittle, 1990), the mouse Ugt1.6 aligns with the rat UGT1.6 (Figure 3), while the rabbit protein aligns with the human UGT1.6. The close similarity in amino acid sequence and functional properties of the rabbit and mouse UGTs to the human and rat phenolic UGTs demonstrates that we have identified the rabbit and mouse phenolic UGT1.6.

The phenolic compounds, as classified by Jackson et al. (1988) and Harding et al. (1988), are conjugated by two

independent UGTs. UGT1.6 is capable of conjugating planar molecules that are ≤6.3 Å in length such as 4-nitrophenol and 1-naphthol. Slightly larger phenolic compounds such as 4-propylphenol and 4-(*t*-butyl)phenol are best conjugated by another phenolic UGT identified as UGT1.7 (Wooster et al., 1991). The human UGT1.6 shares very little amino acid similarity in the amino portion of the protein to the human UGT1.7, yet the overall hydropathy indexes of the two proteins are very similar. Interestingly, neither UGT1.7 nor UGT1.6 is capable of conjugating the slightly larger and bulkier phenols such as 4-hydroxybiphenyl. In rabbits, conjugation of 4-hydroxybiphenyl is catalyzed by UGT2B13 (Tukey et al., 1993), a protein with little relatedness in amino acid sequence or hydropathy similarity to rabbit and human UGT1.6 or human UGT1.7. Therefore, amino acid sequence similarity does not appear to be important in discriminating between minor differences in phenolic structure, but the overall pattern of protein hydrophobicity most likely controls access of the different substrates to the active sites.

While the UGTs do display selective substrate specificities, such as the ability of UGT2B13 to conjugate 4-hydroxybiphenyl (Tukey et al., 1993), there exists considerable substrate overlap in the ability of the different UGTs to conjugate phenolic compounds. This information can be helpful in examining the physical differences between catalytically similar proteins. For example, the rabbit UGT1.6 and mouse Ugt1.6 actively conjugate 4-nitrophenol, 1-naphthol, and 4-methylumbelliferone. These same substrates are also conjugated by UGT2B13 (Tukey et al., 1993). However, 4-hydroxybiphenyl does not serve as a substrate for rabbit UGT1.6 or mouse Ugt1.6. This observation would indicate that the electrostatic interactions and protein structural requirements necessary to hold the small planar phenolic compounds in the active site of the UGT1.6 are similar in UGT2B13. However, the size of the active site or the "pocket" that captures the substrates for conjugation by UGT1.6 may simply be smaller than that of UGT2B13, since the larger planar 4-hydroxybiphenyl serves as a substrate for only UGT2B13. Interestingly, the use of other biphenyl compounds, such as 2-hydroxybiphenyl, which is more compact than 4-hydroxybiphenyl and whose ring structure may be slightly altered as a result of the *o*-hydroxy position, could also be useful in future studies to help predict substrates for this class of protein.

In rats, the UGT1.6 mRNA is enhanced in liver greater than 15-fold following exposure to Ah receptor ligands such as 3MC (Iyanagi et al., 1986; ElAwady et al., 1990). A much smaller but observable induction of mouse Ugt1.6 mRNA can be demonstrated when mice are treated with TCDD (Figure 8). It has been proposed that induction of UGT activities by agents such as polycyclic aromatic hydrocarbons and TCDD are elicited by the Ah receptor (Owens, 1977), a process which has been conclusively shown to transcriptionally activate the mouse *Cyp1a-1* and *Cyp1a-2* genes (Okino et al., 1992). Using cell lines that are deficient in the ability of the Ah receptor to translocate to the nucleus, we have demonstrated that induction of mouse Ugt1.6 is coordinated through the Ah receptor. In rabbits, treatment with Ah receptor agonists such as TCDD induces CYP1A1 and CYP1A2 mRNA (Okino et al., 1985). However, as shown in Figure 8, rabbit UGT1.6 mRNA is not induced following treatment with TCDD. Combined, these observations indicate that the actions of TCDD differentially regulate *UGT1*6* in a species-specific fashion. Since the Ah receptor is functional in rabbits as demonstrated by induction of the *CYP1* genes, the minimal response by TCDD toward the *UGT1*6* gene may indicate that specific allelic differences between legamorphs and rodents exist at the *UGT1*6* locus.

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