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UDP-glucuronosyltransferase activity, expression and cellular localization in human placenta at term

Abby C. Collier[a,*], Natalie A. Ganley[a], Malcolm D. Tingle[a], Marion Blumenstein[b], Keith W. Marvin[b], James W. Paxton[a], Murray D. Mitchell[b], Jeffrey A. Keelan[a]

[a]*Department of Pharmacology and Clinical Pharmacology, University of Auckland Medical School, Private Bag 92019 Auckland, New Zealand*
[b]*The Liggins Institute, University of Auckland, Auckland, New Zealand*

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Abstract

The activity, expression and localization of the UDP-glucuronosyltransferases (UGTs) were investigated in human placenta at term. UGT activity (measured with the substrate 4-methylumbelliferone (4-MU)) was observed in all 25 placentas sampled and maximum velocity (V_{max}) ranged 13-fold from 5.1 ± 0.9 to 66.9 ± 17.5 nmol/min/mg protein (mean $\pm$ SD). Substrate affinity (K_m) ranged 5-fold from 246 ± 24 to 1124 ± 422 μM. Using reverse transcriptase-polymerase chain reaction (RT-PCR), expression of the isoforms UGT2B4, 2B7, 2B10, 2B11 and 2B15 was observed in all (12/12) placentas sampled and expression of UGT2B17 was noted in 8/12 placentas. Northern analysis of the UGT2B7 isoform in 12 placentas revealed a 10-fold difference in expression with RT-PCR variability and the 13-fold variation observed in UGT activity. The presence of UGT2B4 and 2B7 proteins (52 and 56 kDa, respectively) was demonstrated by Western blotting. The sites of placental UGT2B transcription (*in situ* hybridization) and protein expression (immunohistochemistry) were located in the syncytium of the placental trophoblasts bordering the placental villi. UGT1A proteins could not be observed with immunohistochemistry or Western blotting and expression could not be observed with RT-PCR. Our discovery of UGT expression and activity at the site of maternal–fetal exchange is consistent with a role for UGTs in detoxification of exogenous and endogenous ligands and the maintenance of placental function through clearance and regulation of steroid hormones.

Keywords: UDP-glucuronosyltransferase; Placenta; Placental metabolism; UGT gene expression; UGT2B; Immunohistochemistry

1. Introduction

Pregnancy is a dynamic state during which a number of physiological and biochemical changes occur in both the mother and fetus. As all compounds reaching the fetus must first pass through the placenta, understanding the characteristics of placental metabolism and transfer, and defining its role in both protective and harmful consequences for the fetus are essential. Furthermore, a greater understanding of the regulatory processes involved in maintenance and progression of pregnancy such as the steroid metabolizing and endocrine functions of the placenta are of importance in ensuring both maternal and fetal health.

The uridine 5′-diphosphate glucuronosyltransferases (UGTs) are a major class of xenobiotic metabolizing enzymes involved in phase II metabolism. These enzymes are a member of the uridine diphosphate glycosyltransferase superfamily which catalyses conjugation of a glycosyl group from a nucleotide sugar to a small hydrophobic molecule. This is generally considered to be a detoxification reaction producing metabolites from both exogenous and endogenous substrates which are more polar and more readily eliminated [1]. However, in some cases, it is possible to produce metabolites with more potent activity than the parent analogue, the most well known of these metabolites being morphine-6-glucuronide [2]. Other metabolites which are immunoreactive or teratogenic, such

* Corresponding author. Tel.: +64-9373-7599/6417; fax: +64-9373-7556.
E-mail address: a.collier@auckland.ac.nz (A.C. Collier).

Abbreviations: 4-MU, 4-methylumbelliferone; BSA, bovine serum albumin; DEPC, diethyl pyrocarbonate; EDTA, ethylenediaminetetraacetic acid; HRPO, horseradish peroxidase; PBS, phosphate-buffered saline; RT-PCR, reverse transcriptase-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SSC, standard saline citrate; SSPE, standard saline phosphate with EDTA; TBS, Tris-buffered saline; UGT, UDP-glucuronosyltransferase.

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as acylglucuronides and the glucuronides of retinoic acid may also be produced by UGT enzymes [3,4].

To date, in humans, 15 isoforms of UGTs have been identified and classified into two subfamilies; UGT1 and 2. The UGT1 gene is located on chromosome 2 at locus 2q.37 and is encoded by 5 exons [1]. Isoforms are produced from a tandem promoter sequence and differential splicing of exons 2–5 to exon 1 [5]. In contrast the UGT2 subfamily isoforms are encoded by distinct genes on chromosome 4 (each consisting of 6 exons) with each isoform having a different promoter sequence [6,7].

UGT isoforms are constitutively expressed in many tissues including liver, kidney, adipose, adrenal, lung, mammary gland, prostate and skin [8,9], jejunum, ileum, colon [10], pharyngeal mucosa and squamous cancer cells [11] as well as the brain [12].

The study of UGTs in extrahepatic tissues is important for several reasons. For instance some isoforms are expressed exclusively extrahepatically, such as UGT1A8 [10] this may indicate a primary role other than detoxication, for example, in hormonal regulation and response for endocrine organs including the thyroid gland [13,14]. Regulatory roles for UGTs in steroid synthesis, action and clearance in steroid target tissues such as the mammary gland, prostate and testis are also of interest [9,15]. Data on the role of UGTs in metabolism and regulation of endogenous and exogenous compounds in the placenta (a steroid target tissue and endocrine organ) is lacking.

As a major path of xenobiotic and endobiotic detoxification which is not observed in the fetal liver at measurable levels until some time after birth [16,17], the presence of UGTs in the placenta may play a protective role during gestation through metabolism and clearance of compounds. For example, developmental deficiency of bilirubin-UGT is a major cause of jaundice among neonates [18]. Studies have shown that it is the postnatal age, not the gestational age at delivery which affects the development of UGT activity, in that basal levels of UGT at birth were the same in term and pre-term infants and activity developed thereafter at the same rate for both groups [19]. Additionally, evidence for a protective role of UGTs may be inferred from their induction by polyaromatic hydrocarbons found in cigarette smoke [6] and by ethanol [20], both of which have been associated with deleterious consequences (intrauterine growth retardation and fetal alcohol syndrome, respectively) for the fetus. Finally, there are known polymorphisms of the UGT genes in the human population which have functional consequences and associated pathologies, such as Criggler–Najjar syndrome and Gilberts disease [1]. These polymorphisms, if present in placental enzymes, may confer an intrinsically higher risk of adverse consequences to fetuses from xenobiotic exposure during pregnancy. The presence of UGT polymorphisms with functional consequences is increasingly important in light of the current trend towards direct, *in utero* treatment of the unborn fetus for a number of ailments [21].

In this study, the aim is to characterize expression, cellular localization and activity of the UGTs in the human placenta at term.

2. Materials and methods

The following reagents were used: 4-MU (ICN Biomedicals); acrylamide (Biorad); enhanced chemiluminescence reagent and NEN TSA 700A Immunohistochemistry kit (NEN); Hybond-P PVDF, Megaprime™ DNA labeling kit and [α^{32}P]dCTP (Amersham Pharmacia Biotech); uridine diphosphate glucuronic acid (UDPGA), NBT/BCIP tablets and anti-digoxigenin alkaline phosphatase conjugate(Roche Diagnostics Ltd.); secondary biotinylated antibody (donkey anti-rabbit)andnormaldonkeyserum(JacksonLaboratories); 96-well "Spectraplate®" microtitre plates (Whatman); RNA Later® (Ambion); AmpliTaq Gold, Gene Amp PCR Gold Buffer and $MgCl_2$ solutions (Perkin-Elmer Applied Biosystems Ltd.); bromophenol blue, diaminobenzidine, diethyl pyrocarbonate (DEPC) solution, ethylenediaminetetraacetic acid (EDTA), N-laural sarcosine and trisodium citrate (Sigma); agarose (FMC BioProducts); bovine serum albumin (BSA, free fatty acid, fraction V), ethidum bromide, water saturated phenol, guanidine isothiocyanate (GTC) powder and solution, RNA ladder, DNA ladder, RNAseOUT recombinant ribonuclease inhibitor, oligo$(dT)_{12–18}$ primer, dNTP mix and Superscript II RNase H^- reverse transcriptase (Life Technologies). Antibodies to the UGT1A subfamily and UGT2B7 and human lymphoblasts expressing UGT2B7 (Gentest Corporation). Antibody to UGT2B4 was a gift from Jacques Magdalou, Sylvie Fournel-Gigleux (University of Nancy, France) and Anna Radominska Pandya (University of Arkansas, USA) and was generated by Mohamed Ouzzine (University of Nancy, France). UGT2B was a gift from Alain Bélanger (Université Laval, Quebec, Canada). Anti-HCG antibody was a gift from John France, University of Auckland, New Zealand. All other reagents obtained were analytical grade or higher.

2.1. Tissue collection procedure

Placentas were obtained from women with normal pregnancies undergoing elective caesarian section at term for malpresentation or due to previous caesarian section. All tissue was collected with ethical approval from the Auckland Human Ethics Committee and all participants gave informed consent. Villous placental tissue was dissected within 30 min of delivery.

2.2. UGT activity assay

Villous placental tissue was obtained by blunt dissection, washed, placed in ice-cold 67 mM phosphate buffer

with 1.15% KCl, pH 7.4 diluted 1:3 (w:v) and homogenized immediately then centrifuged at 10,000 *g* for 20 min followed by 100,000 *g* for 1 hr to prepare microsomes. Protein content was measured with the bicinchoninic acid method using BSA as the protein standard [22]. UGT activity was measured as previously described [23]. Briefly, a 96-well microtitre plate containing microsomal protein (60 μg), 4-MU (0–1000 μM) final concentration in 0.1 M Tris–HCl, 5 mM $MgCl_2$ and 0.05% BSA buffer, pH 7.4 was placed in a Victor Wallac 1420 Multilabel Counter set to read fluorescence every 2 min for 10 min at 355 nm excitation and 460 nm emission (15 nm bandwidth). The reaction was initiated by addition of the cofactor UDPGA (2 mM final concentration). Results were transformed to nmol/min/mg protein using a standard curve generated with 4-MU ($r^2 = 0.99$). Female human liver microsomes were used as a positive control.

2.3. RNA extraction

Tissue was collected, placed in RNA Later® and stored at 4° until use (for less than 7 days). Total RNA was isolated according to the acid guanidinium–phenol method [24]. Integrity of total RNA was assessed by agarose gel electrophoresis and by the A260/280 absorbance ratio. Total RNA concentrations were evaluated spectrophotometrically at $\lambda = 260$ nm. RNA was stored at −80° until use.

2.4. RT-PCR

The mRNA expression of UGT2B isoforms was assessed in villous trophoblast from 12 individual placentas. UGT2B4 primers were sequenced using the Oligo4s programme and checked for specificity on the NCBI BLAST programme. For the detection of UGT1A, 2B7, 2B10, 2B11 and 2B15 transcripts, isoform-specific oligonucleotides were based on previously published sequences [8,25–28] and custom synthesized by MWG Biotech AG, Germany. Forward and reverse primer sequences to detect UGT2B17 were supplied by Mario Congiu (St. Vincent's Hospital, Fitzroy, Australia). GAPDH was amplified with gene-specific primers to determine successful cDNA synthesis and female human liver used as a positive control for UGT1A. Primer sequences and predicted product sizes are shown in Table 1.

Reverse transcription was performed in a 20 μL reaction volume, containing 2 μg of total RNA, 0.5 μg oligo(dT), 40 U RNAseOUT, first strand buffer (50 mM Tris–HCl, 75 mM KCl and 3 mM $MgCl_2$), 10 mM DTT and 0.5 mM dNTPs. Tubes were incubated for 2 min at 42°, 200 U Superscript II polymerase and DEPC treated water added and the tubes incubated for 50 min at 42°. cDNA was stored at −20° until use.

UGT2B isoforms and the GAPDH positive control were amplified by PCR in a 50 μL PCR reaction volume containing 2 μL cDNA, PCR Gold Buffer (150 mM Tris–HCl, 500 mM KCl), 0.2 mM dNTP mix, 0.25 U AmpliTaq Gold DNA polymerase, 0.5 μM specific primers (sense and antisense) and $MgCl_2$ (1–3 mM optimized for each primer pair). PCR proceeded as follows: initial denaturation at 94° for 10 min followed by 35 cycles at 94° for 1 min, 47–67° for 1 min, 72° for 1 min and final elongation at 72° for 10 min. PCR was performed in a Progene Thermal Cycler (Techne).

PCR products were electrophoretically separated on 2 or 1.5% agarose gel for UGT1A and 2B products, respectively, containing ethidium bromide. Bands were visualized and documented using an Eagle-Eye ultraviolet light box (Stratagene).

Authenticity of gel-purified (Qiagen) PCR products was verified by direct sequencing, performed by the DNA Sequencing Facility at the University of Auckland.

2.5. Northern analysis

Northern blot analysis was used to quantitate mRNA expression of UGT2B7 in term placenta. An amount of 30 μg of total RNA per lane was separated on a formaldehyde agarose gel. RNA was visualized by ethidium bromide and documented as described [29]. The RNA gel was then washed briefly in Milli-Q water, soaked in 10 × standard saline citrate (SSC, 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) for 45 min and transferred to a Gene Screen Plus (NEN Life Sciences) nylon membrane by capillary transfer

Table 1
Forward and reverse primer sequences and predicted sizes of PCR products for the UGT1A subfamily and 2B isoforms[a]

UGT isoform	Primer sequence (5′–3′)	Predicted product size(bp)	Annealing temperature
1A	TCG AAT CTT GCG AAC AAC ACG, ATG AAG GCC ACT GTC AGC ACG	487	59
2B4	TGT TTT CCC CTT TGG TGA GC, CAA CAG GCA CAT AGG AAG GA	134	60
2B7	CAA AGG AGC TAA ACA CCT TCG G, CCG TAG TGT TTT CTT CAT TGC C	407	65
2B10	GCT CACTTATCC TAT CTC CTT GGC, GGG TAG AAG GAT TGG ATG CC	388	67
2B11	TTC CAT TCT TTT TGA TCC CAA TGA TG, TAG GTA TGT AGG AAG GAG GGA AAA TC	407	60
2B15	TTC TGG ATT GAG TTT GAG TA, ATG CTG AAA TAA AGG AGG AG	326	65
2B17	GTG TTG GGA ATA TTC TGA CTA TAA TAT A, CAG GTA CAT AGG AAG GAG GGA A	242	47
GAPDH	CAT CAT CTC TGC CCC CTC TG, CCT GCT TCA CCA CCT TCT TG	437	60

[a] GAPDH was used to confirm first-strand synthesis and PCR integrity.

overnight [30]. After transfer, the membrane was washed in 5 × SSC, the RNA UV cross-linked and left to dry at room temperature.

The UGT2B7 PCR product was separated on an agarose gel, the band of interest excited and extracted using a QIAEX II Extraction kit (Qiagen) and used as a probe for the Northern analysis. The cDNA probe was labeled with [α^{32}P]dCTP by random labeling using a Mega Prime kit (Amersham) as per the manufacturers instructions. Hybridization was performed at 42° for 4 hr in 50% formamide, standard saline phosphate with EDTA (SSPE) (0.15 M NaCl, 0.01 M Na_2HPO_4, 0.75% EDTA), Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone and 0.02% BSA), 3.5% SDS, and fish sperm DNA (100 μg/mL). The next day progressive stringency washing was performed using 2, 0.5 and 0.1 × SSC with 0.1% SDS at 42, 55 and 60°, respectively. Blots were exposed to a storage phosphor screen (Molecular Dynamics) and detection performed with a Storm 860 instrument (Molecular Dynamics). Ribosomal RNA was visualized with ethidium bromide and detected on a Storm 860 scanning instrument in blue excited fluorescence mode and was used as a control for equal loading [29]. Quantitation of fluorescent and phosphor images was performed with ImageQuaNT (Molecular Dynamics) software.

2.6. In situ hybridization

Placental tissue was collected ($n = 3$ placentas), snap frozen in liquid nitrogen, cryosectioned to 15 μm and stored at −20° until use. A digoxigenin-labeled oligonucleotide probe (TAA CAA CAG GTA CAT AGG AAG GAG GGA A), complimentary to a region of the UGT2B coding sequence conserved in all isoforms (613–641 bp) was manufactured by MWG Biotech AG, Germany. The corresponding digoxigenin-labeled sense probe was used as a control. Cryosections from six different placentas were defrosted and fixed in 4% paraformaldehyde for 30 min at room temperature. Sections were washed three times in 1% phosphate-buffered saline (PBS) for 5 min, then pre-hybridized with 50 μL of 50% formamide, 6 × SSC, 5 × Denhardt's solution, 100 μg/mL fish sperm DNA and 0.05% Tween 20 at 37° for 2 hr. Pre-hybridization (50 μL) solution with 100 ng probe was then added and warmed to 37° and incubated overnight. Slides were washed twice in 2 × SSC at room temperature for 15 min, twice in 0.5 × SSC at 55° for 15 min and twice in Tris-buffered saline (TBS, 0.1 M Tris–HCl, 150 mM NaCl, pH 7.5) for 10 min at room temperature. They were then covered with 500 μL TBS containing 0.1% Triton X-100, 2% normal horse serum (NHS) and incubated at room temperature for 30 min in a humid chamber. Solution was removed and sections incubated with anti-digoxigenin alkaline phosphatase conjugate (Fab fragments, 1:500 in the same buffer), for 2 hr at room temperature. Sections were then washed twice in TBS for 10 min, and incubated in 0.1 M Tris–HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5 for 10 min. For color development sections was covered with 200 μL of NBT/BCIP solution with 1 mM levamisole and incubated in a humid, dark chamber overnight. The next day slides were washed in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0 for 30 min and mounted in aquamount mounting media, coverslipped and photographs taken immediately on a Leica DMR-HC microscope (Leica) fitted with a ProgRes 3008 Digital Camera (Kontron Elektronik).

2.7. Western blotting

Placental ($n = 6$), cellular (HEK293, negative control) and liver (positive control) microsomal proteins; 30, 15 and 10 μg, respectively, were resolved on 7.5 and 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels under reducing conditions [31], transferred to Hybond-P PVDF membrane and blocked in 5% non-fat milk powder overnight. Membranes were incubated with primary antibody (1:1000 UGT2B7 and UGT1A and 1:250 UGT2B4 in PBS with 0.05% Tween 20 (PBS-T) and 5% non-fat milk powder) for 2 hr at room temperature, washed and incubated with a secondary, biotinylated antibody (1:10,000 donkey anti-rabbit in PBS-T, 10% non-fat milk powder and 2% NHS) for 1 hr. Antibody detection was subsequently performed using streptavidin–horseradish peroxidase (HRPO) complex (1:3000 in PBS-T), visualised by enhanced chemiluminescence and exposed to X-ray film for 20–40 s.

2.8. Immunohistochemistry

Tissue was fixed in 4% paraformaldehyde for 24 hr, dehydrated through an ethanol gradient and embedded in paraffin. Placental sections (4–7 μm) were cut on a microtome and dried onto polylysine-coated slides. Immunohistochemistry was performed on slides from three different placentas. Sections were dewaxed in xylene, rehydrated through an ethanol gradient and incubated overnight with primary antibody (1:100 in 0.1 M Tris, 0.15 M NaCl (TBS) with 0.5% blocking reagent). The next day, a secondary biotinylated antibody (1:200 in TBS with 0.05% Tween 20) was incubated for 30 min then streptavidin–HRPO (1:100 in TBS with 0.05% Tween 20) for 30 min, followed by tyramide amplification using an NEN 700A Signal Amplification Immunohistochemistry kit as per the manufacturer's instructions. Immunostaining was visualized with diaminobenzidine and counterstaining with Mayers haemalum. A positive control (anti-HCG, 1:500 in TBS with 0.5% blocking reagent) and two negative controls (normal rabbit serum and no primary antibody) were also included. Slides were dried down through an ethanol gradient, mounted with histomount, coverslipped, visualized and photographed with a Leica DMR-HC microscope (Leica) fitted with a ProgRes 3008 Digital Camera (Kontron Elektronik).

3. Results

3.1. Enzyme activity

Using the non-specific substrate 4-MU, UGT activity was observed in all human placental microsomes tested. Kinetic modeling of data was performed by fitting the results to a one-binding site Michaelis–Menten curve with the program GraphPad Prism 3.0 (San Diego, CA, USA) as shown in Fig. 1A. Mean V_{max} (mean of duplicates ± SD) for the 25 placentas sampled was 17.6 ± 12.9 nmol/min/mg protein and ranged 13-fold from 5.1 ± 0.9 to 66.9 ± 17.5 nmol/min/mg protein; the data were positively skewed as demonstrated by the box and whisker plot (Fig. 1B). Substrate affinity (K_m) had a mean of 544 ± 212 μM and ranged 5-fold from 246 ± 24.0 to 1124 ± 422 μM. In contrast, female human liver microsomes showed a mean V_{max} of 23.1 ± 1.05 nmol/min/mg (performed in quadruplicate ± SD) and K_m of 109 ± 48.0 μM (data not shown).

3.2. UGT gene expression

RT-PCR studies revealed bands of appropriate sizes for all UGT2B isoforms investigated. Representative gels of UGT2B PCR products (containing 6 of the 12 placentas tested) are presented in Fig. 2A–G. All 12 placentas tested were positive for UGT2B4, 2B7, 2B10, 2B11, and 2B15 expression. UGT2B17 mRNA was present in only 8 of 12 placentas tested while in all reactions GAPDH (positive control) was detectable. In six of the placentas positive for UGT2B10 expression, only one band of the appropriate size was detected. However, in the remaining six placentas the UGT2B10 PCR product contained an extra, higher molecular weight band. Direct sequence analysis determined this band was derived from non-specific amplification of other UGT isoforms. The UGT2B7, UGT2B10 and UGT2B17 PCR products varied considerably in intensity between placentas which may indicate variable RNA expression although the method was not quantitative.

UGT1A mRNA could not be detected in the human placenta at term by RT-PCR although all placentas tested were positive for GAPDH (Fig. 2H). Female human liver, included as a positive control, showed a band of the appropriate size at 487 bp for UGT1A and was also positive for GAPDH.

Northern analysis for UGT2B7 mRNA expression in 12 placentas revealed the presence of a 2 KB band which showed variable expression in band intensity across different placentas (Fig. 3A). The constitutively expressed ribosomal 18S RNA was used for normalization of UGT2B7 expression. The ratio of UGT2B7:18S varied 10-fold in 12 placentas tested (range 0.009–0.09, Fig. 3B).

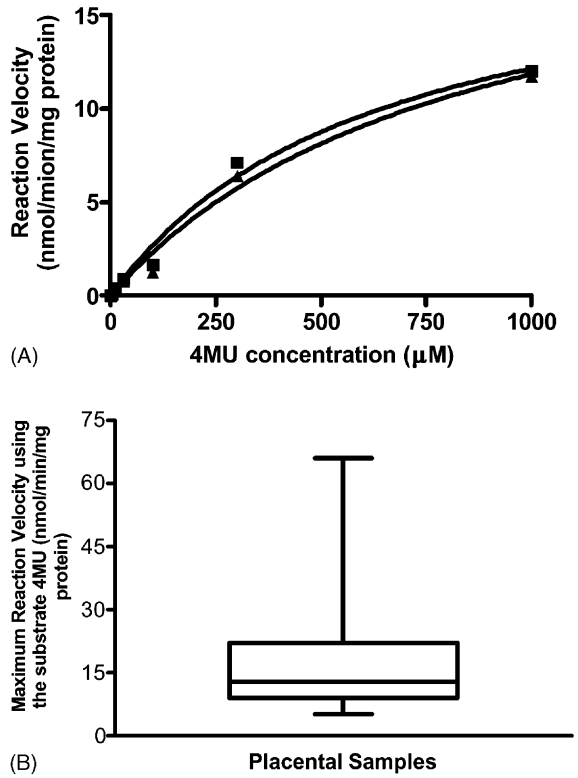

Fig. 1. (A) Representative Michaelis–Menten model from a placenta, performed in duplicate, which has a V_{max} of 16.6 ± 1.4 nmol/min/mg (mean ± SD) and a K_m of 446 ± 45 μM. (B) Box and whisker plot showing a positive skew in maximum reaction velocity of 25 placentas sampled.

3.3. UGT protein expression

Using Western blotting techniques and specific antibodies, protein products of the UGT1A subfamily could not be detected (55 kDa Fig. 3C). Of the UGT2B subfamily isoforms, UGT2B4 (Fig. 3D, 52 kDa) and UGT2B7 (Fig. 3E, 56 kDa) have been positively identified in 6/6 and 5/6 placentas, respectively. UGT2B4 antibody did not detect UGT2B7 protein expressed in human lymphoblasts (provided as a positive control for UGT2B7 by Gentest Corp, data not shown). Inter-individual differences in protein expression (band intensity) was observed in the Western blots for the isoforms UGT2B4 and UGT2B7. Placental protein consistently exhibited slightly retarded SDS-PAGE migration compared with human liver protein possibly reflecting increased glycosylation. However, the band observed on the immunoblots was specific and in the correct molecular weight range.

3.4. Cellular localization of UGT2B expression and proteins

The site of mRNA expression of UGT2B in the human placenta at term, identified by *in situ* hybridization with gene-specific oligonucleotides, was located in the syncytiotrophoblast cells bordering the placental villi (Fig. 4A, antisense probe). A negative control (Fig. 4B, sense probe) gave minimal background staining. UGT proteins, detected by immunohistochemistry, were also localized to the

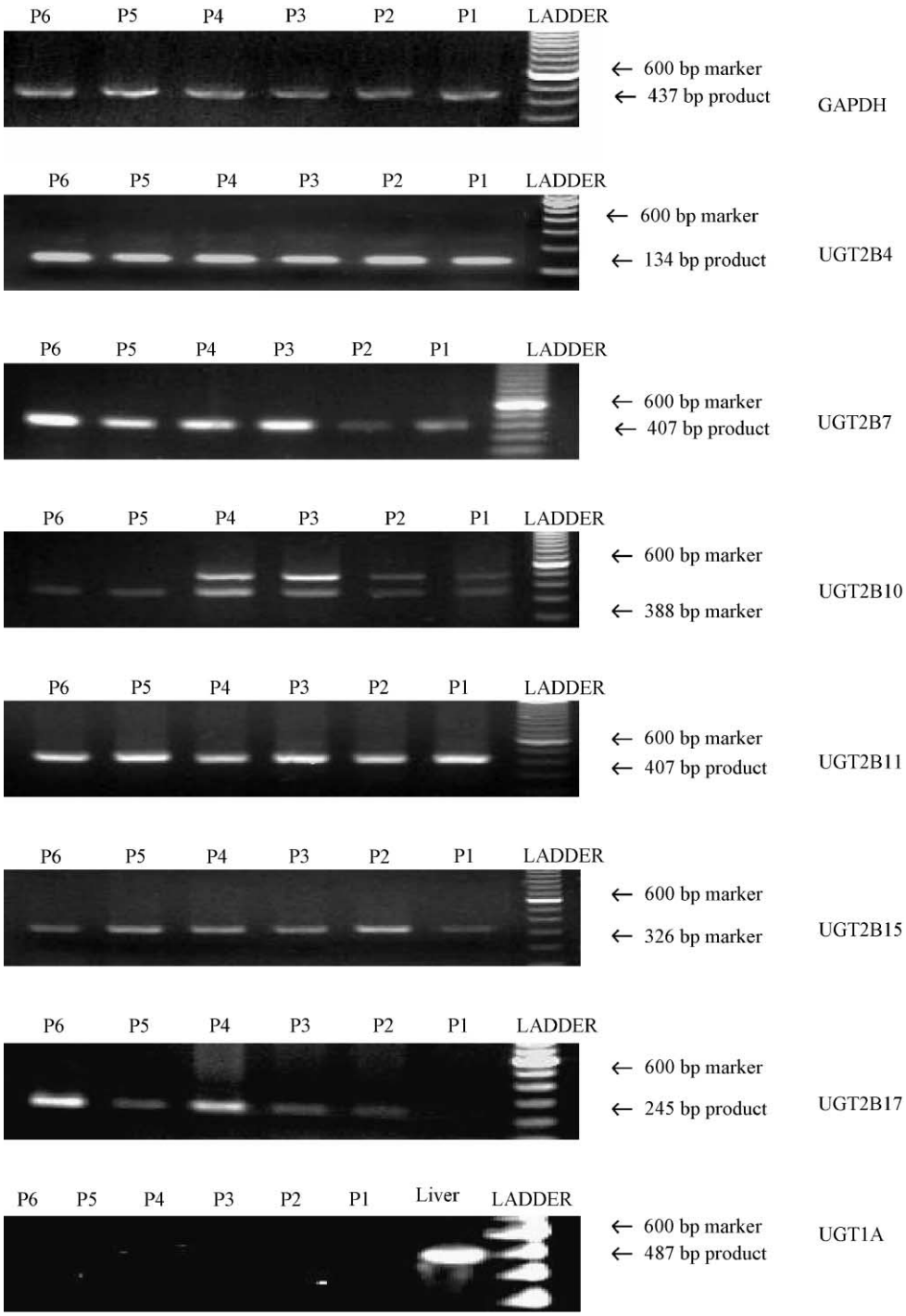

Fig. 2. (A–H) Total RNA was isolated from villous placental tissue at term from 12 placentas and analyzed for the UGT1A subfamily and UGT2B isoform gene expression with specific primers. Representative RT-PCR gels showing expression in six placentas of the positive control (A) GAPDH (B–G) UGT2B isoforms UGT2B4, 2B7, 2B10, 2B11, 2B15 and 2B17, and (H) UGT1A, respectively.

syncytiotrophoblast layer bordering the placental villi. A positive result for the UGT2B subfamily (Fig. 4C) and the isoforms UGT2B4 and UGT2B7 (Fig. 4D and E, respectively) was observed. Conversely, protein from the UGT1A subfamily was absent (Fig. 4F). Postive (anti-HCG, Fig. 4G) and negative (normal rabbit serum, Fig. 4H) controls are included to show representative staining. Some non-specific staining was observed in the mesenchyme of the controls. No specific staining in the placental mesenchyme or fetal endothelium was observed, however, all UGT antibodies showed positive staining in residual red blood. Staining for UGT2B4 was consistently weaker than that noted for UGT2B7 which may indicate lower levels of protein.

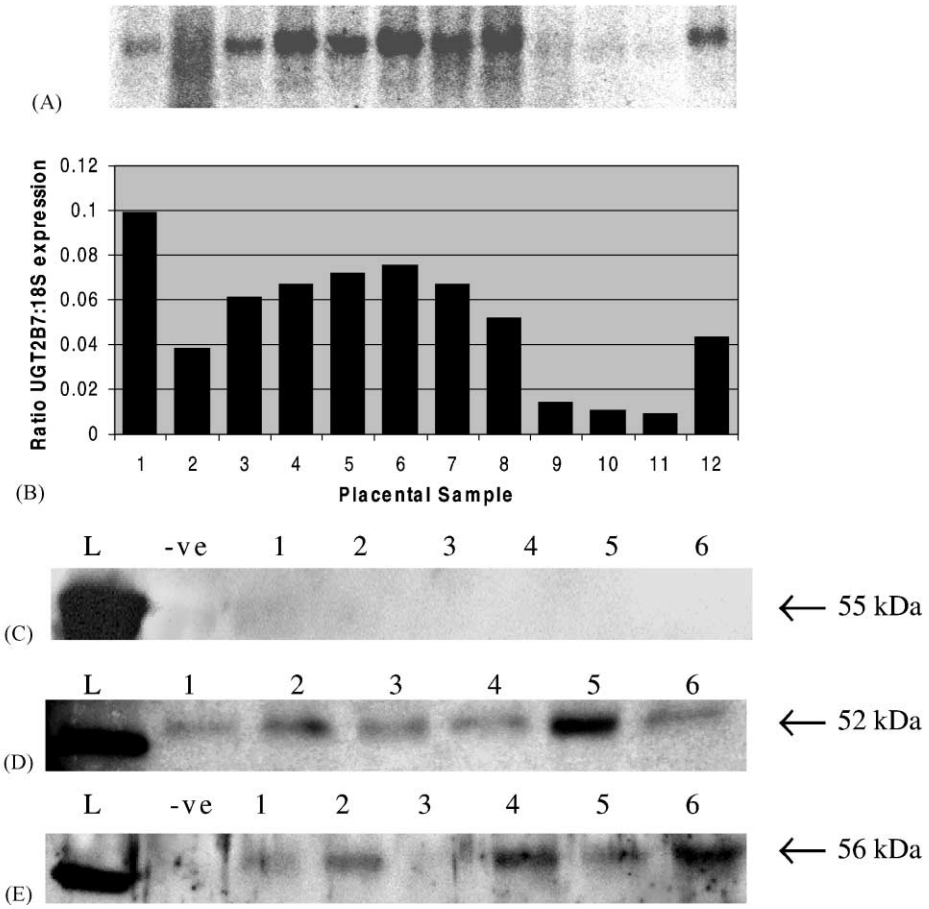

Fig. 3. UGT2B7 expression in human placenta at term determined by Northern analysis (A) normalized to 18S ribosomal RNA (B) and by Western blot (C–E). (A) Total RNA was isolated from villous placental tissue at term and analyzed by Northern blot using [^{32}P]-labeled cDNA as a probe for human UGT2B7. A specific band at 407 bp is noted. (B) Quantitation of UGT2B7 hybridization to the 18S RNA from the gel in 3A, showing 10-fold variation in expression. (C–E) Microsomal protein was isolated from villous placental tissue from six placentas and analyzed by Western blot with specific antibodies for the UGT proteins UGT1A, UGT2B4 and UGT2B7, respectively. Total cellular protein from HEK293 was used as a negative control for UGT1A and UGT2B7 (−ve) and microsomal protein from a female, human liver (L) was used as a positive control for all antibodies.

4. Discussion

We have previously demonstrated, using a sensitive UGT assay developed in our laboratories, that the cultured trophoblast-derived placental cell lines JEG-3, JAr and BeWo exhibit measurable UGT activity *in vitro* but that the amnion-derived cell lines WISH and AV3 do not [23]. In this study, for the first time, we have clearly demonstrated that the UGT2B subfamily is present and active in the human placenta at term. We have, also for the first time, confirmed and quantified gene expression and localized the site of transcription and protein presence in human placenta. In a previous study by Aitio [32], 60% ($n = 40$) of term placentas sampled showed no significant UGT activity while the remaining 40% were highly variable, exhibiting a 16-fold range in constitutive activity (20–327 pmol/min/mg tissue). Using the more sensitive assay we have quantified the activity of UGT in this tissue and have shown activity to be present in all placentas tested. Although, the substrate 4-MU is considered to be relatively ''non-specific'' for UGT activity and has been used as a general screen in these studies, it is metabolized primarily by the UGT1A family including isoforms UGT1A1, 1A6, 1A7, 1A8 and 1A10 [33–35]. Some members of the UGT2B family are also capable of using 4-MU as a substrate although at rates approximately 10-fold lower than the UGT1A isoforms. These include UGT2B4, 2B11 [36] and UGT2B15 [37]. UGT2B7 isoform metabolism of 4-MU *in vitro* varies between laboratories [38–40].

Our results confirm the variation in UGT activity reported previously with similar variation in activity (13-fold, V_{max}) and affinity (5-fold, K_m) in the population sampled ($n = 25$). Interestingly UGT activity towards 4-MU was observed in all placentas sampled, where previously UGT activity towards 4-MU was absent or undetectable in the majority of the placentas sampled [32]. The derived V_{max}:K_m ratio (intrinsic clearance) ranged from 7.5 to 43% that of the female human liver. Microsomal yields from placenta are only approximately 10% that of liver (1 mg microsomes per gram of tissue as opposed to 10 mg of protein per gram of tissue) which would have to be taken into account if comparing whole body clearances.

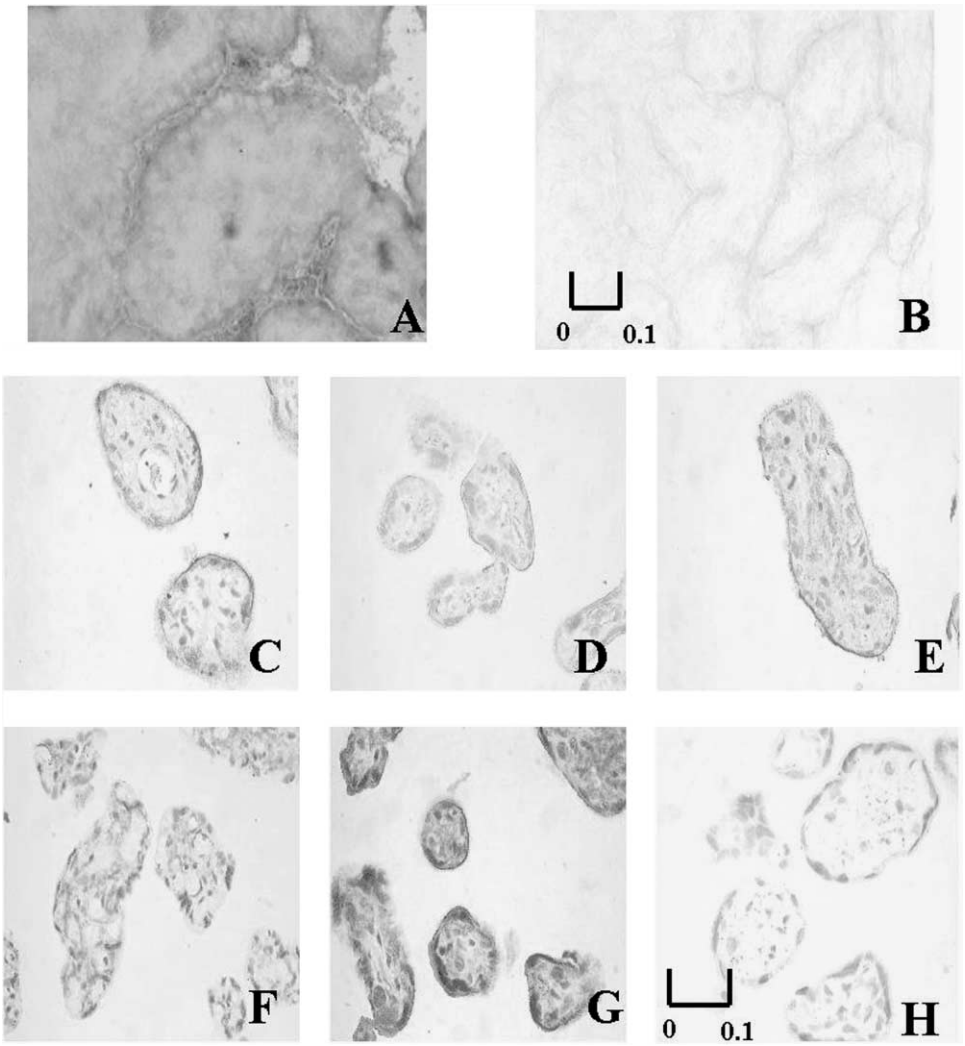

Fig. 4. *In situ* hybridization has demonstrated the site of transcription of UGT2B isoforms (antisense probe (A)) to be in the syncytium of the placental trophoblast. Red blood cells also stain positive for UGT2B transcription. A negative control (sense probe (B)) for *in situ* hybridization is included for comparison. The scale bar illustrated on the negative control represents distance from 0 to 0.1 mm for the *in situ* hybridizations. Immunohistochemistry with specific antibodies has shown that UGT2B family proteins UGT2B, 2B4 and 2B7 are present in the syncytial layer of the placental trophoblast adjacent to placental villi ((C–E), respectively) but UGT1A is not (F). Positive (anti-HCG (G)) and negative (normal rabbit serum (H)) controls show non-specific mesenchymal staining and are included for comparison. The scale bar on the negative control gives distance in millimeter for all immunohistochemistry data.

However, as teratogenesis may be caused by quantitatively minor metabolites, this activity may still be significant in terms of adverse effects on the fetus [41]. Elimination of compounds via the UGT pathway occurs at many other extrahepatic sites in the body such as the biliary tract [42] and the intestine [10].

RT-PCR, revealed that all the UGT2B isoforms studied were expressed in the human placenta at term. However, expression appears to be variable, with isoforms such as UGT2B4 and 2B11 showing stronger band intensities compared to UGT2B10 and 2B15. Although the variation observed in UGT activity with a non-specific substrate was similar to the variability of expression observed for UGT2B7, it is unlikely that UGT2B7 was solely responsible for the variation in activity—it has previously been shown that other UGT isoforms such as UGT2B11 and 2B15 have higher capacity for 4-MU metabolism when expressed in cell lines [36,37]. Differences in expression of UGT isoforms are most likely to be due to intrinsic expression patterns although environmental exposure to compounds may have induced or suppressed UGT. Therefore, interplacental variability in mRNA expression observed in Northern blots is the likely explanation for the up to 10-fold differences in UGT2B7 RNA levels. Although the regulatory mechanisms of UGTs are not fully elucidated, there is some evidence that endogenous bilirubin and other bile acids may regulate their own metabolism through increased transcription of UGTs [43]. It is also well documented that foreign chemicals such as phenobarbital and dioxin can increase UGT expression and activity [44]. Thus, variability of expression and activity in these enzymes is likely to be multifactorial.

Investigation of the expression of isoforms at the protein level is limited by the availability and specificity of

antibodies. Our studies have indicated the presence of protein for UGT2B4 and 2B7, which have been shown previously to be the only UGT2B isoforms to metabolize the bile acid hydrodeoxycholic acid [45]. Although, the two antibodies did not appear to cross-react in immunoblots of UGT2B4 using protein from UGT2B7 expressed in a cell line, the proteins have high primary sequence homology and it is impossible to rule out cross-reactions of the antibodies to expressed protein in tissues. Both isoforms also conjugate simple and complex phenols and estrogenic derivatives [42]. UGT2B7 can also conjugate many steroid and thyroid hormones and xenobiotics [46,47] and has possibly the widest range of substrate affinities of all the UGT2B isoforms. UGT2B4 and 2B7 are highly likely to be involved in clearance of bile acids, specifically hydrodeoxycholic acid, and steroid substrates within the placenta. The staining observed in residual blood in the immunohistochemical studies of UGT is interesting. Human platelets from both umbilical cord samples and maternal blood exhibit UDPGA-dependant bilirubin conjugation activity [48]. The use of tyramide signal amplification may have detected the UGT isoforms involved.

The apparent expression (by RT-PCR) of all the isoforms of the UGT2B subfamily in the human placenta suggests an active and varied role for the enzymes, for example, the clearance of substrates such as hormones, drugs and environmental compounds which come into contact with the placenta. Their site of expression and activity at the syncitial border of the placental villi (the site of maternal–fetal exchange) underlines this concept as all substances which come into contact with the fetus must first pass through the placenta via the maternal blood stream.

The absence of the UGT1A subfamily in the placenta at term is intriguing. The isoforms UGT1A1, 1A4, 1A6, 1A7, 1A8 and 1A10 have been shown to be active on steroid substrates [12,34,49,50]. Furthermore, UGT1A7, 1A8 and 1A10 are thought to be expressed exclusively in extrahepatic tissues [34,42] and could be reasonably expected to be present in the placenta. As the human placenta at term is a functionally end-stage organ, it is possible that the isoforms of UGT1A family are expressed and active at an earlier stage of gestation but have declined towards birth. There is some basis for this assertion as differential expression with gestational age in human placenta has been demonstrated for some isoforms of the cytochrome P450 family [51,52].

As many of the UGT2B isoforms posses steroid metabolizing capacity (UGT2B7 [46], UGT2B15 [53] and UGT2B17 [54]) a role for UGTs in the developing placenta and possible contribution to and maintenance of placental function during pregnancy could be postulated. Furthermore, the production of acylglucuronides and other unstable glucuronides which have the potential to cause deleterious consequences in the developing fetus should also be investigated. Acylglucuronides are capable of undergoing internal rearrangement and hydrolysis of the glucuronide bond to form reactive intermediates and acylation or glycation of macromolecules to form adducts. They have also been shown to be genotoxic *in vitro* [55]. Notwithstanding this, it is probable that the main role of the UGT family in the human placenta is the protection of the fetus through detoxification and elimination of endo- and xenobiotics. The location of UGTs at the site of maternal–fetal exchange strongly supports the theory of a feto-protective role for UGT enzymes in the human placenta.

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