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Identification of Bilirubin UDP-GTs in the Human Alimentary Tract in Accordance with the Gut as a Putative Metabolic Organ

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ABSTRACT. The initial identification of traditionally hepatic enzymes expressed in the gut has led to the hypothesis that the gut may function as a metabolic organ. The UDP glucuronosyltransferases (UDP-GTs) play an important role as phase II metabolizing enzymes. Previously members of this family have been identified in the gut by non-isoform specific immunoreactivity, and a small amount of bilirubin glucuronosyltransferase activity was detected in the colon. Recent reports of gut transplantation to reverse the metabolic defect in Gunn rats raised further interest in the expression and distribution of human bilirubin UDP-GTs (HUG Br 1 and HUG Br 2) in the human alimentary tract. The availability of molecular genetic probes for HUG Br 1 and HUG Br 2 permits the screening of the alimentary tract for the presence of isoform specific message. RNA samples extracted from pinch biopsy specimens of buccal mucosa, esophagus, stomach body, antrum, duodenum, and colon were analyzed for expression of HUG Br 1 and HUG Br 2. HUG Br 1 hybridization was detected in duodenum > colon, whereas HUG Br 2 hybridization was detected in duodenum > esophagus > colon. Immunoreactivity data confirmed the presence of HUG Br 1 protein at low levels in the duodenum, whereas the less abundant HUG Br 2 protein was below the limits of detection of isoform specific anti-peptide antibodies. Bilirubin specific reactivity was demonstrated in duodenal samples but not antrum samples, consistent with the molecular genetic data. The presence of functional bilirubin UDP-GT isoforms in the human alimentary tract supports the notion that the gut may function as a metabolic organ and may have diagnostic and therapeutic implications for disorders of bilirubin metabolism. BIOCHEM PHARMACOL 51;4:483–488, 1996.

KEY WORDS. metabolism; liver; glucuronidation; conjugation; enzyme; detoxification

UDP-GTs§ represent a family of isoenzymes, expressed primarily in the liver, that play an important role as phase II enzymes in metabolizing a wide range of xenobiotics and conjugating bilirubin, a necessary step for the excretion of bilirubin into bile. Genetic defects in the gene locus are responsible for Gilbert's syndrome, Crigler-Najjar syndrome types I and II, and other abnormalities. HUG Br 1 and HUG Br 2 are the major forms of UDP-GTs responsible for conjugation of bilirubin in humans and are part of the *UGT* 1 gene family [1]. Efforts are underway to correct the hyperbilirubinemia of Crigler-Najjar syndrome type I, an often fatal condition, by methods of gene transfer of a functional bilirubin UDP-GT gene [2, 3]. Various approaches of gene transfer are under consideration. Recently, a group demonstrated that the defect could be partially corrected by small bowel transfer in the Gunn rat, a well described animal model for Crigler-Najjar syndrome type I [4]. UDP-GTs have been identified in human small intestine using monoconal antibodies that are not isoform specific [5, 6]. Here, we examine the upper alimentary tract for expression of HUG Br 1 and HUG Br 2 message using DNA probes specific for these isoforms, in addition to demonstrating the presence of isoform specific protein and bilirubin specific enzyme activity. These results may have implications for the role of the alimentary tract mucosa as a metabolic organ.

MATERIALS AND METHODS

Subjects

Alimentary tissue was obtained from four healthy volunteers, 2 male and 2 female, between the ages of 25 and 37 years who had participated in a previous study of cytochrome expression [7]. All were nonsmokers who abstained from caffeinated beverages, tobacco products, and medications 3 days prior to each endoscopy. After an overnight fast, upper endoscopy was performed, and 2-mm pinch biopsies (4–5) were obtained from the buccal mucosa, esophagus, gastric body, gastric antrum, and duodenum distal to the ampulla of Vater for RNA analysis. One volunteer underwent repeat endoscopy to obtain an

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§ *Abbreviations:* UDP-GTs, UDP-glucuronosyltransferases; HUG Br 1 and HUG Br 2, human bilirubin UGT_1 and UGT_2 isoforms; and UGT 1, gene coding UDP-GP isoforms.

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additional four biopsies from the antrum and duodenum for analysis of enzymatic activity. Volunteers also underwent flexible sigmoidoscopy to obtain colonic tissue. Volunteers were sedated with up to 10 mg of intravenous midazolam for endoscopy. The protocol and consent form were approved by The University of Michigan Institutional Review Board.

RNA Extraction, Northern Blot Analysis

RNA was extracted according to the method of Chomczynski and Sacchi [8]. Four biopsy specimens from each tissue were placed in 750 μL of 4 M guanidinium isothiocyanate, 24 mM sodium citrate (pH 7.0), 0.1 M 2-mercaptoethanol, 0.5% sarcosyl and immediately placed in liquid nitrogen for later processing. Total RNA of 30–100 μg per sample was obtained with an A_{260}/A_{280} ratio of greater than 1.8. Ten micrograms of total RNA was separated in 2.2 M formaldehyde, 1% SeaKem agarose gels (FMC Biochemical, Rockland, ME), transferred to Hybond-N membranes (Amersham Corp., Arlington Heights, IL) by capillary transfer, and baked to the membrane at 80° under vaccuum for 2 hr.

UDP-GT molecular probes used for analysis were HUG Br 1 and HUG Br 2 specific, a *Bam*H I/*Eco*R I digest of pcDNA HUG Br 1 and a *Bam*H I/*Dra* III digest of pcDNA HUG Br 2 [2, 3]. These probes are specific for the first exons of the *UGT* 1 locus, 1A and 1D, which encode the variable region of HUG Br 1 and HUG Br 2, respectively. In contrast to their conserved carboxy termini, there is striking dissimilarity (≈44%) between the amino termini of HUG Br 1 and HUG Br 2 from which the probes used in this study were derived [9]. For duodenal samples, a human villin cDNA probe, provided by Dr. Kenneth Lown (University of Michigan), was used as a positive control to ensure adequate integrity of the duodenal RNA samples and to ensure equivalent loading of RNA into the small bowel lanes. Villin is expressed constitutively by villous gut tissue such as the small intestine enterocyte [10]. Equivalent loading of the lanes with RNA for other tissues was ensured by ethidium bromide staining of ribosomal RNA. The cDNAs were isolated from their plasmid vectors by gel purification and radiolabeled with [^{32}P]dCTP (Amersham) using a random-primed oligonucleotide labeling method as previously described [2]. Unincorporated nucleotides were removed from the labeled DNA by size exclusion chromatography using a Sepharose 4B matrix. The membranes were prehybridized, hybridized at 42°, and washed as previously described [2]. Autoradiography was performed with Kodak X-Omat film and an intensifying screen at −70°; image quantitation was performed using a Molecular Dynamics phosphor imager.

Enzyme Assay

Four pinch biopsies from the antrum and duodenum of each volunteer were frozen in liquid nitrogen for later processing. The samples were homogenized in a 2-mL Dounce homogenizer and then centrifuged at 10,000 *g* at 4° for 5 min. Assays were performed and analyzed by HPLC as previously described using the specified quantities of protein [2, 11–13].

COS Cell Transfections

The previously acknowledged incomplete HUG Br 2 cDNA was repaired using the overlapping oligonucleotide primers GATCCATGGCCAGAGGACTCCAGGTTCCCCTGCCGC and GGCAGGGGAACCTGGAGTCCTCTGGCCATG and ligating into the SAC II site of HUG Br 2 [2, 3]. The expression vectors pcDNA HUG Br 1 and pcDNA HUG Br 2 were fully sequenced as described [2, 3] prior to expression in COS cells. Cells were transfected with 10 μg of pcDNA HUG Br 1 or 10 μg of pcDNA HUG Br 2 per 15-cm dish using the DEAE dextran method. Cells were harvested 48 hr after transfection and analyzed by immunotransblot as described below.

Western Blot Hybridization

Peptides were designed to isoform specific sequences contained in the unique amino terminus regions of HUG Br 1 and HUG Br 2 (HUG Br 1 as described [2] and HUG Br 2, WTQKEFDRVTLGYTQGFFETEHLLKRYSRSMAIMNNV coupled to keyhole limpet hemacyanin). Antibodies used in these studies were raised in rabbits by injecting the peptides with Titer max adjuvant, phlebotomizing, and checking antibody titer by ELISA assay. Antibody reactivity and specificity were confirmed in lysates of COS 1 cells transformed to express either HUG Br 1 or HUG Br 2 to demonstrate isoform specific reactivity.

Using these two primary anti-peptide antibodies, western blots were performed as previously described [14], first comparing human liver microsomes, lysate from COS cells transfected with HUG Br 1, and lysate from COS cells transfected with HUG Br 2 to confirm isoform specificity of the antibodies. Next, tissue samples from liver microsomes, antral mucosa and duodenal mucosa were compared. Uniformity of protein loading was confirmed visually by staining the proteins transferred to the nitrocellulose filter with Ponceau S [3-hydroxy-4-(2-sulfo-4-[4-sulfophenylazo]-phenyl-azo)-2,7-napthalenedisulfonic acid] (Sigma, St. Louis, MO), prior to immunologic probing.

RESULTS

Expression of human bilirubin UDP-GTs in the gut was examined using tissue obtained by endoscopic biopsy from six discrete sites: buccal mucosa, esophagus, stomach body, antrum, duodenum, and colon. In addition, control normal liver tissue obtained at surgical resection was analyzed as a positive control. Three independent methods of analysis were used to analyze gene expression: RNA hybridization, immunotransblot, and functional enzymatic assay.

The gut was first screened for expression of HUG Br 1 and HUG Br 2 by RNA blot hybridization. HUG Br 1 and HUG Br 2 probes were shown previously to be isoform specific by hybridization with 3T3 cell total cellular RNA obtained following transduction with HUG Br 1 and HUG Br 2 retrovirus expression vectors; no cross-reactivity was observed between HUG Br 1 probe and HUG Br 2 message or HUG Br 2 probe and HUG Br 1 message (data not shown). High purity, undegraded mRNA was obtained from endoscopic pinch biopsies as reflected by spectrophotometric and electrophoretic analysis (OD 260 nm/280 nm > 1.8, intact ribosomal RNA on dena-

turing gel electrophoresis). Since the RNA appeared pure and undegraded, it presented an appropriate target for molecular genetic probes to examine which isoforms, if any, of the bilirubin UDP-GT enzymes were expressed and their distribution throughout the gut. Uniformity of loading of RNA was confirmed by hybridization with a control villin probe as well as ethidium bromide staining. RNA blot hybridization using the Br 1 cDNA specific probe revealed a single ≈2.1 kb RNA band in the duodenum of all four volunteers as represented by a single volunteer in Fig. 1A. This is the anticipated band size for transcripts of human HUG Br 1 as reflected in the control liver RNA contained in the lane marked "L." On multiple RNA hybridization experiments from different volunteers, the relative abundance of HUG Br 1 mRNA in the gut was consistently duodenum > colon, which was statistically significant with none detected in other sites in the gut. RNA blot hybridization using a HUG Br 2 specific probe also showed a single ≈2.1 kb RNA band in the duodenum of all four volunteers as represented by a single volunteer in Fig. 1B. This was also the band size for transcripts of human HUG Br 2 as shown by hybridization to control liver (Fig. 1B, lane L). RNA hybridization experiments from different volunteers demonstrated that the relative abundance of HUG Br 2 mRNA in the gut was consistently duodenum > esophagus > colon. Although differences in levels among these three groups were not statisticaly significant, none was detected in other sites in the gut, as summarized in Table 1.

Antibody isoform specificity was confirmed by transfecting separate dishes of COS cells with either HUG Br 1 or HUG Br 2 pcDNA expression vectors and performing immunotransblot analysis. The cell lysates were electrophoresed on two gels along with a human liver microsome control, transferred, and probed with either HUG Br 1 or HUG Br 2 rabbit anti-peptide polyclonal antibodies (Fig. 2). No cross-reactivity was observed between the anti-HUG Br 1 antibodies and the expressed HUG Br 2 protein or the anti-HUG Br 2 antibodies and the HUG Br 1 protein, suggesting that these antibodies are appropriate reagents for differentiating the expression of specific bilirubin UDP-GT isoforms.

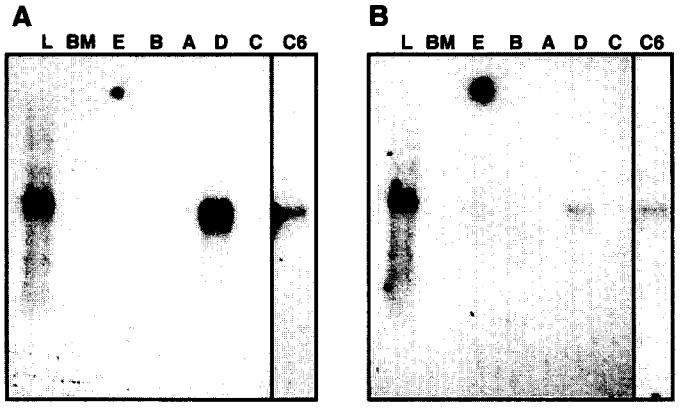

FIG. 1. RNA blot hybridization of total cellular RNA from human liver (L), buccal mucosa (BM), esophagus (E), stomach body (B), antrum (A), duodenum (D) and colon (C) at 24-h exposure and colon (C6, inset) at 6-day exposure probed for bilirubin UDP-GTs. Panel A is representative of a blot probed with a HUG Br 1 specific probe. After stripping the blot shown in panel A and exposing the blot to film for 6 days to document adequacy of HUG Br 1 probe removal, the blot was rehybridized with the HUG Br 2 probe. Panel B is representative of a blot probed with an HUG Br 2 specific probe. Each lane contains 10 μg of total cellular RNA. Note sequence specific hybridization to HUG Br 1 and HUG Br 2 in liver and duodenal samples on the 24-hr exposures, with colon RNA developing a positive signal on longer 6-day exposure.

TABLE 1. HUG Br 1 and HUG Br2 RNA levels in biopsy samples from various specified regions of the human alimentary tract

	HUG Br 1 probe (%)*	HUG Br 2 probe (%)*
1. Liver (Control)	100	100
2. Buccal mucosa	0	0
3. Esophagus	0	19 ± 10
4. Stomach body	0	0
5. Stomach antrum	0	0
6. Duodenum	97 ± 34	38 ± 24
7. Colon	7 ± 5	15 ± 7

HUG Br 1 and HUG Br 2 total cellular RNA levels in gut biopsy samples from the specified regions of the human alimentary tract were determined using isoform specific ^{32}P-labeled probes, quantified on phosphor image analysis, and reported as the percentage of the signal from a lane loaded with control human liver total cellular RNA.

* % = mean energy of tissue sample/energy of liver control. Values are means ± SD (N = 4 in all tissues except the liver); the SD of the mean energy from tissue samples/energy of liver control is reported as percent of liver control.

Expression of HUG Br 1 and HUG Br 2 proteins in the gut was assessed by immunotransblot analysis (Fig. 3). Antibodies used in this assay were engineered against unique amino acid sequences in the amino terminus of the HUG Br 1 (Fig. 3A) or HUG Br 2 (Fig. 3B) isoforms. Following confirmation of uniform loading of the wells by Ponceau S staining, specific reactivity of normal human liver microsomes (L) to HUG Br 1 and HUG Br 2 antibodies was observed. Antral tissue, which did not express HUG Br RNA, was used as a negative control. HUG Br 1 reactivity in duodenum was near the limit of sensitivity for this antibody with a faint band detected at the appropriate size for HUG Br 1 (arrow, Fig. 3A). HUG Br 2 reactivity in duodenum was below the level of sensitivity of detection (Fig. 3B). Specific band sizes in the human liver and duodenum were ≈52–54 kDa, as reflected by comparison to prestained molecular weight markers consistent with the predicted size of HUG Br 1 and HUG Br 2 [9].

Enzyme activity was measured in the liver, antrum, and duodenum using an assay specific for bilirubin UDP-GT activity. Figure 4 is a representative HPLC tracing of bilirubin UDP-GT reaction products prepared from (A) liver microsomes, (B) antral biopsy lysates, and (C) duodenal biopsy lysates. Peaks corresponding to bilirubin diglucuronides (BDG), bilirubin monoglucuronides (BMG), and unconjugated bilirubin (UCB) were clearly identified and resolved. Stringent measures were taken to avoid the formation of photobilirubin, and a photobilirubin peak was never defined on hundreds of assays. Duodenal biopsy samples uniformly dem-

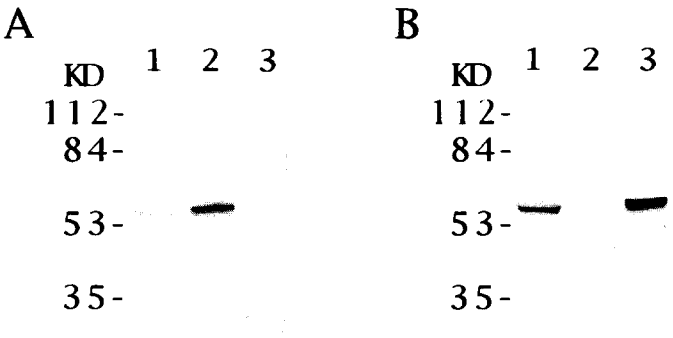

FIG. 2. Immunotransblot of COS cell lysates for HUG Br 1 (A) or HUG Br 2 (B) protein. Each lane contains 20 μg of protein, and panels A and B contain identical quantities of the same samples. Lane 1 contains human liver microsomes, lane 2 contains lysate from cells transfected with pc DNA HUG Br1, and lane 3 contains lysate from cells transfected with pc DNA HUG Br 2. In the panel on the left (A), anti-HUG Br 1 peptide antibody was used to probe the samples. Both a band in human liver microsomes and a band in the HUG Br 1 expressing cells were observed. Note that no background or HUG Br2 cross-reacting signal is seen in lane 3. In the panel on the right (B), anti-HUG Br 2 peptide antibody was used to probe the samples, and appropriate isoform specificity was similarly demonstrated. The indistinct background bands from 60–70 kDa most likely represent non-specific recognition of contaminating keratins. Pre-stained molecular weight markers are shown on the left (112,000; 84,000; 53,000; 35,000).

onstrated bilirubin UDP-GT activity, whereas antral tissue did not express reactivity toward bilirubin.

DISCUSSION

Two previous studies had demonstrated immunoreactivity using non-isoform specific antibodies to the common region of UDP-GTs and a small quantity of bilirubin reactivity in human colon [5, 6]. The cloning of human bilirubin UDP-GTs has made a bilirubin UDP-GT isoform specific survey of the gut possible. Prior studies had not surveyed the distribution of bilirubin UDP-GTs throughout the gut nor defined the presence of bilirubin specific isoforms. Using sequence specific probes, the gut was first surveyed by screening RNA samples obtained from endoscopic pinch biopsy. Pinch biopsy as opposed to cadaveric or brain dead donor sampling permits the assay of fresh tissue samples, particularly important for gut tissue, which is highly susceptible to ischemic injury.

The presence of HUG Br 1 protein in duodenal lysates is consistent with the RNA studies. The presence of a stronger signal in the liver lane is consistent with the known subcellular localization of UDP-GT activity in microsomes and their concentration in the liver microsomal preparation as opposed to the lysate preparation from duodenal pinch biopsies. The ability to detect HUG Br 1 but not HUG Br 2 protein in duodenal samples is not surprising given that the abundance of HUG Br 1 RNA is greater than HUG Br 2 RNA and the sensitivity of the immunotransblot assays. The demonstration of bilirubin specific reactivity in duodenum was an important finding as it clearly demonstrates a functional correlate consistent with the molecular genetic data.

The observation that the highest levels of gut expression are seen in the duodenum is consistent with other metabolic enzymes expressed in liver and gut [15]. One putative role of metabolic enzymes in the gut is to metabolize potential toxins to protect the organism. Bilirubin is a breakdown product of heme, which needs to be removed from the body, and detoxification of ingested bilirubin in red meats may be the function of these enzymes in the gut. More recently, estradiol reactivity of expressed bilirubin UDP-GTs has been demonstrated, and detoxification of other substances may also be important [16]. Expression of bilirubin UDP-GTs at low levels was consistent with prior observations; the presence of HUG Br 2 RNA in the esophagus was unanticipated. The presence of greater HUG Br 1 expression than HUG Br 2 expression parallels expression levels in liver [9] and is consistent with HUG Br 1 being the more physiologically important bilirubin glucuronosyltransferase isoform [17, 18].

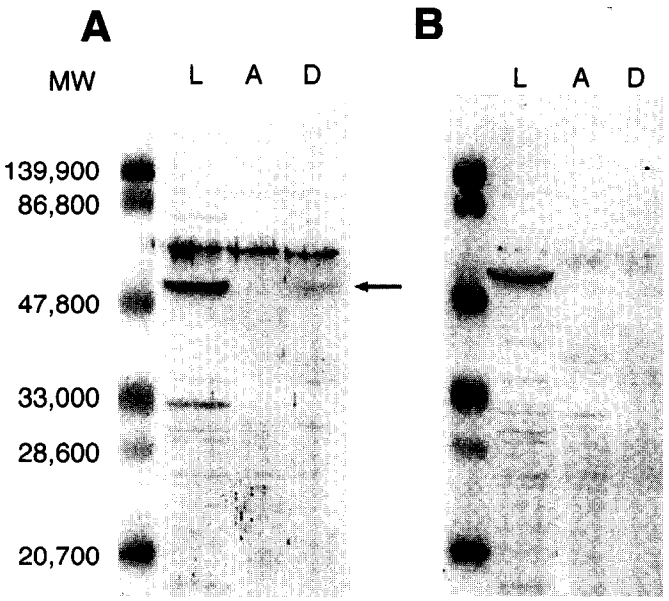

FIG. 3. Immunotransblot of human liver microsomes (L), antral biopsy (A) and duodenal biopsy (D) samples for human bilirubin UDP-GT protein. Each lane contains 20 μg of the specified protein, and panels A and B contain identical quantities of the same preparations from the same patient. In the panel on the left (A), anti-HUG Br 1 peptide antibody was used to probe the samples. Both a strong band in human liver microsomes and a faint band of the corresponding size in human duodenum homogenate (arrow) were observed. In the panel on the right (B), anti-HUG Br 2 peptide antibody was used to probe the samples. Immunotransblot assays were not sensitive enough to detect HUG Br 2 protein in antrum or duodenum. The indistinct background bands from 60–70 kDa most likely represent non-specific recognition of contaminating keratins. Pre-stained molecular weight markers are shown on the left (139,900; 86,800; 47,800; 33,300; 28,600; 20,700).

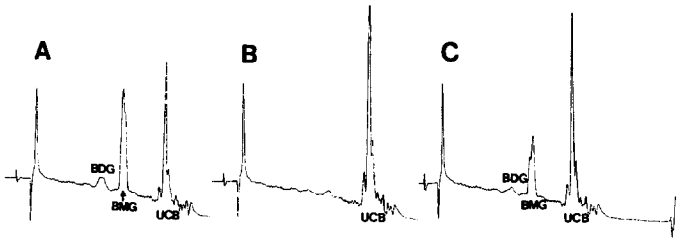

FIG. 4. HPLC analysis of bilirubin UDP-GT activity in liver microsomes, and antral and duodenal homogenates. The panel on the left (A) is a representative HPLC tracing of reaction product from human liver microsomes (200 μg protein). The initial peak at approximately 5.6 min was a solvent injection peak. Bilirubin diglucuronides (BDG) are eluted at approximately 15.5 min, followed by bilirubin monoglucuronides (BMG) at approximately 18.4 min and finally the unconjugated bilirubin (UCB) peak at approximately 24 min. The panel in the middle (B) is a representative HPLC tracing of reaction product from assays of antral mucosa (200 μg protein). Note the presence of only the solvent injection and UCB peaks. The panel on the right (C) contains a representative HPLC tracing of reaction product from human duodenal pinch biopsies. Note the presence of solvent injection, BDG, BMG, and UCB peaks in this sample. Photobilirubin peaks were not observed, as avoiding light exposure was fastidiously controlled.

The recent demonstration that gut transplantation from a wild-type Wistar rat to a Gunn rat can correct the genetic defect in Crigler-Najjar syndrome raises the prospect that segmental gut transplantation might be employed to treat this disease. The localization of significant quantities of human bilirubin UDP-GT activity in the gut is encouraging. The presence of bilirubin UDP-GT activity in the human gut is of interest because previous reports of kidney transplantation to reverse the metabolic defect in Gunn rats are not applicable to humans, as human kidneys do not possess bilirubin UDP-GT activity [19]. The challenges of gut transplantation are nontrivial so it is not likely that gut transplantation will be useful in the immediate future. Endoscopic duodenal biopsy as opposed to liver biopsy may prove to be a reasonable approach to screen Crigler-Najjar syndrome patients or their families by the relatively less morbid procedure, but this remains to be seen.

These studies demonstrate functional and genetic evidence of bilirubin UDP-GT expression in the human alimentary tract, greatest in the duodenum. The presence of duodenal bilirubin UDP-GT activity may have diagnostic or therapeutic utility. These studies lend further support to the definition of the gut as a potentially significant metabolic organ [7, 10, 15, 20].

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