0026-895X/00/070089-09$3.00/0
MOLECULAR PHARMACOLOGY

MOL 58:89–97, 2000 /13136/831470

Characterization of the *UDP-Glucuronosyltransferase 1A* Locus in Lagomorphs: Evidence for Duplication of the *UGT1A6* Gene

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Received December 1, 1999; accepted March 20, 2000

This paper is available online at http://www.molpharm.org

ABSTRACT

The *UGT1* locus is felt to be highly conserved between species, as is evident from the characterization of the locus in rodents and humans. In rabbits, cDNAs encoding proteins homologous to human UGT1A4, UGT1A6, and UGT1A7 have previously been identified. Here we demonstrate by Southern blot analysis, using exon 1 divergent 5′ segments from rabbit UGT1A4 and UGT1A6 cDNAs, the existence of a cluster of highly related genes that are homologous to each of these exon 1 sequences. In comparing rabbit and human, it is evident that the *UGT1A4* and *UGT1A6* gene clusters in rabbit have undergone gene duplication. This is particularly evident with rabbit *UGT1A6*. The human *UGT1A6* cDNA anneals to only a single gene fragment, as displayed by Southern blot analysis, indicating that the *UGT1A6* exon 1 sequence is highly conserved. However, up to six rabbit *UGT1A6* genes could be predicted from Southern blot analysis. To examine the potential linkage of the rabbit *UGT1A6* genes, multiple *UGT1A6* exons were identified from genomic DNA by extended polymerase chain reaction techniques and cloning of the *UGT1A6* exon 1 sequences. Five unique *UGT1A6* exon 1 gene sequences were characterized that could be predicted to encode proteins that are 98% similar in amino acid structure. Using a conserved region of the rabbit *UGT1A6* cDNA as a probe to screen cDNA libraries, we identified a second *UGT1A6* cDNA, termed *UGT1A6*α. In addition, a cDNA that encodes a protein similar to human UGT1A3 was also cloned. Characterization of UGT1A6α demonstrated the protein to be 98.9% identical to UGT1A6. The expression of rabbit UGT1A3, UGT1A4, and UGT1A6 displayed catalytic activities similar to their human orthologs. However, UGT1A6α was catalytically divergent from UGT1A6, indicating that UGT1A6 and UGT1A6α do not arise from allelic polymorphism. These results demonstrate that lagomorphs have evolved at least five additional *UGT1A*6 genes, an event that is not duplicated in rodents or humans.

The UDP-glucuronosyltransferases (UGTs; EC 2.4.1.17) catalyze by glucuronidation many endogenous agents, such as steroids, bile acids, and bilirubin, in addition to a vast number of xenobiotics and dietary related by-products (Dutton, 1980). The transfer of glucuronic acid from UDP-glucuronic acid to the aglycones, which is catalyzed by the UGTs, renders the products more polar, thereby facilitating excretion of the glucuronides from the cell. The diversity in the selection of numerous structurally diverse compounds for glucuronidation results from a relatively large superfamily of UGTs (Tukey and Strassburg, 2000). Cloning studies using cDNA constructed from primates, rodents, and lagomorphs have resulted in the identification of several dozen novel UGT isoforms. Based upon overall structural similarities, the known UGTs have been classified into either the *UGT1* or *UGT2* gene families (Mackenzie et al., 1997; Tukey and Strassburg, 2000). The *UGT2B* RNAs are transcribed from independent structural genes, all of which appear to be located on chromosome 4 in humans (Monaghan et al., 1992, 1994). The human UGT1A proteins are encoded from a single locus on chromosome 2 (Harding et al., 1990; Moghrabi et al., 1992). The organization of the *UGT1A* locus is unique in displaying up to 12 divergent exon 1 sequences (Ritter et al., 1992; Strassburg et al., 1997b), organized in tandem and each encoding approximately 285 amino acids of the amino-terminal portion of the proteins. The remainder of the gene is composed of common exons 2–4, which encode the carboxyl 246 amino acids of each UGT1A protein. The unique UGT1A transcripts are produced by exon shuffling, resulting in RNA transcripts that encode divergent amino-terminal regions with identical carboxyl portions.

The general organization of the *UGT1A* locus is conserved among different species. For example, the *UGT1A* locus in rodents is represented by at least nine flanking exon 1 sequences (Emi et al., 1995). Exon 6 encodes rodent UGT1A6, and this protein displays a high catalytic turnover for small phenolic substrates like 4-nitrophenol and 1-napthol. An ortholog to human UGT1A6 has also been identified in rabbits and was shown to have catalytic activities similar to those of

This work was supported in part by U.S. Public Health Service Grant GM49135.

ABBREVIATIONS: UGT, UDP-glucuronosyltransferase (EC 2.4.1.17); P450, cytochrome P450; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; LC-PCR, long range PCR; TLC, thin-layer chromatography; UDPGA, UDP-glucuronic acid.

rodent UGT1A6 (Lamb et al., 1994). DNA sequence analysis of genomic DNA from human and rat clearly demonstrates the existence of a single exon 1 sequence encoding UGT1A6, with flanking divergent exon 1 sequences that encode UGT1A5 and UGT1A7. In human, UGT1A7 is uniquely expressed in extrahepatic tissues such as gastric epithelium (Strassburg et al., 1998b), whereas rodent UGT1A7 is found to be expressed in liver only after treatment with polycyclic aromatic hydrocarbons, but is also expressed constitutively in several extrahepatic tissues (Kessler and Ritter, 1998). It would appear that some degree of tissue-specific *UGT1A* regulatory control between rodents and human is maintained. The structural conservation, catalytic uniqueness, and regulatory similarities of the *UGT1A* locus between humans and rodents indicate that these genes must be highly conserved between other mammals.

Rabbits have served as an excellent animal model for the study of the biochemical and molecular properties of many proteins involved in the pharmacology of Phase I and Phase II metabolism. Several important similarities have been shown to exist between rabbits and human when the catalytic properties associated with glucuronidation have been examined. For example, rabbits and humans efficiently glucuronidate tertiary and quaternary amines, a catalytic process that is absent in rodents. However, rabbits are much more efficient in this metabolic process (Coughtrie and Sharp, 1991), possibly because two of the UGT1A proteins, UGT1A4 and UGT1A7, carry out this catalytic process (Bruck et al., 1997). In examining the catalytic potential between rabbit and human liver microsomal preparations, it was clear that rabbits have a much greater capacity to glucuronidate simple and complex phenols as well as quaternary amines, a result that supports previous observations comparing these activities in microsomes from rabbit and human liver samples (Dulik and Fenselau, 1987; Coughtrie and Sharp, 1991). For this reason, experiments were undertaken to further define the genetic diversity of the *UGT1A* locus in rabbits and compare this diversity to that of the human *UGT1A* locus. Results presented indicate that the rabbit *UGT1A6* (phenol) gene has undergone extensive duplication, an event that has not occurred in rodents or humans.

Experimental Procedures

Materials. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). Oligo(dT)-cellulose was purchased from Boehringer Mannheim (La Jolla, CA). Nitroplus 2000 nitrocellulose hybridization paper was purchased from μm Separations Inc. (Westwood, MA). The Erase-a-Base DNA sequence kit was obtained from Promega (Madison, WI), and DNA sequencing kits were purchased from United States Biochemical (Cleveland, OH). DNA nick translation kits, [α-^{32}P]dCTP (3000 Ci/mmol), and [^{35}S]dATPαS (400 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, IL). Uridine diphosphate glucuronic acid [glucuronyl-^{14}C(U)] (225 mCi/mmol, ammonium salt) and Trans^{35}S-label ([^{35}S]L-methionine, [^{35}S]L-cysteine) were obtained from ICN Radiochemicals (Costa Mesa, CA). The eukaryotic expression vector pSVL was purchased from Pharmacia (Piscataway, NJ). Aglycone substrates, uridine 5′-diphosphoglucuronic acid (triammonium salt), and formalin-fixed *Staphylococcus aureus* cells were obtained from Sigma (St. Louis, MO). Glass-backed linear-K thin-layer chromatography (TLC) plates with preabsorbent strips were purchased from Whatman Labsales, Inc. (Hillsboro, OR). The SuperScript Preamplification System for First Strand cDNA Synthesis was purchased from Life Technologies (Gaithersburg, MD). Cloned *Pfu* DNA polymerase was purchased from Stratagene (La Jolla, CA). Bio-X-ACT DNA polymerase was obtained from ISC BioExpress (Kaysville, UT). All oligonucleotides were manufactured with an Applied Biosystems model 380B DNA synthesizer from the UCSD Cancer Center Molecular Biology Core laboratory.

Southern Blot Analysis. Genomic DNA was isolated from rabbit liver or human peripheral blood samples by the method of Blin and Stafford (1976), and approximately 10 μg of DNA was digested with restriction endonucleases. The digested DNA fragments were electrophoresed in a 1.0% agarose gel containing 50 mM Tris-HCl (pH 8.4), 20 mM sodium acetate, 18 mM sodium chloride, and 2 mM EDTA. After denaturing and neutralizing, DNA was transferred from the gel to MSI NitroPlus 2000 nitrocellulose filters. Filters were prehybridized and then hybridized at 42°C for 18 h with 10^7 cpm/ml ^{32}P-labeled probe DNA in 6× SSC (1× SSC: 15 mM sodium citrate, 150 mM sodium chloride, pH 7.0), 5× Denhardt's solution (1× Denhardt's solution: 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin fraction V), 100 mg/ml denatured salmon sperm DNA, 0.1% sodium dodecyl sulfate (SDS), and 50% deionized formamide. The filter was washed twice at room temperature in 2× SSC and 0.1% SDS for 5 min, then twice at 55°C in 0.1× SSC and 0.1% SDS for 30 min. The filter was exposed to X-ray film with intensifying screens at −70°C.

Long-Range Polymerase Chain Reaction (LR-PCR). LR-PCR analysis was undertaken to determine whether the rabbit *UGT1A6*-like exons were linked. Two oligonucleotides, 5′-gagcgggcctcagacggaatacaggaataa-3′ (1A6-SL), which corresponded to bases 396–425, and 5′-gagcgagcctcagacggaatacaggaaact-3′ (1A6-AL), which corresponded to bases 563–534 of the rabbit UGT1A6 cDNA (Lamb et al., 1994), were synthesized and used as primers to amplify rabbit genomic DNA. LR-PCR was performed with a kit from ISC BioExpress (Kaysville, UT). The 50-μl reaction mixture included 10 ng of rabbit genomic DNA, 1× OptiPerform (ISC) buffer, 2 mM magnesium chloride, 250 mM dNTPs, and 200 nM 1A6-SL and 1A6-AL oligonucleotides. The reaction profile included 2 min at 94°C, 25 cycles at 94°C for 10 s, then 65°C for 30 s, followed by 68°C for 15 min. The amplified products were electrophoresed in a 0.6% agarose gel.

Genomic Cloning of Rabbit *UGT1A6*-like Genes. To amplify rabbit *UGT1A6*-like genes from genomic DNA, oligonucleotides 5′-actggatccaggatggcctgcctg-3′ and 5′-agacctcgagtccggcttcttgcagttga-3′, which matched the 5′ and 3′ regions of the coding region represented by rabbit *UGT1A6* exon 1, were synthesized and used as primers for PCR. There are no restriction *Bam*HI or *Xho*I endonuclease-specific sites, so recognition sites for these enzymes were introduced into the 5′ ends of these two oligonucleotides. In a 100-μl reaction buffer containing 20 mM Tris-HCl (pH 7.8), 2 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 200 nM primers, 200 mM dNTPs, 500 ng of rabbit genomic DNA, and 5 units cloned *Pfu* DNA polymerase was denatured at 94°C for 1 min, annealed at 58°C for 1 min, and polymerized at 72°C for 1 min, and the reaction was repeated for 30 cycles. The PCR products were purified and digested with *Bam*HI and *Xho*I and cloned into these same sites in pBluescript-KS+. More than 20 recombinants were sequenced using primers that recognized the T3 and T7 promoter regions.

Screening Rabbit Liver cDNA Library. The New Zealand White adult rabbit λ-ZAP cDNA library, previously constructed in this laboratory (Tukey et al., 1993), was screened with a ^{32}P-labeled 700-bp 3′ (*Bsa*I-1294/*Xmn*I-1943) conserved portion of the rabbit *UGT1A6* cDNA. The cDNA probe was labeled by nick translation to an approximate specific activity of 1×10^8 cpm/μg of DNA. The cDNA library was plated on 100-mm LB plates containing ampicillin at a density of $2–3 \times 10^3$ pfu/plate. Each filter was annealed with 5×10^6 cpm/ml of labeled DNA at 42°C for 16 h in 50% deionized formamide, 5× SSC, 100 mg/ml sonicated salmon sperm DNA, and 20 mM potassium phosphate buffer (pH 7.4). The filters were first washed at room temperature in 2× SSC containing 0.1% SDS for 15

min, followed by washing in 0.1× SSC and 0.1% SDS for 2 h, with several changes of this wash solution. Each positive cDNA recombinant was isolated and placed in 0.5 ml of water and rescreened as outlined. This procedure was repeated until each λ-ZAP cDNA was demonstrated to be 100% pure, as judged by positive hybridization. Those positive clones with inserted DNA larger than 1600 bp were chosen for further studies. As outlined by Stratagene, each recombinant λ-ZAP cDNA clone was recovered as a double-stranded plasmid. Using both T3 and T7 primers, we sequenced the plasmids at their 5′ and 3′ regions, as outlined in the protocols from the United States Biochemical DNA sequencing kits. The cDNA clones of interest were selected for further characterization.

DNA Sequence Analysis. The cDNA fragments were individually sequenced using primers that recognized the T3 and T7 promoter regions. In addition, a series of clones with progressively overlapping deletions from the 5′ end were constructed by removing portions of the cDNA by exonuclease III and S_1-nuclease digestion, as outlined with the Erase-A-Base kit supplied by Promega. Colonies were prepared using conventional mini plasmid preparations, and each clone was sequenced using T7 or T3 primers.

Construction of Plasmids for Expression in COS-1 Cells. The entire coding region of rabbit *UGT1A3* cDNA was removed by digesting the plasmid with *Xba*I and *Eco*RV, and then cloning this fragment into the *Xba*I and *Sma*I sites in the eukaryotic expression vector pSVL. This recombinant plasmid was identified as rabbit p1A3.SVL. Furthermore, the coding region of rabbit *UGT1A6α* cDNA was removed from pBluescript plasmid by digestion with *Xho*I and *Bam*HI, and then subcloned into the same sites in pSVL, and the plasmid was referred to as rabbit p1A6α.SVL. The expression vectors p1A4.SVL and p1A6.SVL have previously been constructed (Lamb et al., 1994; Philipp et al., 1994).

The Expression of Rabbit UGTs in COS-1 Cells. The rabbit expression vectors, p1A3.SVL, p1A4.SVL, p1A6.SVL, and p1A6α.SVL, were transfected into COS-1 cells as outlined (Zuber et al., 1986). COS-1 cells were grown to 50% confluency in Dulbecco's modified Eagle's medium in 150-mm diameter plates, supplemented with 5% fetal calf serum and 5% Nu-serum. After being washed twice with phosphate-buffered saline (PBS), the cells were transfected by incubation with Dulbecco's modified Eagle's medium, 5 mg/ml plasmid DNA, 0.25 mg/ml DEAE-dextran (MW 500,000) and 20 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (pH 7.5). The transfection medium was aspirated and cells were incubated for 5 h in medium containing 100 mM chloroquine. The cells were washed twice with PBS and further incubated for 48 h in culture medium.

^{35}S-Labeling and Immunoprecipitation. Forty-eight hours after transfection, COS-1 cells from a single 35-mm tissue culture plate were washed three times with Hanks' balanced salt solution and incubated for 4 h in methionine-free modified Eagle's medium supplemented with 100 mCi/ml Trans^{35}S-label. The cells were washed twice in PBS, and the cells were lysed on ice in 600 μl of RIPA solution containing 1% Triton X-100, 1% sodium deoxycholate, 1% SDS, 150 mM sodium chloride, 1 mM phenylmethanesulfonyl fluoride, and 50 mM Tris-HCl (pH 7.5). Cellular DNA was broken by shearing through a 25-gauge needle and was removed with other cellular debris by centrifugation at 16,000*g*. The cleared supernatant was incubated for 4 h at 4°C with 22 mg of sheep anti-rabbit UGT1A6 Ig (Lamb et al., 1994), followed by 80 μl of a 10% suspension of formalin-fixed *S. aureus* cells. After the cells were washed four times in RIPA and once in TSA buffer [50 mM Tris-HCl (pH 7.5), 150 mM sodium chloride], the *S. aureus*. cells were suspended in 100 μl of loading buffer containing 63 mM Tris-HCl (pH 6.8), 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. The immunoprecipitated proteins were analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gel was soaked with 1 M sodium salicylate for 30 min before drying, and the radiolabeled bands were detected by fluorography at −70°C overnight.

UDP-Glucuronosyltransferase Activity Assays. UGT activities were determined according to the method of Bansal and Gessner (1980), as modified (Tukey et al., 1993). Transfected COS-1 cells were homogenized in 5 volumes of ice-cold 50 mM Tris-HCl (pH 7.5) containing 10 mM $MgCl_2$. In a total volume of 100 μl, 100 μg of cell extract, 100 μM UDP-glucuronosyltransferase activity (UDPGA), and 0.04 mCi of UDP-[^{14}C]glucuronic acid, 10 mg of phosphatidylcholine, and 100 mM substrate in 50 mM Tris-HCl (pH 7.5) containing 10 mM $MgCl_2$ were incubated at 37°C for 1 h and then extracted with 200 μl of 100% ethanol. The protein was removed by centrifugation at 10,000 rpm for 10 min in an Eppendorf centrifuge, and the supernatant was dried and resuspended in 30 μl of methanol for application to a Whatman glass-backed linear K preabsorbent TLC plate. Chromatography was performed in a mixture of 1-butanol/acetone/acetic acid/water (35:35:10:20). The TLC plate was dried, sprayed with a thin layer of EN^{3}HANCE, and exposed to X-ray film at −70°C. The appropriate portions of the TLC cellulose that represented the glucuronides were scraped into glass scintillation vials and dissolved in 0.5 ml of water, and then 12 ml of EcoLite scintillation liquid was added. The vials were shaken and counted in a Beckman scintillation counter. For each substrate, control experiments were conducted with mock-transfected COS-1 cells, and the area of the TLC plate corresponding to the Rf value of the glucuronide from transfected COS-1 cells was scraped and quantitated. Those values were subtracted from the values obtained from transfected cells. We observed no detectable glucuronidation for any of the substrates in nontransfected cells.

Results

Genetic Analysis of the Rabbit *UGT1A* Locus. The human *UGT1A* locus has evolved several conserved clusters of exon 1 sequences that are encoded by *UGT1A1, UGT1A3-UGT1A5, UGT1A6,* and *UGT1A7-UGT10*. The exon 1 sequences within each cluster are more conserved in DNA sequence than between the different clusters. Recombinant cDNAs that encode rabbit UGT1A4, UGT1A6, and UGT1A7 have been identified from rabbit liver cDNA libraries (Lamb et al., 1994; Philipp et al., 1994; Bruck et al., 1997). We have recently demonstrated that rabbit UGT1A7 is most homologous to human UGT1A7 (Bruck et al., 1997). In continuing experiments to examine the genetic composition of the rabbit *UGT1A* locus, genomic DNA was digested with a panel of restriction endonucleases and subjected to Southern blot analysis using ^{32}P-labeled cDNA fragments from the first exon sequences of rabbit *UGT1A4* and *UGT1A6*. Most of the restriction enzymes that were selected do not contain cleavage sites within the exon sequences encoded by the cDNA probes. Thus the appearance of multiple genomic fragments indicates the identity of highly related genes. Each rabbit genomic Southern blot was directly compared to Southern blots, using human genomic DNA that was probed with comparable exon 1 sequences from human *UGT1A4* and *UGT1A6* cDNAs (Figs. 1 and 2). As predicted, rabbit *UGT1A4* identifies several related gene fragments, most likely those that encode *UGT1A3*, *UGT1A4,* and *UGT1A5*. However, the banding patterns associated with UGT1A4 in rabbits indicate a greater multiplicity of homologous genes than observed in human. This result indicates that the rabbit

UGT1A4 gene has undergone additional gene duplication events.

In contrast, the exon sequence of human *UGT1A6* recognizes only single gene fragments, consistent with the finding that other *UGT1A* exon sequences are not closely related to the more divergent *UGT1A6* exon (Fig. 2). This finding is in direct contrast to those results from rabbit *UGT1A6* cDNA (Fig. 2), where anywhere from five to nine individual gene fragments can be identified relative to the different restriction endonucleases. This result indicates that the location of the *UGT1A6* exon on the *UGT1A* locus has undergone repetitive duplication events.

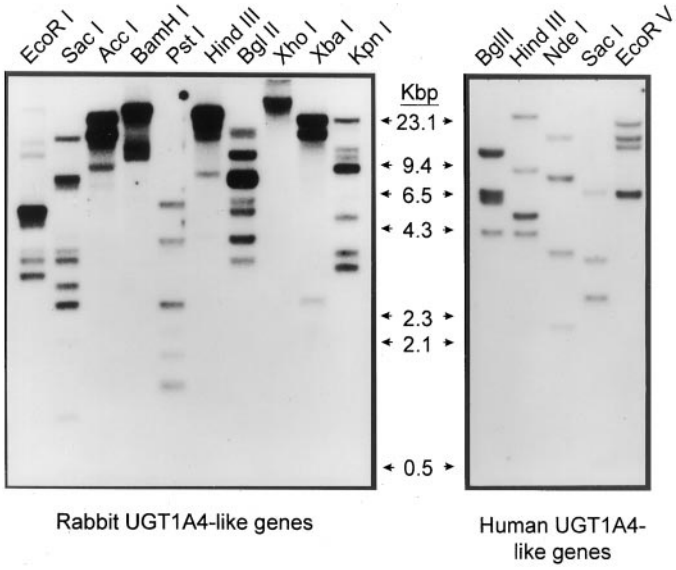

Fig. 1. The genetic diversity of rabbit and human UGT1A4, as detected by Southern blot analysis. Rabbit and human liver DNA was isolated and digested with a panel of restriction enzymes. After transfer of the DNA to nitrocellulose, the rabbit DNA was hybridized with an *Eco*RI fragment isolated from the rabbit *UGT1A4* cDNA that corresponds to bases 1–668. The filter containing the human DNA was hybridized with a human *Nsi*I *UGT1A4* cDNA fragment corresponding to bases 1–781. Neither of the restriction enzymes that were used to digest rabbit or human DNA was recognized within the cDNA fragments used for hybridization. The patterns of hybridization demonstrated in this figure are reproducible with DNA isolated from other rabbit and human tissue. Thus the incidence of the multiplicity as observed most likely is not the result of incomplete restriction enzyme digestion.

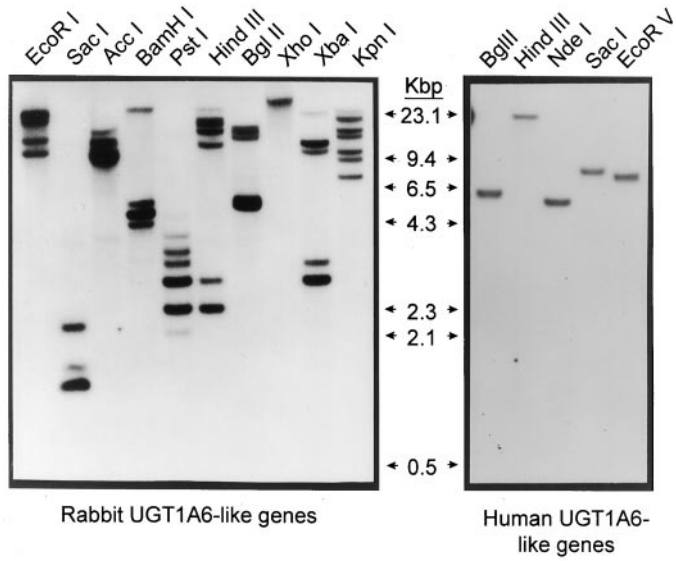

Fig. 2. The genetic diversity of rabbit and human UGT1A6 as detected by Southern blot analysis. The Southern blot containing the rabbit DNA was hybridized with an *Sac*I fragment isolated from the rabbit *UGT1A6* cDNA that corresponds to bases 1–796. The human DNA was hybridized with a *Pvu*II cDNA fragment isolated from the human *UGT1A6* cDNA that corresponds to bases 1–778. The human *UGT1A4* cDNA used in Fig. 1 and the *UGT1A6* cDNA employed in this figure have been isolated previously in this laboratory from human liver mRNA by reverse transcription, followed by polymerase chain reaction using specific *UGT1A4* and *UGT1A6* oligonucleotides.

Identification of *UGT1A6* Exon 1 Gene Sequences. To examine the potential linkage of the *UGT1A6* exons, extended PCR reactions were developed with a pair of sense (1A6-SL) and antisense oligonucleotide primers (1A6-AL). The positions of these oligonucleotides are in divergent regions of the *UGT1A6* exon 1. We predicted that if the *UGT1A6* exons are arranged in tandem, sense and antisense primers generated to similar regions of the exon would prime and extend the sense position of one exon to the antisense position of the flanking exon. With the pair of primers (sense and antisense) made to exon 1 of *UGT1A6*, PCR analysis using rabbit genomic DNA generated three DNA transcripts ranging from approximately 9 to 15 kb (Fig. 3). Similar size transcripts were generated when primers made to the 5′ and 3′ regions of the *UGT1A6* exon 1 cDNA were used. When we eluted and purified these larger bands from the agarose gels and used the DNA as a substrate, PCR experiments using 5′ and 3′ oligonucleotides to *UGT1A6* resulted in the generation of UGT1A6 exon fragments, as confirmed by DNA sequence analysis. These results provide indirect evidence that the *UGT1A6*-like exons are located on both sides of these large DNA fragments and support the Southern blot analysis, indicating the existence of multiple *UGT1A6*-like exon sequences.

The exon sequences were further characterized, generating a library of *UGT1A6* exon 1 fragments by PCR, using rabbit genomic DNA and primers to the 5′ and 3′ positions of the *UGT1A6* exon 1 sequence. DNA sequence analysis from over 20 genomic clones identified five unique exons, one of which encoded the original rabbit *UGT1A6* cDNA (Lamb et al.,

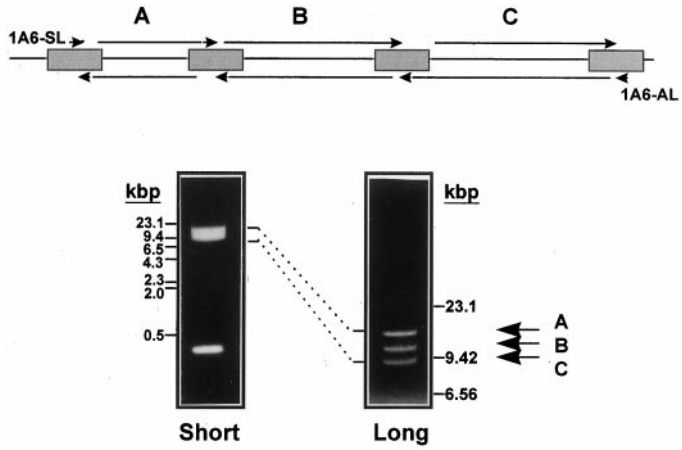

Fig. 3. Long-range polymerase chain reaction (LR-PCR) of the rabbit *UGT1A6* gene. Two oligonucleotides specific to rabbit *UGT1A6*, the sense 1A6-SL and antisense 1A6-AL (described in *Experimental Procedures*), were used in a PCR reaction using rabbit liver DNA. The reaction profile for LR-PCR included 2 min at 94°C followed by 25 cycles that included 10 s at 94°C, 30 s at 68°C, and 15 min at 68°C. The amplified products were subjected to electrophoresis in a 0.6% agarose gel. Shown in the figure are photographs taken during short and extended electrophoresis.

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1A6    M A C L L S A A Q R A S A G V L F V A L W G T V L -25
1A6α                                                   
1A6β                                                 V 
1A6λ                                                 V 
1A6δ                                                 V 

1A6    G D R L L V V P Q D G S H W L S M Q D I V E A L G -50
1A6α                                                   
1A6β                                                   
1A6λ                                                   
1A6δ                                                   

1A6    A R G H E I V V L V P E V N L L L R E S R F Y T R -75
1A6α                                                   
1A6β                                                   
1A6λ                                                   
1A6δ                                                   

1A6    R I Y P V P F D Q E E Q S Y R Y R T F G E K H F T -100
1A6α                                                   
1A6β                                                   
1A6λ                                                   
1A6δ                                       S           

1A6    D R S W L S G P Q T E Y R N N M V V I D M Y F I N -125
1A6α              E             K L                    
1A6β                              L              H     
1A6λ                                                   
1A6δ              E             K L                    

1A6    C Q S L L R H G D T L D F L R A G K F D A L F T D -150
1A6α                                                   
1A6β                                                   
1A6γ                              K                    
1A6δ                                                   

1A6    P A L P C G V I L A E Y L G L P S V Y L F R G F P -175
1A6α     F                                             
1A6β     F                                             
1A6λ     F                                             
1A6δ     F                                             
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1A6    C S L E H G F G G S P N P V S Y I P R C Y T K F S -200
1A6α                                                   
1A6β           L                                       
1A6λ           L                                       
1A6δ                                                   

1A6    D Q M S F P Q R V V N F L V N L L E V P L F Y C L -225
1A6α                              S                    
1A6β                                                   
1A6λ                                                   
1A6δ             H                S                    

1A6    Y S K Y E D L A V E L L K R E V D L P T L F Q K D -250
1A6α                                                   
1A6β                              K                    
1A6λ                              K                    
1A6δ                                                   

1A6    P V W L L R Y D F V F E Y P R P V M P N M V L I G -275
1A6α                                                   
1A6β                                                   
1A6λ                                                   
1A6δ                                                   

1A6    G I N C K K P                                   -282
1A6α                                                   
1A6β                                                   
1A6λ                                                   
1A6δ                                                   
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Fig. 4. Alignment of the predicted amino acid sequences of the rabbit *UGT1A6* exon sequences. Using rabbit DNA and oligonucleotides (see *Experimental Procedures*) specific for the *UGT1A6* exon 1 sequence, we cloned PCR products into Stratagene pBluescript II KS+ plasmid. From a library of recombinants, 20 randomly selected plasmids were purified, and the DNA inserts were sequenced. These recombinants were grouped into one of five categories based upon their sequence. Shown in the figure is the predicted amino acid sequence of these five recombinant subgroups. The original UGT1A6 cloned in this laboratory is shown on top, and those amino acids that differ from this sequence are shown.

1994). Their predicted amino acid sequences are shown in Fig. 4. Each of the predicted UGT1A6 sequences is more than 98% homologous. Because we obtained five exon sequences generated by PCR analysis and three different genomic fragments, it can be predicted that at least four of the *UGT1A6* exons are located in tandem and separated by 9–15 kb of DNA, as shown in Fig. 3.

Identification of Additional *UGT1A* cDNAs. To determine whether additional *UGT1A6* RNA transcripts are transcribed from the *UGT1A6* exons in rabbit liver, a New Zealand White adult rabbit λ-ZAP cDNA library constructed from the sample of tissue used for the Southern blot analysis was screened with a radioactive probe encoding common exons 2–5. This probe would identify all of the *UGT1A* RNAs represented in the cDNA library. The positive clones with inserts larger than 1600 bp were sequenced with T3 and T7 primers to identify 5′ and 3′ DNA sequences. Recombinant cDNAs encoding the previously identified UGT1A4 (Philipp et al., 1994), UGT1A6 (Lamb et al., 1994), and UGT1A7 (Bruck et al., 1997) were identified. In addition, a cDNA that encoded UGT1A3 and a UGT1A6 cDNA that was identical in sequence to exon *UGT1A6α* (Fig. 4) were also identified. The UGT1A3 cDNA is 2017 bases in length and contains an open reading frame that encodes a protein of 533 amino acids. The deduced amino acid sequence of UGT1A3 is 80.1, 78.9, and 77.7% similar to those of human UGT1A3, UGT1A4, and UGT1A5, respectively, and less than 70% homologous to the other UGT1A isoforms. The predicted amino acid sequence of UGT1A6α is 98.2% similar to that of UGT1A6. This latter result demonstrates that multiple UGT1A6-like gene products are transcribed from the *UGT1A6* locus in rabbit liver.

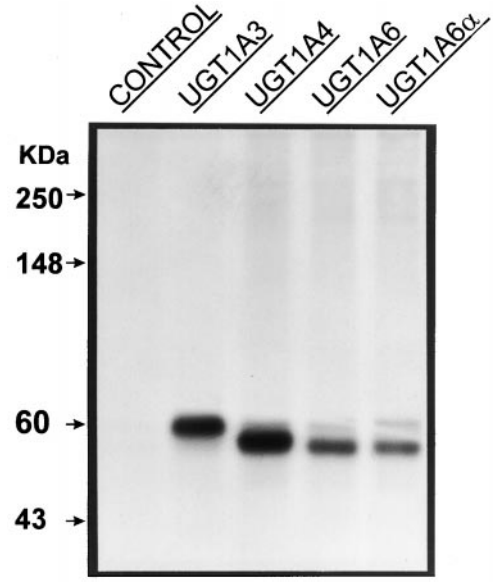

Fig. 5. Immunoprecipitations of radiolabeled COS-1 cell extracts. pSVL expression plasmids containing rabbit *UGT1A3, UGT1A4, UGT1A6*, and *UGT1A6α* were transfected in COS-1 cells. Control cells were transfected with only the pSVL plasmid. Approximately 48 h after transfection, the cells were labeled for 4 h with 100 μCi/ml [^{35}S]methionine/cysteine, as previously outlined (Lamb et al., 1994). The cells were lysed, and the extracts were incubated with antibody. The antigen/antibody complexes were then incubated with *S. aureus* cells, and the complex was precipitated and extensively washed. The cell pellet was boiled, and a portion was subjected to SDS-PAGE on an 8% acrylamide gel. The gel was soaked in 1 M sodium salicylate as a fluorographic enhancer, dried, and exposed to X-ray film overnight at −70°C.

To examine the functional properties of UGT1A6 and UGT1A6α, the cDNAs were recloned into eukaryotic expression plasmid pSVL, and the cDNAs were transfected transiently into COS-1 cells. For comparison, enzyme analysis was also conducted in COS-1 cells that had been transfected with the rabbit UGT1A3 and UGT1A4 cDNAs. To examine the expression levels, transfected cells were labeled with [^{35}S]methionine/cysteine, followed by immunoprecipitation with a polyclonal anti-UGT antibody (Lamb et al., 1994), to confirm that each of the cells transfected with the cDNAs was manufacturing UGTs of the appropriate length (Fig. 5). Total cell extracts were used to examine phenolic, steroid, and quaternary amine glucuronidation patterns (Table 1). UGT1A3 was active toward bulky phenolic compounds such as 4-hydroxybiphenyl, octylgallate, and propylgallate, as well as several steroids like β-estradiol, estriol, and androstane. Like previous reports on the activity of human UGT1A3 (Mojarrabi et al., 1996), rabbit UGT1A3 was also active toward estrone. UGT1A4 catalyzed the glucuronidation of androstane, estriol, and androsterone, but was not active toward estrone. We have previously demonstrated that rabbit UGT1A4 catalyzes the glucuronidation of tricyclic antidepressants such as imipramine (Bruck et al., 1997), but it is also active in the presence of other tertiary amines such as amitriptyline. The catalytic profiles of rabbit UGT1A3 and UGT1A4 are similar to those observed for their human orthologs.

UGT1A6 is most active toward small phenolic substrates such as 1,5-isoquinoline, 2-hydroxybiphenyl, 4-isopropylphenol, 4-methylumbelliferone, and 4-*tert*-butylphenol (Table 1). Although UGT1A6 and UGT1A6α are more than 98% homologous in amino acid sequence, UGT1A6α metabolized these phenolics poorly in comparison to the catalytic activities attributed to UGT1A6. Interestingly, bulky phenols such as octylgallate and propylgallate as well as flavones such as 7-hydroxyflavone are excellent substrates for glucuronidation by UGT1A6α, although being poor substrates, in comparison, for UGT1A6. Figure 6 demonstrates this dramatic difference, as observed for glucuronide formation by TLC analysis. Plasmids were transfected into COS-1 cells, and UGT analysis was conducted in the presence of substrate and 100 μM UDPGA. When these experiments are carried out with 1 mM UDPGA, dilution of the labeled UDPGA in the reaction leads to a dramatic reduction in detectable 2-hydroxybiphenyl or 4-isopropylphenol catalyzed by UGT1A6, yet significant 4-methylumbelliferone glucuronide is still observed. Likewise, when 1 mM UDPGA is used to assay UGT1A6α catalysis, only octylgallate and 7-hydroxyflavone glucuronide formation is detectable (data not shown). This result, along with the data presented in Table 1 and Fig. 6, clearly demonstrates that the six amino acid differences between UGT1A6 and UGT1A6α dramatically affect the efficiency of glucuronidation and substrate specificity of these two highly homologous rabbit UGTs.

Discussion

Southern blot analysis comparing rabbit UGT1A4 and UGT1A6 to human UGT1A4 and UGT1A6 indicates that the rabbit *UGT1A* locus is more complicated in composition than

TABLE 1

The catalytic activities of COS-1 expressed rabbit UGT1A3, UGT1A4, UGT1A6, and UGT1A6α

One hundred micrograms of COS-1 cell extracts that have been transfected with expression plasmids containing recombinant UGT1A3, UGT1A4, UGT1A6, and UGT1A6α were used in each assay with substrate concentrations of 100 μM. The ^{14}C-labeled glucuronides were detected after thin-layer chromatography by visualization on X-ray film, and the products were removed and analyzed by scintillation counting. The activities were calculated from duplicate experiments and expressed as picomoles of glucuronide formed per minute per milligram of protein.

Substrates	Activity			
	UGT1A3	UGT1A4	UGT1A6	UGT1A6α
	pmol glucuronide/min/mg protein			
Phenolic structures				
1,5-Isoquinolinediol	0.44	ND	3.30	0.40
2-Hydroxybiphenyl	ND	ND	11.80	0.72
4-Hydroxybiphenyl	0.44	ND	ND	ND
4-Isopropylphenol	ND	ND	13.60	0.90
4-Nitrophenol	ND	ND	34.80	12.50
4-*tert*-Butylphenol	ND	ND	5.70	ND
Acetaminophenol	ND	ND	ND	ND
Octylgallate	1.11	ND	3.60	44.90
Propylgallate	0.12	ND	0.70	4.90
Vanillin	ND	ND	27.60	16.90
Flavonoids				
7-Hydroxyflavone	1.07	ND	0.82	6.68
Chrysin	2.11	ND	3.89	10.30
Naringenin	1.87	ND	1.60	5.39
Coumarin structures				
4-Methylumbelliferone	ND	ND	22.10	2.20
Napthol structures				
α-Naphthol	0.12	ND	83.10	51.80
β-Naphthol	ND	ND	81.80	11.60
Steroid hormone structures				
Androsterone	0.16	3.88	ND	ND
β-Estradiol	0.69	0.23	ND	ND
Estriol	0.36	0.24	ND	ND
Estrone	1.63	ND	ND	ND
Tertiary Amines				
Amitriptyline	ND	0.74	ND	ND
Imipramine	ND	1.82	ND	ND

ND, no detectable activity above background.

the human *UGT1A* locus, as schematically shown in Fig. 7. For example, using as a probe the human UGT1A4 cDNA that encodes exon 1, four related gene products can be identified from human DNA. This is not surprising, inasmuch as a substantial DNA sequence exists between exons *UGT1A2*, *1A3*, *1A4*, and *1A5*. Yet with the rabbit UGT1A4 cDNA, DNA fragments indicating a gene cluster anywhere from six (with *Pst*I) to nine (with *Sac*I) related genes can be inferred from Southern blot analysis using rabbit DNA. A similarly dramatic representation of rabbit *UGT1A* gene multiplicity was observed in Southern blots that compared human and lagomorph *UGT1A6*. With a human UGT1A6 cDNA as a probe and stringent conditions of hybridization, only a single human gene was identified with each of the five restriction enzymes used. The human *UGT1A6* exon 1 sequence is significantly divergent from the other *UGT1A* sequences, so that selective hybridization to a single allele occurred. However, the rabbit UGT1A6 cDNA identified five to nine separate rabbit gene fragments, which were confirmed by cloning and characterization of five genomic exon 1 sequences. Although we cannot be precise in determining the exact number of duplicated exon 6 sequences in rabbit DNA, these experiments indicate that at least five highly homologous exon 1 elements have evolved in this species.

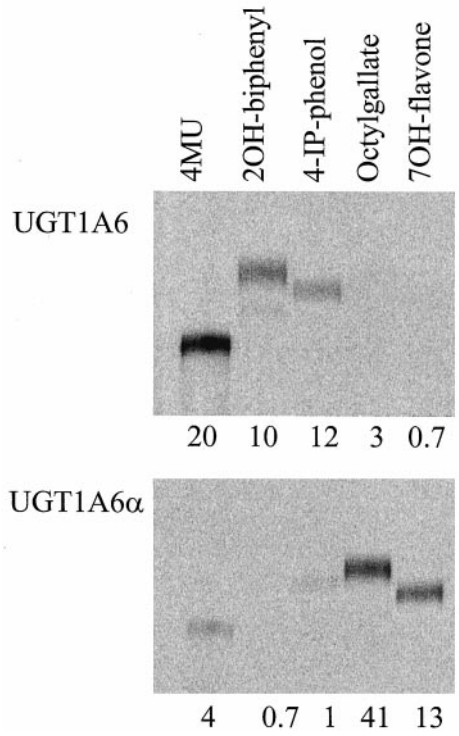

Fig. 6. Differential expression of UGT1A6 and UGT1A6α. The expression plasmids encoding UGT1A6 and UGT1A6α were transiently transfected into COS-1 cells, and total cell extracts were used to analyze patterns of glucuronidation, using 4-methylumbelliferone (4 MU), 2-hydroxybiphenyl (2OH-biphenyl), 4-isopropylphenol (4-IP-phenol), octylgallate, and 7-hydroxyflavone (7OH-flavone). Assays were conducted as outlined in *Experimental Procedures*, using similar concentrations (100 μg) of cell extract. The specific activities (pmol/mg protein/min) are shown beneath the respective lanes of the TLC. These experiments were run independently from those reported in Table 1.

It has recently been demonstrated that the human phenol UGT1A6 transferase exists as a variant that is composed of two missense mutations, resulting in codon differences at amino acids 181 and 184 (Ciotti et al., 1997). Both the wild-type UGT1A6 cDNA and the variant were isolated from the same λ-ZAP cDNA libraries. These differences have been attributed to allelic polymorphisms at the *UGT1A6* locus. Unlike the result obtained in humans, rabbits have duplicated the *UGT1A6* locus. When a rabbit liver cDNA library was screened, the original UGT1A6 cDNA, as well as the UGT1A6α cDNA that matched one of the cloned exon sequences, was characterized. UGT1A6α displayed amino acid differences from the wild type at G107E, N114K, N115L, A152F, N215S, and L285V. These amino acid changes have a significant impact on the ability to conjugate phenols. Small phenolic substrates, represented by 4-methylumbelliferone and 4-nitrophenol, which are excellent substrates for UGT1A6, serve as poor substrates for UGT1A6α, whereas bulkier phenolics, such as octylgallate and propylgallate, are very good substrates for UGT1A6α and poor substrates for the wild-type UGT1A6. Conceivably, the amino acid differences could have an impact on substrate access to the UGTs or influence substrate coordination within the active site. A slightly larger active site in UGT1A6α could explain these differences, allowing for the more efficient metabolism of bulkier phenolics. Analysis of UGT activity between rabbit and human liver microsomal protein demonstrates that rabbits have a greater capacity to conjugate phenols than do humans. It is possible that this increased capacity in rabbit liver is due to an enhancement of the microsomal UGT1A6-like protein in this species, a result that could be attributed

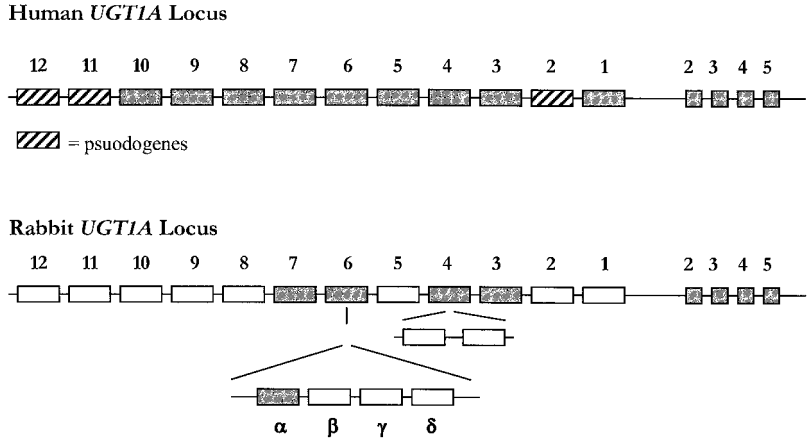

Fig. 7. Graphic representation of the human and rabbit *UGT1A* locus. The human *UGT1A* locus has been characterized from genomic DNA, and the represented gene products shown in solid blocks have been cloned from different human tissues. The patterned blocks encode nonfunctional exon sequences. The diagram of the rabbit *UGT1A* locus is speculative. The exon sequences encoded in solid blocks have all been cloned from rabbit liver RNA, and the expressed proteins have been shown to be active. We predict that the *UGT1A4* allele may have been duplicated, and this is represented by the addition of two additional *UGT1A4*-like exon sequences. The *UGT1A6* allele is encoded by four additional exons, the predicted amino acid sequences of which are shown in Fig. 4. The open boxes indicate that the respective gene products have not yet been identified.

to gene duplication and expression of the multiple alleles associated with the *UGT1A6* locus.

Orthologous forms of UGT between human and rabbit share some similarities in substrate specificities. Rabbit and human UGT1A4 (Green et al., 1995) catalyzed the glucuronidation of several tertiary amines, such as amitriptyline and imipramine, whereas rabbit and human UGT1A3 (Mojarrabi et al., 1996) catalyzes the glucuronidation of estrone. More extensive analysis of substrate specificities carried out with human UGT1A3 indicates that this protein also glucuronidates tertiary amines (Green et al., 1998). However, rabbit UGT1A3 was unable to glucuronidate the prototypical tertiary amines amitriptyline and imipramine, indicating that structural differences between rabbit and human UGT1A3 underlie the differences in the glucuronidation of tertiary amines by this isozyme. Another example of evolutionary differences in catalytic activity is represented by rabbit liver UGT1A7 (Bruck et al., 1997), an isoform similar to human UGT1A7 and UGT1A10 (Strassburg et al., 1998a). Human UGT1A7 and UGT1A10 share a wide range of substrate specificities and are capable of glucuronidating coumarins, flavonoids, phenolic compounds, and steroids through *O*-linked glucuronidation, but not drugs that contain tertiary amine nitrogens. However, rabbit UGT1A7 efficiently glucuronidates tertiary amines such as imipramine and amitriptyline. UGT1A7 and possibly other isozymes related to UGT1A4 may be responsible for the efficient glucuronidation of tertiary amines by rabbits.

The most complete understanding of the organization of the *UGT1A* locus has been obtained through characterization of the human *UGT1A* locus, in addition to the identification of the UGT1A cDNAs in different human tissues (Strassburg et al., 1997b). The human *UGT1A* locus is composed of 12 exons, yet only nine are transcribed into functional protein (Ritter et al., 1992; Mackenzie et al., 1997). All of the exon sequences in the form of cDNAs that encode the different UGTs have been identified from various human tissues. In human liver, only UGT1A1, 1A3, 1A4, 1A6, and 1A9 are expressed (Strassburg et al., 1997a,b). Biliary epithelium from liver tissue also expresses UGT1A1, 1A3, 1A4, and 1A6, but does not express UGT1A9. Extrahepatic tissues such as bilary epithelium (Strassburg et al., 1997b), colon (Strassburg et al., 1998a), esophagus (Strassburg et al., 1999), and small intestine (C.P. Strassburg and R.H. Tukey, unpublished observations) express UGT1A10, indicating that this gene transcript may be selectively expressed in most extrahepatic tissues. Colon epithelium expresses seven transferases in UGT1A1, 1A3, 1A4, 1A6, 1A8, 1A9, and 1A10 (Strassburg et al., 1998a). Interestingly, UGT1A7 has been identified only in gastric tissues of the gastrointestinal tract (Strassburg et al., 1998b). Clearly, a strict pattern of tissue-specific expression of the *UGT1A* locus is in place throughout the gastrointestinal tract, predictably to catalyze the glucuronidation of the many different dietary constituents. Because of the abundance of the UGTs in the gastrointestinal tract, it could be perceived that they play an important role in metabolism during absorption, contributing to first-pass metabolism in intestinal tissue as well as through enterohepatic metabolism.

Ecological studies demonstrate that rabbits and rodents were distinct from each other at the very early stages of mammalian evolution (Colbert, 1980; Novacek, 1989). Most pronounced in the development of rabbits is the tandem arrangement of the front incisor teeth used for gnawing and the unique premolar cheek teeth used for grinding. Although both rodents and rabbits in the wild are considered to be primarily herbivorous, some genera of rodents are carnivorous. There is no evidence in the evolution of lagomorphs that rabbits were meat eaters. The unique expression patterns of the *UGT1A* locus in the human intestinal tract suggest that the evolution of these genes has facilitated the metabolism of dietary constituents. Because it is clear that lagomorphs have evolved with a diet that is entirely vegetable matter, it is possible to speculate that the unique expansion of the *UGT1A6* and *UGT1A4* genes in rabbits has occurred as a result of selective pressure to metabolize the dietary constituents present in a purely herbivorous diet.

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