Isolation and characterization of the monkey *UGT2B30* gene that encodes a uridine diphosphate-glucuronosyltransferase enzyme active on mineralocorticoid, glucocorticoid, androgen and oestrogen hormones

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The present study reports the genomic organization and the characterization of a novel cynomolgus monkey UDP-glucuronosyltransferase (UGT) enzyme, UGT2B30. UGT enzymes are microsomal proteins that catalyse the transfer of the glucuronosyl group from UDP-glucuronic acid (UDPGA) to a wide variety of lipophilic compounds, namely hormonal steroids. The 15 kb *UGT2B30* gene amplified by PCR showed a genomic organization similar to those encoding UGT2B human enzymes. The cDNA encoding UGT2B30 was isolated from a cynomolgus monkey prostate cDNA library, and the deduced amino acid sequence showed an identity of 94 % with UGT2B19, a monkey isoform previously characterized. Stable expression of UGT2B30 protein in human kidney 293 (HK293) cells was assessed by Western-blot analysis and its conjugating activity was screened using 39 potential substrates. The UGT2B30 enzyme is active on many compounds of different classes, including testosterone, dihydrotestosterone, 5α-androstane-3α,17β-diol, androsterone, oestradiol, tetrahydroaldosterone and tetrahydrocortisone, with glucuronidation efficiencies (V_{max}/K_m ratios) ranging from 0.6 to 8.8 $\mu l \cdot min^{-1} \cdot mg$ of $protein^{-1}$. Reverse-transcriptase-PCR analysis revealed that the UGT2B30 transcript is expressed in several tissues, including prostate, testis, mammary gland, kidney, adrenals and intestine. The relative activity of UGT2B30 in comparison with other simian UGT2B isoforms, as well as its large variety of substrates, strongly suggest that this enzyme is essential to inactivation of several steroids.

Key words: glycosyltransferase, glucuronidation, steroid metabolism, relative activity, genomic structure.

INTRODUCTION

Glucuronidation, which is catalysed by uridine-diphosphoglucuronosyltransferase (UGT) enzymes, involves the transfer of the sugar moiety from UDP-glucuronic acid (UDPGA) to small hydrophobic molecules having functional groups of oxygen, nitrogen and sulphur [1]. Glucuronidated products are more polar and thus more easily excreted by hepatic or renal systems. UGT enzymes catalyse the conjugation of numerous endogenous and exogenous compounds, including steroid hormones, biogenic amines, fat-soluble vitamins, bile acids, bilirubin, various drugs and carcinogens [1–3].

While the major role of the liver in glucuronidation is widely accepted, recent studies have demonstrated the importance of extrahepatic conjugation [4–6]. For instance, UGT2B17, an enzyme that conjugates androgens, was strongly expressed in prostate, whereas it showed low expression in the liver [7]. The high expression of UGT2B15 and UGT2B17 in the human prostate, was in agreement with the presence of 5α-reduced C_{19}-steroid glucuronides, namely androsterone glucuronide (ADT-G), in this tissue [8]. Finally, tissues from the gastrointestinal tract, which are first-pass organs for potentially toxic exobiotics, express UGT1A7, UGT1A8 and UGT1A10, which are not detected in liver [8–10].

Human and monkey are unique in having high levels of circulating ADT-G and 5α-androstane-3α,17β-diol glucuronide (3α-Diol-G), two major androgen metabolites of testosterone (Testo) and dihydrotestosterone (DHT), whereas in other species, such as rabbit, rat, mouse, bovine and dog, the plasma levels of these conjugates are undetectable [11]. These results suggested that monkey is the more relevant animal model to study glucuronidation of steroids. Moreover, previous characterization of the 6 UGT1A and 5 UGT2B simian enzymes showed that amino acid sequences and substrate specificities are quite similar to those of human enzymes, and that monkey UGT mRNAs are, as observed in the human, expressed in numerous extrahepatic tissues, including steroid target tissues. In fact, the 5 monkey UGT2B isoforms recently characterized, UGT2B9 [12], UGT2B18 [13], UGT2B19 [14], UGT2B20 [15] and UGT2B23 [16], shared at least 75 % sequence identity with human isoforms and showed overlapping substrate specificity mainly on androgens, bile acids and exogenous compounds.

In the present study, we report the isolation and characterization of UGT2B30, a novel monkey UGT2B enzyme which is active on androgens, oestrogens, mineralocorticoids and glucocorticoids. Its expression in the majority of extrahepatic steroid target tissues, in addition to its large substrate specificity, strongly suggest a major role of UGT2B30 in steroid metabolism.

Abbreviations used: UDPGA, uridine diphosphoglucuronic acid; UGT, uridine diphosphoglucuronosyltransferase; -G, -glucuronide; HSD, hydroxysteroid dehydrogenase; Testo, testosterone; DHT, dihydrotestosterone; DHEA, dehydroepiandrosterone; ADT, androsterone; 3α-Diol, 5α-androstane-3α,17β-diol; 3β-Diol, 5α-androstane-3β,17β-diol; Δ5-Diol, androst-5-ene-3β,17β-diol; Etio, etiocholanolone; E_1, oestrone; E_2, oestradiol; E_3, oestriol; HDCA, hyodeoxycholic acid; THE, tetrahydrocortisone; RT-PCR, reverse transcription-polymerase chain reaction; TLC, thin layer chromatography; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FBS, fetal bovine serum.

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UGT2B30 sequences have been submitted to the GenBank databases under accession number AF401657.

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Moreover, we established the structure of the UGT2B30 gene, which represents the first monkey gene to be analysed.

EXPERIMENTAL

Materials

UDPGA and all aglycones were obtained from Sigma (St. Louis, MO, U.S.A.) and ICN Pharmaceuticals, Inc. (Québec, Canada). Radiolabelled steroids [^{3}H]testosterone, [^{3}H]DHT, [^{3}H]3α-Diol and [^{3}H]ADT and [^{14}C]UDPGA were purchased from NEN Life Science Products (Boston, MA, U.S.A.). Radioinert steroids were purchased from Steraloids Inc. (Wilton, NH, U.S.A.). Blasticidin-S-HCl and ExGEN reagent for transfections were obtained from Invitrogen (Carlsbad, CA, U.S.A.) and Euromedex (Strasbourg, France), respectively. Protein assay reagents were purchased from Bio-Rad Laboratories, Inc. (Richmond, CA, U.S.A.). Restriction enzymes and other molecular-biology reagents were from Pharmacia LKB Biotechnology Inc. (Milwaukee, WI, U.S.A.), Stratagene (La Jolla, CA, U.S.A.) and Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.). SuperScript II reverse transcriptase was from Life Technologies (Burlington, ON, Canada) and AmpliTaq DNA polymerase from Perkin-Elmer Cetus (Branchburg, NJ, U.S.A.). Human embryonic kidney 293 (HK293) cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.).

RNA and cDNA isolation

Total RNA was isolated from *Macaca fascicularis* mammary gland, epididymis, prostate, kidney, testis, adrenal, bile duct, small intestine, brain, cerebellum, lung, colon, spleen, liver, ovary, seminal vesicle, thyroid, vagina, HK293 cells and HK293 cells stably expressing UGT2B30 according to the Tri-reagent acid phenol protocol as specified by the supplier (Molecular Research Center Inc., Cincinnati, OH, U.S.A.). The mRNAs obtained from monkey prostate and liver were isolated by affinity chromatography through oligo(dT)–cellulose (Pharmacia, Milwaukee, WI, U.S.A.) and used to construct a cDNA library in the lambda ZAP express system phage (Stratagene). The UGT2B30 cDNA clone was excised from the pBK-CMV vector using a helper phage (Stratagene). The UGT2B30 cDNA clone was isolated and sequenced in both directions using UGT-specific oligonucleotides [7].

In vitro transcription/translation of the UGT2B30 cDNA

To confirm the ability of the UGT2B30 clone to encode a protein, the cDNA was transcribed and translated *in vitro*. The T7 RNA polymerase, in the presence of [^{35}S]methionine, was used according to the transcription/translation-coupled rabbit reticulocyte lysate system from Promega Corp. (Madison, WI, U.S.A.). The protein product was separated on a SDS/polyacrylamide (10 % gel) and exposed on Hyperfilm-MP for 1 h.

Stable expression of UGT2B30

In order to benefit from the selective properties of blasticidin, the UGT2B30 cDNA was transferred into the pcDNA6 vector (Invitrogen). HK293 cells were grown in Dulbecco's modified Eagle's medium containing 4.5 g/l glucose, 10 mM Hepes, 110 μg/ml sodium pyruvate, 100 units of penicillin/ml, 100 μg/ml of streptomycin and 10 % (v/v) fetal bovine serum (FBS) in humidified incubator, with an atmosphere of 5 % CO_2, at 37 °C. Two μg of pcDNA6-UGT2B30 were used to transfect HK293 cells using ExGEN reagent according to the manufacturer's instructions and a stable transfectant was selected in media containing 10 μg/ml blasticidin, as previously reported [15].

Microsomal protein isolation

HK293 cells stably expressing UGT2B9, UGT2B18, UGT2B19, UGT2B20, UGT2B23 and UGT2B30 were harvested and homogenized in a buffer (0.1 M K_2HPO_4, 0.1 M KH_2PO_4, pH 7.4, 20 % (v/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol, 2.5 μg/ml pepstatin and 0.5 μg/ml leupeptin) using a Potter-Glass-col (Terre Haute, IN, U.S.A.) type homogenizer with a Teflon pestle at 4 °C. Cell homogenates were centrifuged at 12000 ***g*** and 4 °C for 20 min. Supernatants were centrifuged at 105000 ***g*** for 1 h at 4 °C to isolate microsomal proteins. Microsome protein pellets were resuspended in 0.2 ml of homogenization buffer, pooled, and quantified using the Bradford method, with BSA to create standard curves [17]. Samples were kept at −80 °C until Western-blot analysis or glucuronidation assays.

Western-blot analyses

Fifteen μg of microsomal proteins from untransfected HK293 cells or from HK293 cells stably expressing UGT were loaded on to a 10 % SDS-polyacrylamide gel. After migration, the gel was transferred on to a nitrocellulose membrane (Xymotech, Montreal, Canada), and probed with the EL-93 antibody, a specific anti-UGT2B antibody. An anti-rabbit IgG antibody conjugated with peroxidase (Amersham, Ontario, Canada) was used as the second antibody, and the resulting immunocomplexes were visualized using the Western-Blot Chemiluminescence Reagent Plus as specified by the manufacturer (NEN Life Science Products, Boston, MA, U.S.A.) and quantified by BioImage Visage 110s from Genomic Solution Inc. (Ann Arbor, Michigan, U.S.A.).

Glucuronidation assays using microsomal preparations

To screen for substrates that react with UGT2B30, 11.0 μg of microsomal proteins, extracted from HK293 cells stably expressing UGT2B30, were incubated in the presence of 15 μM [^{14}C]UDPGA, 85 μM unlabelled UDPGA with 200 μM of various potential substrates in a final volume of 100 μl. Assays were performed in 50 mM Tris/HCl buffer (pH 7.5), 10 mM $MgCl_2$, 8.5 mM saccharolactone, 10 μg/ml phosphatidylcholine, 2.5 μg/ml pepstatin and 0.5 μg/ml leupeptin, for 16 h at 37 °C, and were terminated by adding 100 μl of methanol. Products were subsequently loaded on to TLC plates and migrated in a toluene/methanol/acetic acid (7:3:1, by vol) solvent for 2 h. In a second set of experiments, compounds that demonstrated reactivity with UGT2B30 in this screening were reassayed to assess activity in the same buffer, containing 15 μM [^{14}C]UDPGA, 500 μM unlabelled UDPGA and 200 μM aglycone, for 4 h at 37 °C. Glucuronide product formation was quantified by Phosphorimager (Molecular Dynamics, Sunnyvale, CA, U.S.A.). Time-course experiments using 3α-Diol (200 μM) as substrate, in presence of 500 μM UDPGA, showed that the enzyme reaction is linear for 18 h.

Relative activity of each monkey UGT2B isoform was ascertained using microsomal proteins extracted from HK293 cells stably expressing UGT2B9, UGT2B18, UGT2B19, UGT2B20, UGT2B23 and UGT2B30 as described above. The reactivity of

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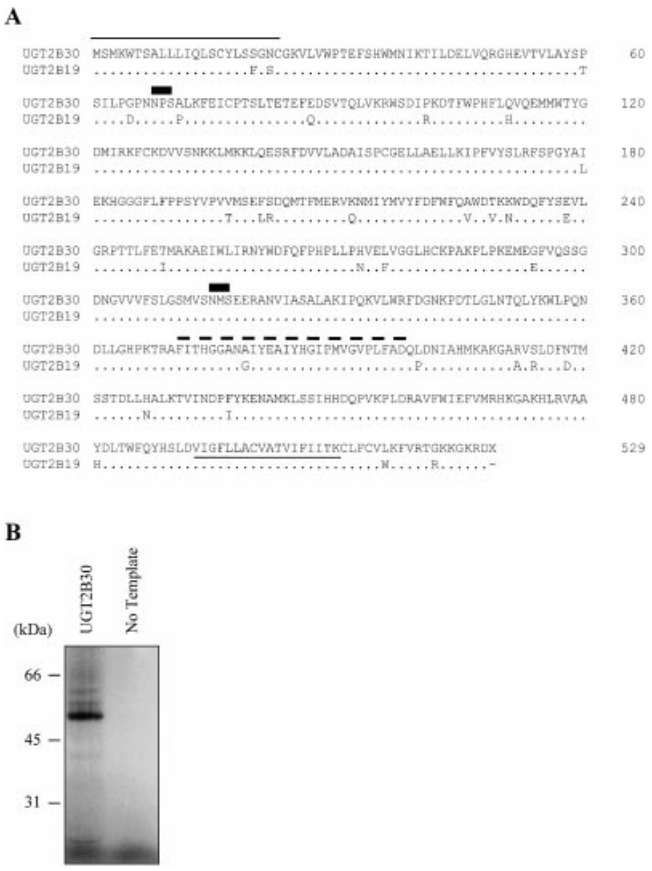

Figure 1 Amino acid sequence of UGT2B30 and alignment with UGT2B19 (A), and *in vitro* transcription/translation of UGT2B30 (B)

(**A**) The putative membrane insertion signal is indicated by the upper line and the sequence of the putative membrane-anchoring domain is underlined. The dashed upper line denotes the consensus sequence of UGT enzymes and the upper lines in bold show the potential asparagine-linked [NX (S/T)] glycosylation sites. (**B**) The *in vitro* transcription/translation product of the UGT2B30 cDNA was separated by SDS/PAGE (10% gel). The protein of 51 kDa, produced from UGT2B30 cDNA, is not seen in the control (No Template).

each monkey UGT2B isoform was determined using 6.74 μg of microsomal proteins in the presence of 15 μM [^{14}C]UDPGA, 500 μM unlabelled UDPGA and 200 μM Testo, DHT, 3α-Diol and ADT. The reactions were carried out for 1 h at 37 °C and steroid-glucuronide formation was analysed and quantified as described above. Glucuronidation activities of each monkey UGT2B isoform were subsequently normalized with the level of protein expression in the corresponding microsomal preparation, as determined in Western-blot experiments.

Kinetic analyses using microsomal preparation

The K_m values were determined by incubation of microsomes (11.0 μg) from HK293 cells stably expressing UGT2B30 with 1 to 20 μM of oestradiol, 5α-tetrahydrocortisone (THE) or 5β-THE in presence of 15 μM [^{14}C]UDPGA, 500 μM unlabelled UDPGA at 37 °C for 4 h. V_{max} and K_m were calculated using double-reciprocal plots (Lineweaver–Burk) and values represent the mean of three separate experiments, each performed in duplicate. Error bars represent the standard deviation of the mean.

Apparent K_m determination in intact cells

Apparent K_m determination in intact cells was performed by incubating HK293 cells, stably expressing UGT2B30, with radiolabelled steroid substrates. Cells were plated at a density of 10^5 cells/well in 24-well plates and exposed for 6 h to concentrations ranging from 0.2 to 20 μM of unlabelled testosterone, DHT, 3α-Diol or ADT, in addition to concentrations ranging from 9.3 to 12.5 nM of the corresponding radiolabelled substrate. Previous experiments have demonstrated that treatment for 6 h are linear. The medium was then removed and analysed for glucuronide formation by organic extraction and scintillation counting as described previously [18]. Total cellular protein concentrations were determined using the Bradford method (Bio-Rad, Hercules, CA, U.S.A.) [17].

Genomic DNA purification and UGT2B30 gene structure analysis by specific PCR amplification

Genomic DNA (136.5 μg) was isolated and purified from 10 ml of cynomolgus monkey whole blood with the QIAamp DNA Blood Maxi Kit, as specified in the manufacturer's handbook (Qiagen, Mississauga, ON, Canada). For PCR amplification, a set of specific oligonucleotides corresponding to exon sequences were designed by comparing the nucleic acid sequence of UGT2B30 and UGT2B4, a human isoform encoded by a gene previously characterized [19]. The UGT2B30-specific binding of each primer pair was assessed by PCR using both the UGT2B30 and the homologous UGT2B19 cDNAs as templates, and with increasing annealing temperatures. The lowest temperature leading to a PCR fragment with the UGT2B30 cDNA and not with UGT2B19 was used for the genomic analysis. Amplifications of UGT2B30 introns were accomplished using the Advantage Genomic PCR Kit from Clontech (Palo Alto, CA, U.S.A.) for 30 cycles: 20 s at 94 °C, 20 s at corresponding annealing temperature (from 50–60 °C) and 5–12 min at 68 °C. Exon/intron junctions of the *UGT2B30* gene were determined by direct sequencing of PCR products using the Thermo Sequenase Radiolabelled Terminator Cycle Sequencing Kit from USB (Cleveland, OH, U.S.A.). One-sixth of each PCR was electrophoresed on an ethidium bromide-stained 0.7 %-agarose gel, and markers from MBI Fermentas (Burlington, ON, Canada) were used to evaluate their sizes.

Reverse transcriptase (RT)-PCR analysis

The tissue distribution of the UGT2B30 expression was determined using a RT-PCR approach as previously reported [14], using 5 μg of total RNA from cynomolgus monkey tissues, HK293 cells stably expressing UGT2B30, and untransfected cells. Reverse transcription reactions were performed using 500 pmol of the UGT2B30-specific antisense primer 5′-CACTGTAAAACTGATCCCACTTCTTCG-3′ and 2 pmol of the antisense glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer 5′-CCCAGCGTCAAAGGTCC-3′, in the presence of 200 units of SuperScript II reverse transcriptase, according to the manufacturer's instructions (Life Technologies). The PCR reactions were carried out with one-tenth of the RT products and 100 pmol of the specific sense primer 5′-GAAACTGAGTTTGAGGATAGCGTCACA-3′ and 100 pmol of the UGT2B30 specific antisense primer described above, using ampliTaq DNA polymerase. The PCR was performed for 30 cycles (1 min at 94 °C, 1 min at 67 °C, 1 min at 72 °C). One-fifth of the PCR products were electrophoresed on an ethidium bromide-stained 1 %-agarose gel. The sense primer for GAPDH, 5′-TGGGTGT-

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GAACCATGAG-3′, with the GAPDH specific antisense primer described above were used as controls for each PCR reaction. The identity of PCR products was verified by direct sequencing [20].

RESULTS

Isolation of the UGT2B30 cDNA

Cynomolgus monkey prostate and liver cDNA libraries were screened with a combination of probes corresponding to the full-length human cDNAs of UGT2B7, UGT2B10 and UGT2B15. Twenty positive clones were isolated, and sequence analysis led to isolation of six novel cDNAs: UGT2B9 [12], UGT2B18 [13], UGT2B19 [14], UGT2B20 [15], UGT2B23 [16] and UGT2B30. The cDNA encoding UGT2B30 is 1915 bp long with an open reading frame of 1587 bp. The protein primary structure, which consists of a 529-amino-acids sequence predicted from the nucleotide sequence, is characteristic of UGT2B enzymes. It contains a hydrophobic signal peptide from residues 1–23, a putative membrane-anchoring domain from 493–510 and a consensus signature sequence found in all UGT enzymes (371–399) [1]. Two potential sites of *N*-glycosylation [NX(S/T)] are present at positions 68–70 and 315–317 (Figure 1A). The predicted coding region of UGT2B30 cDNA was confirmed by *in vitro* transcription/translation that lead to the expression of a 51 kDa protein, which is consistent with other characterized UGT proteins (Figure 1B).

Amino acid sequence comparison with other UGT2B isoforms showed that UGT2B30 is highly homologous with the monkey UGT2B19 (94 % of amino acid sequence homology) and shares between 74 and 80 % of sequence identity with other cynomolgus monkey proteins (Table 1). Interestingly, UGT2B30 is also highly homologous with the Rhesus monkey UGT2B33 and UGT2B9*2 proteins, with 78 % and 80 % homology respectively (GenBank AF294902 and AF294901, respectively). As shown in Table 1, UGT2B30 is highly similar to the human UGT2B4 enzyme, with an overall homology of 87 %, while it shares between 76 and 80 % homology with other human UGT2B proteins. Homology between UGT2B30 and monkey UGT2B proteins is high in the carboxy-terminal domain (residues 291–529), which is suggested to be the region that binds UPDGA. For instance, UGT2B19 contains 11 amino acid residues which are different from those in the UGT2B30 sequence in this domain, and 20 different amino acids in the amino-terminal domain (1–290).

UGT2B30 gene structure

PCR amplifications using specific primers were performed through the *UGT2B30* gene to determine the general intron–exon structure of the gene. As expected, the *UGT2B30* gene is composed of 6 exons and 5 introns, spanning approximately 15.3 kb (Figure 2A). As deduced from electrophoresis of PCR fragments (Figure 2B), the introns 1 to 5 are 2.9, 4.3, 2.9, 1.2 and 2.4 kb in size, respectively. All the exon/intron junctions agree with the ''GT-AG'' rule as described by Breathnach and Chambon [21] (Figure 2C). The *UGT2B30* gene structure is most similar to those of rat isoforms *UGT2B1* and *UGT2B2*, and of human *UGT2B4* and *UGT2B7* (Figure 2D). In contrast, the *UGT2B30* gene is shorter than those of human *UGT2B15* and *UGT2B17*. However, despite the fact that the size of exons is conserved in all the mammalian *UGT2B* genes characterized to date, intron sizes vary greatly. Interestingly, the *UGT2B30* gene introns 1 and 3 were 2-fold longer and shorter respectively, in comparison with those of other rat and human *UGT2B* genes, with the exception of the *UGT2B15* gene, which demonstrated similar-size introns (Figure 2D). The size of intron 5 is more variable among all genes, ranging from 2.4 kb for *UGT2B30* to 12.0 kb for *UGT2B17*.

Table 1 Homology between the deduced amino acid sequence of UGT2B30 and other mammalian UGT2B isoforms

Protein sequences were obtained from GenBank database. The sequence identity of the amino-terminal domain (residues 1–290), the carboxy-terminal domain (residues 291–529), and the entire protein is as indicated. The steroid specificity of each isoenzyme is also indicated. UGT2B19 and UGT2B4, which share the most sequence identity with UGT2B30, are shown in bold. 4-OHE_1, 4-hydroxyoestrone; DHT, dihydrotestosterone; ADT, androsterone; 3α-Diol, 5α-androstane-3α,17β-diol; Etio, etiocholanolone; T, testosterone; E_3, oestriol; 2-OHE_3, 2-hydroxyoestriol; E_2, oestradiol; E_1, oestrone. Unpublished work (B. Dean, S. Zhao and C. King).

			UGT2B30 amino acid identity (%)			
Source	Enzyme	Substrate specificity	Amino-terminal domain	Carboxy-terminal domain	Overall	References
Cynomolgus monkey	UGT2B9	4-OHE_1, DHT, ADT	78	82	80	[12]
	UGT2B18	ADT, 3α-Diol, Etio	78	83	80	[13]
	UGT2B19	T, Etio, 4-OHE_1	**93**	**95**	**94**	[14]
	UGT2B20	3α-Diol, T, DHT	68	79	74	[15]
	UGT2B23	Etio, E_3, 3α-Diol, ADT	76	80	78	[16]
Rhesus monkey	UGT2B9*2		78	81	80	unpublished
	UGT2B33		77	80	78	unpublished
Human	**UGT2B4**	4-OHE_1, 3α-Diol	**84**	**92**	**87**	[41]
	UGT2B7	4-OHE_1, E_3, 2-OHE_3	78	84	80	[43]
	UGT2B10	Not detected	74	83	79	[44]
	UGT2B11	Not detected	75	85	80	[45]
	UGT2B15	3α-Diol, DHT, T	71	79	76	[46]
	UGT2B17	DHT, ADT, 3α-Diol, T	71	79	76	[7]
Rat	UGT2B1	T, DHT, E_2	61	72	66	[47]
	UGT2B2	ADT, Etio	58	69	63	[23]
	UGT2B3	T, DHT, E_2	60	67	64	[48]
	UGT2B6	E_2	59	67	63	[48]
	UGT2B12	No steroid	62	70	66	[49]

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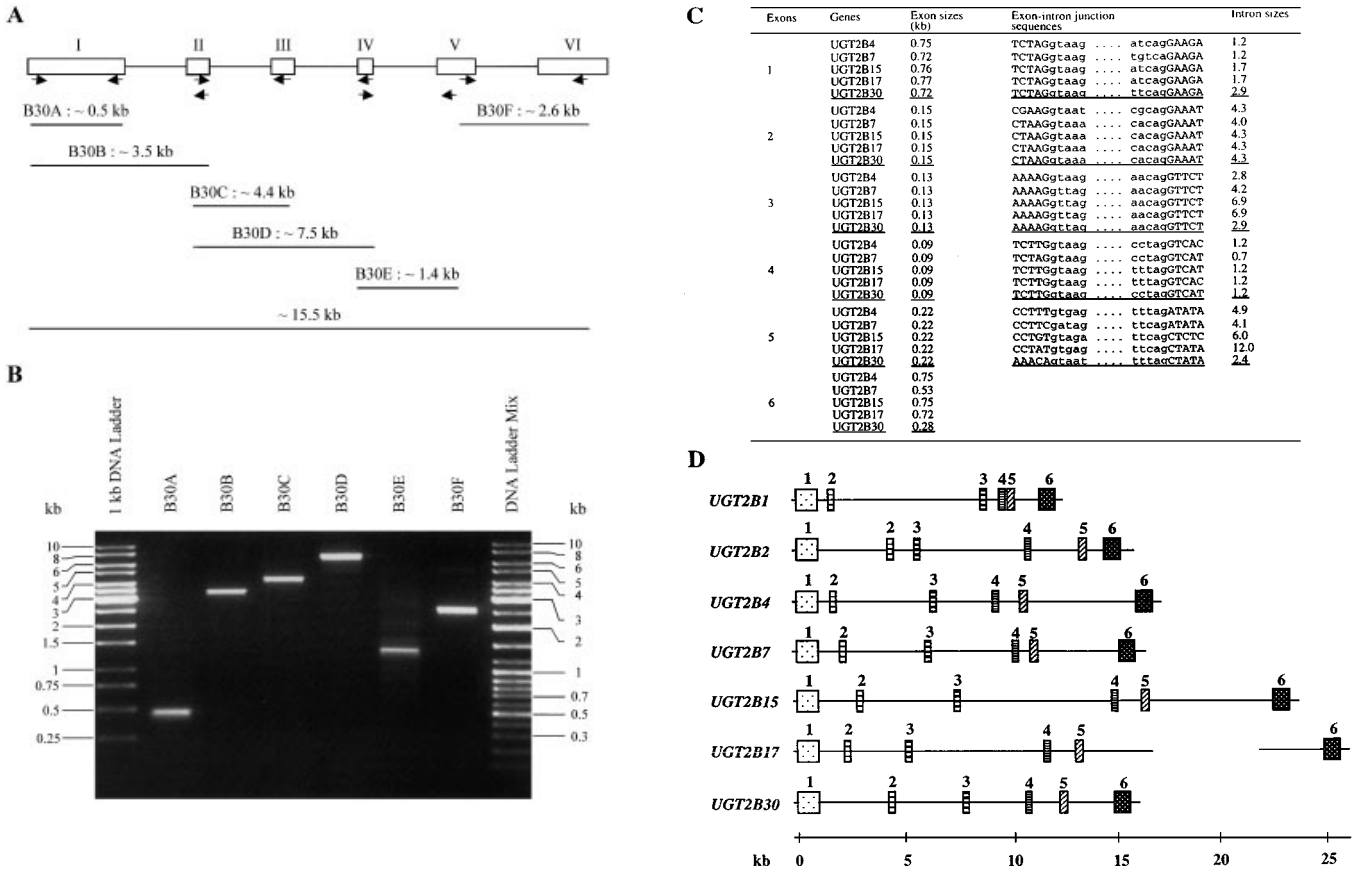

Figure 2 Exon-intron structure of the *UGT2B30* gene

(**A**) The positions and relative sizes of exons (boxes) and introns (connecting lines) of the monkey UGT2B30 gene are shown. The arrows indicate the location of hybridization of PCR primers used for determination of intron lengths. (**B**) The PCR fragments were separated on a 0.7%-agarose gel stained with ethidium bromide and their sizes were estimated using the GeneRuler™ 1 kb DNA ladder (left) and the GeneRuler™ DNA ladder mix (right) markers. (**C**) The exact sizes of the exons and the approx. sizes of the introns of the human genes and the UGT2B30 gene are indicated. The exon–intron junction sequences are represented with upper-case and lower-case letters respectively. (**D**) The organization of all *UGT2B* genes characterized to date in different species is represented in a schematic view.

Characterization of the UGT2B30 protein

To further characterize UGT2B30, HK293 cells were transfected with the UGT2B30-pcDNA6 expression vector and selected for using blasticidin-S-HCl. The stable expression of UGT2B30 protein from 19 clones was verified by Western blotting using the anti-UGT2B EL-93 antibody [22]. An immunoreactive protein of 51 kDa was detected in a microsomal preparation of the stable cell lines, while no antigenic protein was observed in microsomes of untransfected HK293 cells (results not shown). The 10 clones which demonstrated the highest protein expression levels were tested for their glucuronidation activity using 3α-Diol and eugenol as substrates (results not shown). The clone demonstrating the highest expression and activity was used for the following experiments. Using this clone, the optimal conditions for the glucuronidation assay were determined. Preliminary experiments using 3α-Diol (200 μM) as substrate demonstrated that UGT2B30 activity is linear for up to 18 h and that 515 μM UDPGA is saturating (results not shown).

To ascertain the substrate specificity of UGT2B30, 39 endogenous and exogenous compounds were tested (Table 2). This enzyme conjugated 24 compounds, including androgens, oestrogens and progestins, in addition to glucocorticoid and mineralocorticoid metabolites. The major glucuronidation activity was obtained with 3α-Diol and its 5β epimer (5β-androstane-3α,17β-diol). Etiocholanolone, *p*-nitrophenol, 1-naphthol, tetrahydroaldosterone, 5β-tetrahydrocortisone (5β-THE), oestradiol and androsterone were also glucuronidated to a high velocity (Figure 3). C_{19} steroids having an hydroxyl group on 3α position (3α-Diol, etiocholanolone and ADT) were the more reactive endogenous substrates for UGT2B30, whereas 3β-hydroxyandrogens (epiandrosterone, dehydroepiandrosterone (DHEA), androst-5-ene-3β,17β-diol (Δ5-Diol) and 3β-Diol) were not conjugated. Interestingly, UGT2B30 presented a 3- and 4-fold higher glucuronidation velocity for 3α-Diol than for the potent androgen (DHT) or its precursor testosterone, respectively. UGT2B30 also conjugated the potent oestrogen (oestradiol) and its metabolite (oestriol). The glucuronidation of several catecholoestrogens, C_{21} steroids, such as 5α-pregnan-3α-ol-20-one and 5α-pregnane-3α,20α-diol, and hyodeoxycholic acid (HDCA) was also catalysed by this UGT isoenzyme, but to a lower extent. Interestingly, UGT2B30 was not active on aldosterone and cortisol, whereas it demonstrated activity for their respective metabolites, tetrahydroaldosterone and 5α- and 5β-THE.

Kinetic analysis of UGT2B30 conjugation of testosterone, DHT, 3α-Diol and ADT was performed using intact HK293 cells stably expressing UGT2B30 (Figures 4A and 4C). While the analyses using intact cells represent a composite of various factors, the apparent K_m values provide more physiological indication of the cellular glucuronidation efficiency of UGT2B30.

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Table 2 Reactivity of endogenous and exogenous compounds with UGT2B30 expressed in HK293 cells

The initial screening on potential substrates was performed using 15 μM [^{14}C]UDP-glucuronic acid (UDPGA), 100 μM unlabelled UDPGA, 200 μM various aglycons, and 11.0 μg of HK293-UGT2B30 microsomal preparations. Compounds that demonstrated reactivity were reassayed, under saturating and linear conditions, in the presence of 15 μM [^{14}C]UDPGA, 500 μM unlabelled UDPGA and 200 μM various aglycons, for 4 h at 37 °C. (–) indicates no reactivity detected at initial screening, and N.Q. (not quantifiable) indicates that the extent of glucuronidation was too low to be quantified. Activity values presented are the mean of two independent experiments each performed in duplicate.

Endogenous compounds	Glucuronide formation (pmol/min per mg of protein)
C_{19} Steroids	
Testosterone	117.0 ± 1.5
Dihydrotestosterone	107.1 ± 7.9
Androsterone	142.0 ± 3.4
Epiandrosterone	–
Dehydroepiandrosterone	–
Etiocholanolone	332.7 ± 33.6
Androst-5-ene-3β,17β-diol	–
5α-Androstane-3α,17β-diol	432.2 ± 58.4
5α-Androstane-3β,17β-diol	–
5β-Androstane-3α,17β-diol	411.2 ± 17.0
C_{18} Steriods	
Oestrone	–
Oestradiol	128.0 ± 5.5
Oestriol	158.8 ± 7.0
1,3,5,10-Oestratriene-2,3-diol-17-one	–
1,3,5,10-Oestratriene-3,4-diol-17-one	33.7 ± 0.6
1,3,5,10-Oestratriene-2,3,17β-triol	28.9 ± 0.5
1,3,5,10-Oestratriene-3,4,17β-triol	51.1 ± 13.9
C_{21} Steriods	
Pregnenolone	–
17-OH-Pregnenolone	–
5α-Pregnane-3α-ol-20-one	29.9 ± 1.3
5α-Pregnane-3α,20α-diol	24.7 ± 4.8
Aldosterone	–
Tetrahydroaldosterone	11.3 ± 0.7
Cortisol	–
11-Deoxycortisol	–
5α-Tetrahydrocortisone	115.3 ± 14.0
5β-Tetrahydrocortisone	177.0 ± 26.1
Bile acids	
Chenodeoxycholic acid	N.Q.
Cholic acid	N.Q.
Lithocholic acid	
Hyodeoxycholic acid	12.8 ± 1.1
Others	
Retinoic acid	–
Triiodothyronine	–
Thyroxine	–
Exogenous compounds	
4-Methylumbelliferone	81.3 ± 6.1
p,*p*′-Biphenyl	63.9 ± 3.3
Eugenol	90.3 ± 20.3
1-Naphthol	183.2 ± 31.7
p-Nitrophenol	257.9 ± 5.4

Furthermore, previous reports suggested that microsome assays and intact-cell experiments yielded comparable apparent K_m values for C_{19}-steroid glucuronidation by monkey UGT2B enzymes [12,13]. The apparent K_m values were in the micromolar range, as determined by the double-reciprocal plots (Lineweaver–Burk). K_m value for conjugation of ADT (0.7 μM) was more than 2-fold lower than for 3α-Diol, DHT and testosterone (1.4, 1.4 and 1.5 μM respectively). However, the apparent maximal velocity (V_{max}) value obtained for ADT was 2-fold higher than for 3α-Diol, thus the V_{max}/K_m ratios demonstrate similar glucuronidation efficiencies for both substrates. Experiments performed using microsomal proteins showed K_m values of 20.2, 15.6 and 7.0 μM for oestradiol, 5α-THE and 5β-THE respectively (Figures 4B and 4D). The V_{max}/K_m ratio was 2.5-fold higher for 5β-THE than for 5α-THE, while the V_{max} was similar for these two metabolites.

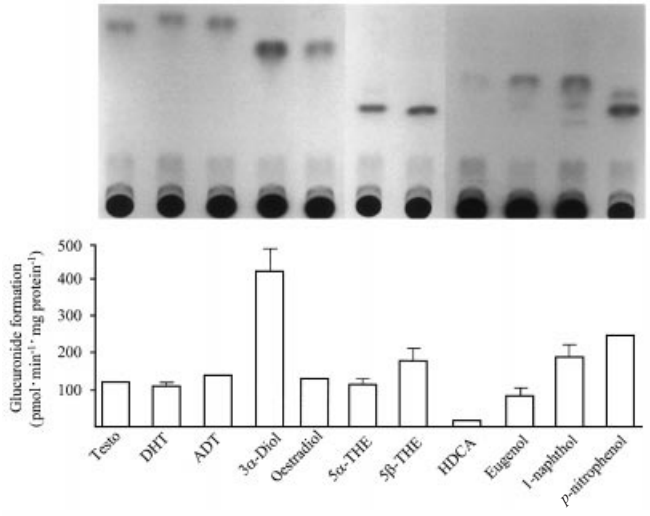

Figure 3 Thin layer chromatography (TLC) of glucuronidated products by UGT2B30

Assays were performed using HK293-UGT2B30 cell microsomes in the presence of 15 μM [^{14}C]UDPGA, 500 μM unlabelled UDPGA, and 200 μM various aglycons, for 4 h at 37 °C. The free [^{14}C]UDPGA (at the bottom) and labelled conjugated product were separated by TLC using a solvent system of toluene/methanol/acetic acid (7:3:1, by vol.) and the plate was exposed on Hyperfilm^{TM}-MP for 14 d. Error bars represent the standard deviation of the mean. V_{max} values of the corresponding substrates are presented.

Relative activity of monkey UGT2B isoforms on androgens

Glucuronidation of the four major androgens, namely testosterone, DHT, 3α-Diol and ADT, was evaluated using microsomal proteins purified from HK293 cells stably expressing UGT2B9, UGT2B18, UGT2B19, UGT2B20, UGT2B23 or UGT2B30. To ensure that the difference observed between the glucuronidation activity of C_{19} steroid by monkey UGT2B enzymes does not reflect their different expression levels in UGT-HK293 cell lines, the UGT2B protein contents were determined by Western-blot experiments using the anti-UGT2B EL-93 antibody (Figure 5). Immunoreactive proteins were quantified, and the androgen-conjugating activity of each enzyme was therefore corrected with the amount of proteins contained in each extract. Our results show that testosterone is glucuronidated preferentially by UGT2B9, UGT2B19 and UGT2B20 (Figure 5A), whereas UGT2B9 is the most active on DHT (Figure 5B). Thus, conjugation of the two major androgen metabolites found in monkey serum as glucuronide-conjugated (3α-Diol and ADT) is mainly catalysed by UGT2B30 and UGT2B9 for 3α-Diol (Figure 5C), and by UGT2B18 and UGT2B9 for ADT (Figure 5D). Interestingly, UGT2B30 activity is very high for these four androgens. Considering the low level of UGT2B23 protein expression in HK293 cells, the precise role of this isoform in androgen metabolism remains to be assessed.

Tissue distribution of the UGT2B30 transcript

To determine in which tissues UGT2B30 is expressed, RT-PCR analysis using specific primers was performed on total RNA

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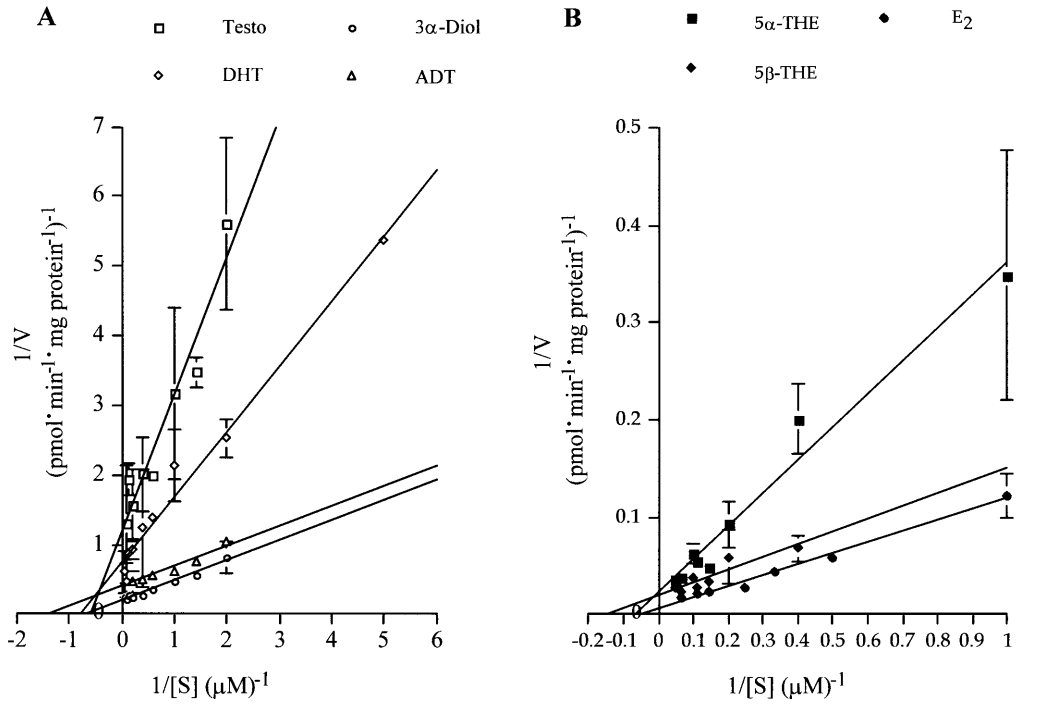

C

Substrates	Km (app)	Vmax (app)	Vmax/Km
	μM	pmol • min^{-1} • mg protein^{-1}	μl•min^{-1}•mg protein^{-1}
Testo	1.47±0.70	0.88±0.05	0.598
DHT	1.35±0.68	1.66±0.59	1.229
3α-Diol	1.41±0.24	5.17±0.13	3.666
ADT	0.66±0.06	2.42±0.12	3.666

D

Substrates	Km (app)	Vmax (app)	Vmax/Km
	μM	pmol • min^{-1} • mg protein^{-1}	μl•min^{-1}•mg protein^{-1}
E_2	20.2±9.0	177.3±63.4	8.798
5α-THE	15.6±2.0	45.8±4.6	2.933
5β-THE	7.0±3.0	52.9±11.9	7.534

Figure 4 Lineweaver–Burk plots and kinetic analysis for glucuronidation of testosterone (Testo), dihydrotestosterone (DHT), 5α-androstane-3α,17β-diol (3α-Diol), androsterone (ADT), oestradiol (E_2), 5α-tetrahydrocortisone (5α-THE) and 5β-tetrahydrocortisone (5β-THE) by UGT2B30

Experiments were performed using intact cells (**A, C**) and microsomal preparations (**B, D**) of HK293 cells stably expressing the UGT2B30 enzyme as described in the Experimental section. Values of apparent K_m and V_{max} were deduced from three separate experiments each performed in duplicate.

isolated from monkey tissues. Oligonucleotides used for the PCR reactions were shown to be specific to UGT2B30 mRNA and did not amplify the previously characterized monkey UGT2B transcripts. Direct sequencing confirmed the identity of the 465 bp PCR product as being UGT2B30. This product, demonstrating the expression of the UGT2B30 mRNA, was found in RNA purified from monkey mammary gland, prostate, kidney, testis, adrenal, bile duct, small intestine, brain, colon, liver, ovary, seminal vesicle and vagina (Figure 6). However, the UGT2B30 mRNA was not detected in epididymis, cerebellum, lung, spleen and thyroid gland. As expected, untransfected HK293 cells did not express UGT2B30, while the 465 bp product was found in the UGT2B30-HK293 cells. The integrity of each RNA sample was verified by amplification of the GAPDH transcript using specific oligonucleotides.

DISCUSSION

To investigate further the use of cynomolgus monkey as the best animal model for the study of the physiological role of extrahepatic steroid glucuronidation, monkey prostate and liver cDNA libraries were screened to isolate UGT2B enzymes. A novel monkey enzyme, UGT2B30, active on the major metabolites of all classes of steroid hormones has been characterized. The gene encoding simian UGT2B30 revealed a general organization of six exons spanning approximately 15 kb, which is

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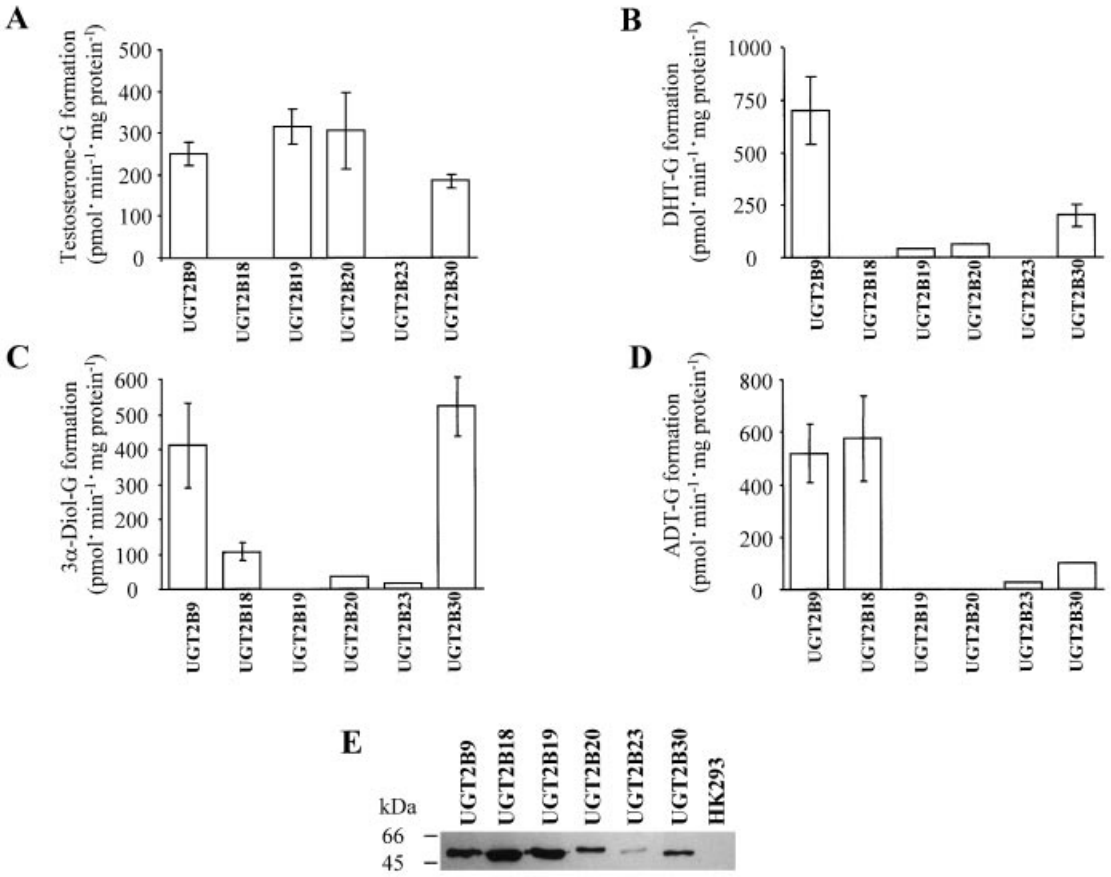

Figure 5 Relative activity of monkey UGT2B enzymes toward androgenic substrates

Microsomal preparations of HK293 cells expressing each monkey UGT2B isoform were tested for their reactivity on testosterone (**A**), dihydrotestosterone (**B**), 3α-Diol (**C**) and androsterone (**D**). Proteins were incubated for 1 h at 37 °C in the presence of 15 μM [^{14}C]UDPGA, 500 μM unlabelled UDPGA and 200 μM potential substrate. Results represent two independent experiments performed in duplicate. Glucuronide formation activities were normalized by the level of protein expression in the corresponding transfected cell line as determined by Western-blot analysis (**E**).

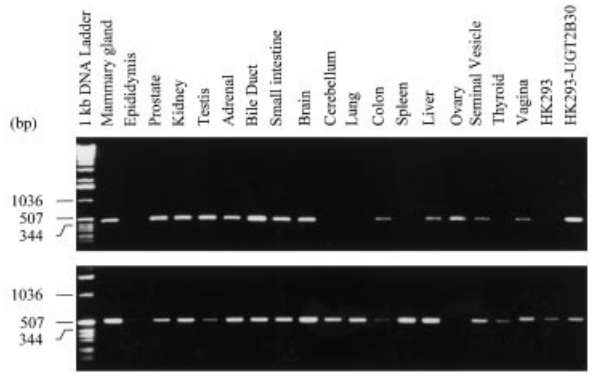

Figure 6 Tissue distribution of the UGT2B30 transcript

Total RNA isolated from several cynomolgus monkey tissues, non-transfected HK293 cells and HK293 cells stably expressing UGT2B30 was analysed by specific RT-PCR. One fifth of each PCR product was separated on a 1%-ethidium-stained agarose gel. The 465 bp PCR product represents amplification of the UGT2B30 transcript, as confirmed by direct sequencing. The integrity of each RNA sample was verified by amplification of the GAPDH transcript using specific oligonucleotides.

similar to that of rat genes *UGT2B1* and *UGT2B2* [23,24], and human *UGT2B4*, *UGT2B7*, *UGT2B15* and *UGT2B17* [19,25–27]. In addition to amino acid sequence homology, the genomic structure is an important factor in discriminating between UGT1A and UGT2B isoforms. In rats and humans, UGT1A proteins are encoded by a single gene composed of 17 different exons. The first 13 exons encode for the amino-terminal parts of the 13 different mRNAs, and are named exons 1A1 to 1A13, while the remaining 4 exons encode the common carboxy-terminal half of UGT1A proteins [28,29]. In contrast, rat and human UGT2B enzymes are encoded by separate genes each composed of 6 exons. While the structure of the gene encoding cynomolgus monkey UGT1A is still not determined, the similarity between human and monkey *UGT2B* genes suggests a similar organization of the monkey *UGT1A* and *UGT2B* genes. Moreover, the high degree of homology in the genomic organization of *UGT2B* genes, as observed in the similar exon sizes, supports the hypothesis that *UGT2B* genes originate from a common ancestral gene that was conserved during evolution.

The product of the *UGT2B30* gene catalyses the glucuronidation of various exogenous and endogenous compounds, such as androgens, oestrogens, progestins, mineralocorticoids and glucocorticoids. Of the 34 endogenous compounds tested as potential substrates, the more reactive substrates were C_{19} steroids, which can be glucuronidated on the hydroxyl group at both 3α or 17β positions. Interestingly, the glucuronidation of the 3β-hydroxylated androgens was not detected, thus suggesting a specificity of UGT2B30 for different stereoisomers of the C_{19} steroid molecule. UGT2B30 conjugates 5β and 5α-reduced androgens

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with the same velocity, and is in contrast with monkey UGT2B19 enzyme, which is more active on 5α-reduced compounds [14].

Whereas UGT2B enzymes catalyse preferentially the conjugation of C_{19} steroids, the glucuronidation of oestrogens and their metabolites is generally associated with UGT1A isoforms. Thus, oestradiol-conjugating activity of human and monkey UGT1A1 [30], UGT1A3 [31] and UGT1A9 [32] has been demonstrated and no human UGT2B enzyme, except UGT2B7 which shows a low activity with oestradiol, is active on the potent oestrogen (oestradiol, E_2) and its precursor (oestrone, E_1) [6]. Interestingly, the two monkey enzymes, UGT2B9 and UGT2B30, are able to efficiently conjugate oestradiol.

UGT2B30 is also active on other steroids, including some pregnanes such as mineralocorticoid (tetrahydroaldosterone) and glucocorticoid metabolites. This isoenzyme is involved in the conjugation of 5α-THE and 5β-THE, which are the two major cortisol metabolites found in urine [33]. Cortisol is metabolized by several enzymes, including irreversible inactivation by A-ring reductase, while two isoforms of 11β-hydroxysteroid dehydrogenase (11β-HSD) catalyse the interconversion of hormonally active cortisol and inactive cortisone [34,35]. As for other hormones, glucuronidation is an important metabolic pathway for hydroxylated cortisone derivatives. In fact, more than 91% of cortisol metabolites present in human urine correspond to conjugated metabolites, and mainly to glucuronidated products [33]. Furthermore, elevated urinary levels of glucuronidated metabolites of cortisol and cortisone could be associated with Cushing's syndrome [36–38]. As in the human, cortisol metabolites are mainly excreted as glucuronide derivatives in the cynomolgus monkey [39]. UGT2B30 conjugates 5β epimer of THE with a 3-fold higher efficiency (ratio V_{max}/K_m) than 5α-THE; this observation is in agreement with the observation that, in humans, 5β-THE-glucuronide is the predominant conjugated form of cortisol metabolites found in urine [33].

In addition to the structural similarity of the gene encoding UGT2B4 and UGT2B30, the two proteins share more than 85% of their amino acid composition. Comparison of the amino-terminal portion of the proteins, which has been proposed to contain the aglycone-binding domain, reveals that UGT2B30 is 84% identical with UGT2B4. In contrast, the substrate specificity of UGT2B30 toward androgens is more similar to those of the human enzyme UGT2B17 [7], which also conjugates C_{19} steroids at their hydroxyl group at position 3α or 17β, but not at 3β. In fact, UGT2B4 and UGT2B30 present important differences in their ability to conjugate steroid substrates. The human enzyme UGT2B4 catalyses only the 3α-hydroxyandrogen glucuronidation and only one catecholoestrogen, the 4-hydroxyoestrone [40,41]. Furthermore, bile acids are important substrates for UGT2B4, while they are poorly metabolized by UGT2B30 [40,41]. These differences between primary-structure homology and substrate specificities of UGT2B proteins suggest that only a few amino acid changes could markedly affect their enzymatic properties.

UGT2B30 shares 94% amino acid sequence identity with UGT2B19, a simian enzyme previously characterized [14]. Despite similar substrate specificities on catecholoestrogens, UGT2B30 conjugates a wider range of steroids such as androsterone, oestradiol, oestriol, some pregnanes and bile acids, whereas UGT2B19 is almost limited to androgen substrates (testosterone, etiocholanone and 3α-Diol). The UGT2B30 substrate specificity appears quite similar to that of UGT2B9 [12], which has only 80% sequence identity with UGT2B30. According to their stereo-specificity for androgen glucuronidation, the 6 monkey UGT2Bs characterized to date could be divided into three categories. UGT2B9 and UGT2B30 catalyse the glucuronidation of both the 3α- and 17β-hydroxy positions on the androgen molecule [12]. UGT2B18 and UGT2B23 are more specific for glucuronidation of 3α-hydroxyandrogens [13,16], while UGT2B19 and UGT2B20 glucuronidate androgens more specifically at the 17β-hydroxy position [14,15].

To evaluate the role of each monkey UGT2B isoform in target tissues, their relative activity was determined using the main four androgen substrates (testosterone, DHT, ADT and 3α-Diol). The glucuronidation of each C_{19} steroid was performed by a distinct set of isoenzymes, with different velocities. UGT2B23 expression in the derived HK293 cell line is very low, and thus the exact role of this enzyme in androgen metabolism could be under-rated. To extrapolate these results *in vivo*, respective expression levels of isoforms in the given tissues must be taken in account. For instance, relative activity experiments showed a similar activity of UGT2B9, UGT2B19, UGT2B20 and UGT2B30 for testosterone. Since UGT2B19 is not expressed in testis [12], UGT2B9, UGT2B20 and UGT2B30 would be responsible for the glucuronidation of testosterone in this tissue. Moreover, relative activity for the monkey UGT2B enzymes demonstrated that UGT2B9 is the principal isoform implicated in glucuronidation of DHT. As well, UGT2B9 and UGT2B30 are the most important UGTs for the elimination of 3α-Diol, whereas for ADT, UGT2B9 and UGT2B18 are the two major isoforms. In the mammary gland, where androgens may regulate the oestrogen receptor and serve as precursors for oestrogen synthesis, UGT2B9 and UGT2B18 mRNAs were not detected [4]. Thus, UGT2B30 could be the major UGT2B isoform involved in the conjugation of the active androgen and its reduced metabolites in breast tissue.

RT-PCR analysis showed that the UGT2B30 transcript is expressed in many extrahepatic tissues, including steroid target tissues. The expression of UGT2B30 in tissues such as kidney, adrenals, intestine, liver and pancreas is consistent with its high reactivity toward glucocorticoids and mineralocorticoids, in addition to xenobiotics. In prostate, where DHT is the main androgen, all monkey UGT2B isoforms are expressed [4]. The presence of UGT2B30 in monkey prostate as demonstrated by RT-PCR analysis, combined with its reactivity toward the active hormone testosterone and its metabolites, further suggest an intracrine role of this enzyme in androgen catabolism in this tissue [42].

In conclusion, we report in this study the isolation and characterization of a novel UGT2B monkey isoform, UGT2B30, which is expressed in many extrahepatic tissues. This enzyme is active on all classes of steroid hormones, namely metabolites of glucocorticoids and mineralocorticoids. In addition, we assessed the structural similarities of the UGT2B30 gene to rat and human *UGT2B* genes, which suggest a conserved genomic structure among mammalian species.

We thank Dr Pei Min Rong for its technical assistance in DNA sequencing and Western-blot analysis. This work was supported by the Canadian Institutes of Health Research (CIHR) and by the Fonds pour la formation de chercheurs et l'aide à la recherche-Fonds de la recherche en santé du Québec (FCAR-FRSQ).

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Received 30 October 2001/11 February 2002; accepted 15 April 2002

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