

# Discrimination between Crigler–Najjar Type I and II by Expression of Mutant Bilirubin Uridine Diphosphate-Glucuronosyltransferase

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## Abstract

Crigler–Najjar (CN) disease is classified into two subtypes, type I and II. The molecular basis for the difference between these types is not well understood.

Several mutations in the bilirubin UDP-glucuronosyltransferase (B-UGT) gene of six CN type I and two CN type II patients were identified. Recombinant cDNAs containing these mutations were expressed in COS cells. B-UGT activity was measured using HPLC and the amount of expressed protein was quantitated using a sandwich ELISA. This enabled us to determine the specific activities of the expressed enzymes. All type I patients examined had mutations in the B-UGT1 gene that lead to completely inactive enzymes. The mutations in the B-UGT1 gene of patients with CN type II only partially inactivated the enzyme. At saturating concentrations of bilirubin (75  $\mu\text{M}$ ) CN type II patient A had  $4.4 \pm 2\%$  residual activity and CN type II patient B had  $38 \pm 2\%$  residual activity.

Kinetic constants for the glucuronidation of bilirubin were determined. The affinities for bilirubin of B-UGT1 expressed in COS cells and B-UGT from human liver microsomes were similar with  $K_m$  of  $5.1 \pm 0.9 \mu\text{M}$  and  $7.9 \pm 5.3 \mu\text{M}$ , respectively. B-UGT1 from patient B had a tenfold decreased affinity for bilirubin,  $K_m = 56 \pm 23 \mu\text{M}$ .

At physiological concentrations of bilirubin both type II patients will have a strongly reduced conjugation capacity, whereas type I patients have no B-UGT activity. We conclude that CN type I is caused by a complete absence of functional B-UGT and that in CN type II B-UGT activity is reduced. (*J. Clin. Invest.* 1994. 94:2385–2391.) Key words: hereditary diseases • site directed mutagenesis • enzyme-linked immunosorbent assay • enzyme kinetics • enzyme activity

## Introduction

Crigler–Najjar disease (CN)<sup>1</sup> is a form of familial hyperbilirubinemia characterized by high serum levels of unconjugated

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1. Abbreviations used in this paper: B-UGT, bilirubin UDP-glucuronosyltransferase; CN, Crigler–Najjar disease; DOPC, dioleoyl phosphatidylcholine; UGT1, human UGT1 gene.

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bilirubin (1). The disease is caused by an absence or strong reduction of the activity of the hepatic enzyme bilirubin UDP-glucuronosyltransferase (B-UGT) (EC 2.4.1.17). The CN syndrome can be divided into two subtypes; the more severe type I with high levels of serum bilirubin and the milder type II with lower levels of serum bilirubin and a reduction of these levels of more than 30% upon phenobarbital treatment (2–10). Bile of type II patients contains bilirubin glucuronides, though much less than bile of healthy controls (2–4).

Recently the human *UGT1* gene has been characterized at the molecular level (11). At least four different transcripts are generated from a single gene by a mechanism of alternative splicing. This mechanism produces isoforms of UGT with transferase activity towards bilirubin and phenolic substrates. All *UGT1* gene products share an identical carboxyl terminal domain. This constant domain probably contains the UDP-glucuronic acid binding site and the putative transmembrane helix. Mutations located in this constant part of the *UGT1* locus will affect all isoenzymes. The *UGT1* gene products have a unique amino terminal domain, termed exons *UGT1A* to *UGT1F*, which are responsible for the substrate specificity (11, 12).

Two UGT isoforms (B-UGT1 and B-UGT2, the products of the *UGT1A* and *UGT1D* exons) have been postulated to mediate bilirubin glucuronidating activity (11).

To investigate the molecular basis for the difference between CN type I and II we identified mutations in the variable and the constant part of the bilirubin *UGT1* gene of CN type I and II patients (13–15) and expressed these mutant enzymes in COS cells.

## Methods

### Patients

Two CN type II patients and six CN type I patients were studied. Clinical features of these patients are described below and are summarized in Table I. Mutations present in these patients are described in Table I.

*CN type II patients.* Patient A was diagnosed as a type II and described in reference 15. The patient is a 40-yr-old male. 1 mo after birth his serum bilirubin level was 85  $\mu\text{M}$ , 3 mo after birth his serum bilirubin had risen to 194  $\mu\text{M}$ . At the ages of 29 and 40 yr serum bilirubin levels were 286 and  $342 \pm 23 \mu\text{M}$ , respectively. When fasting for 24 h serum bilirubin was increased with 43%. Phenobarbital treatment resulted in a 36% decrease in serum bilirubin. Bilirubin decreased from 279 to 218  $\mu\text{M}$  when treated for 1 wk with 50 mg phenobarbital/d and from 218 to 179  $\mu\text{M}$  on a dose of 100 mg/d during 1 wk.

Patient B became jaundiced directly after birth. Phototherapy reduced the serum bilirubin from 470 to 200  $\mu\text{M}$ . A duodenal bile sample was collected at an age of 5 wk. The bile was analyzed using HPLC. Unconjugated and conjugated bilirubin were detected, the composition was 5% bilirubin diconjugate, 30% bilirubin mono conjugate and 65% unconjugated bilirubin. Total bilirubin concentration in the sample, which contained ~ 50% bile, was 36  $\mu\text{M}$ .

The patient was treated with phenobarbital at an age of 1 yr, which

resulted in a moderate response. When a dose of 5 mg/kg body wt was given for 1 wk serum bilirubin dropped from 120 to 87  $\mu\text{M}$ . A week after the treatment was halted serum bilirubin was 92  $\mu\text{M}$ .

At an age of 4 yr the patient receives 4 h of light therapy per d with 12 20-W tubes, with this treatment serum bilirubin levels range between 100 and 130  $\mu\text{M}$ . Since her serum bilirubin is kept at a relatively low level with only limited treatment and because a significant amount of bilirubin glucuronides were detected in bile she was classified as type II, despite the limited phenobarbital response.

*CN type I patients.* Patient C at the age of 16, underwent an orthotopic liver transplantation. Before transplantation bilirubin levels were around 400  $\mu\text{M}$  and she did not respond to phenobarbital treatment (13). A liver specimen did not contain detectable B-UGT activity (16).

Patients D and E both underwent orthotopic liver transplantation (14). Diagnosis of CN type I was based on high serum bilirubin levels, 460  $\mu\text{M}$  in patient D and 430  $\mu\text{M}$  in patient E. There was no response to phenobarbital treatment. Bile of patient E did not contain detectable amounts of bilirubin conjugates. Liver specimens did not contain any detectable B-UGT activity.

A clinical and immunological description of patients F and G was given (16), where these patients were termed C and D, respectively. Both patients underwent orthotopic liver transplantation. Patient F was a 3-yr-old (at the time of study) female with an average serum bilirubin of 430  $\mu\text{M}$ . Patient G was a 9-yr-old (at the time of study) female with an average serum bilirubin of 420  $\mu\text{M}$ . Liver specimens of both patients did not contain detectable B-UGT activity. Diagnosis of CN type I was based on high serum bilirubin levels and lack of phenobarbital response.

Patient H is a 16-yr-old boy in whom jaundice was noted soon after birth. On phototherapy, serum bilirubin levels varied from 340 to 425  $\mu\text{M}$ . At the age of 15, he started experiencing difficulty in learning and had subtle motor abnormalities. There was no response to phenobarbital. He underwent orthotopic liver transplantation, which reversed the neurological abnormalities. Hepatic B-UGT activity was undetectable.

### Chemicals

DOPC was obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). UDP-glucuronic acid and Triton X-100 from Boehringer (Mannheim, Germany). Bilirubin and cell dissociation solution from Sigma Chemical Co. (St Louis, MO). Nitro blue tetrazolium, 5-bromo, 4-chloro, 3-indoyl phosphate, acrylamide, bis acrylamide, goat anti-mouse alkaline phosphatase, goat anti-rabbit alkaline phosphatase, and goat anti-mouse peroxidase from Biorad (Veenendaal, The Netherlands). pSVK3 and DEAE dextran were from Pharmacia (Uppsala, Sweden). Nitrocellulose membranes 0.45  $\mu\text{m}$  pore size from Schleicher and Schuell (Dassel, Germany). 3',3',5',5' Tetramethylbenzidine was from Fluka Chemie AG (Buchs Switzerland). Cromspher 5, C18, 100  $\times$  10 mm, reverse phase column was from Chrompack (Middelburg, The Netherlands). Slide Write plus 5, from Advanced Graphics Software, Inc. (Carlsbad, CA).

All other chemicals were of analytical grade and obtained from E. Merck (Darmstadt, Germany).

### Identification of mutations, site directed mutagenesis, and expression of recombinant B-UGT cDNAs in COS cells

Identification of mutations was performed as described previously (13–15). B-UGT1 cDNAs were cloned in the EcoRI and ApaI restriction sites of pSVK3. Site-directed mutagenesis was performed as described in (17). Plasmids were isolated using Qiagen and sequenced completely to confirm the introduction of the mutations and to exclude abnormalities.

COS cells were transfected using DEAE dextran. Cells grown in 162-cm<sup>2</sup> culture bottles were incubated 4 h at 37°C in a 10% CO<sub>2</sub> atmosphere with 9 ml optimum medium containing 2 mg/ml DEAE dextran, 100  $\mu\text{M}$  chloroquine, and 10  $\mu\text{g}$  of plasmid DNA. The medium was removed and the cells were treated for 2.5 min with 10% DMSO in PBS. The cells were washed twice with DME and grown for 2–3 d in DME containing 10% fetal calf serum. Cells were harvested using cell dissociation solution and washed twice with 5 mM Mops pH 7.0, 250 mM mannitol, and 2 mM EGTA. The cells were incubated for 15

min at 4°C in this buffer and disrupted with 25 strokes of a dounce. Nuclei and large debris were removed by centrifugation for 5 min at 7,000 g. The microsomal fraction was pelleted by centrifuging for 1 h at 100,000 g. COS cell microsomes were resuspended in 50 mM Hepes pH 7.8, 2 mM EDTA, and 20% glycerol (buffer A) and stored at –20°C.

Human liver microsomes were isolated as described (18), resuspended in buffer A and stored at –70°C.

### Quantitation of B-UGT expression

Microtiter plates were coated overnight with the monoclonal antibody WPI (18) diluted 1:4000 in PBS. The plates were blocked with 1% bovine serum albumin in PBS for 2 h at 37°C. Different UGT containing preparations were solubilized with 1% Triton X-100 in deionized water for 1 h at 4°C, diluted 10 times with 0.1% Triton X-100, 1% BSA in PBS (buffer B), and applied to the microtiter plate for further dilution. Microsomal samples were diluted 20–320 times in buffer B, all samples were applied in triplicate. Standard immunopurified UGT was processed in the same way except that twofold dilutions from 20 to 20,960 times were used.

Binding was allowed to proceed for 2 h at 37°C. The plate was washed with PBS containing 0.05% Tween 20. As secondary antibody a polyclonal serum raised against immunopurified human UGT (16) was used. The serum was diluted 1:500 in PBS 0.1% Triton X-100, 1% BSA, and incubated for 2 h at 37°C. The plate was washed with PBS, 0.05% Tween 20. The presence of the polyclonal antibody was detected with goat anti-rabbit immunoglobulins conjugated with horseradish peroxidase. This antibody conjugate had been previously incubated with the monoclonal antibody WPI to minimize the background. The conjugate was detected with 0.1 mg/ml 3,3',5,5'-tetramethylbenzidine, 0.0036% H<sub>2</sub>O<sub>2</sub> in 0.1 M NaHCOOH pH 5.5 and quantified using a EASIA reader, (Medgenix Diagnostics BV, Soesterberg, The Netherlands). The amount of UGT1 present in the samples was calculated by constructing a standard curve from the titration of the isolated human UGT and fitting this curve with the softmax program, supplied with the EASIA reader.

### Assay of B-UGT activity

DOPC liposomes were prepared in 10 mM Hepes pH 7.8, 150 mM NaCl, by sonication (19), centrifuged at 4000 g for 10 min to remove titanium particles, stored at 4°C under argon, and used within 2 wk.

Human liver microsomes or COS cell microsomes (100–300  $\mu\text{g}$  protein) were incubated with DOPC liposomes for 30 min at 4°C before the assay. Concentrations in the reaction mixture were 50 mM Tris HCl pH 7.8, 5 mM MgCl<sub>2</sub>, 3.5 mM UDPGA, 1 mM 1,4 saccharolactone, 2.5 mg/ml DOPC SUV, and 2.5–75  $\mu\text{M}$  bilirubin in a total volume of 400  $\mu\text{l}$ . Incubations were performed under argon in the dark at 37°C for 10 min up to 4 h. All manipulations with bilirubin were performed in the dark or under dimmed light. Bilirubin solutions were prepared as follows: 2.9 mg of bilirubin was dissolved in 1.25 ml 50 mM NaOH by vortexing until the solution was optically clear, and neutralized by the addition of 3.75 ml of 100 mM Tris HCl pH 7.8. Bilirubin solutions were used within 5 min after preparation. For the determination of initial rates of bilirubin glucuronide formation a concentration range of 2.5–40  $\mu\text{M}$  bilirubin was used. Maximal 15% of bilirubin was glucuronidated at the lowest concentrations of bilirubin employed. Reactions were stopped and bilirubin and conjugates were extracted by a modification of an existing procedure [20]. First a dash of ascorbic acid was added to the reaction vessels, subsequently 1.4 ml of chloroform/ethanol 1:1 (vol/vol) and 1 ml of 0.4 M HCl adjusted to pH 1.8 with solid glycine was added and the mixture was vortexed vigorously. The tubes were centrifuged at 3000 rpm for 5 min. The lower colored phase was aspirated and transferred to a glass tube. The chloroform was immediately evaporated under a stream of nitrogen. Samples were stored under argon in the dark at –20°C for up to 2 d. The time needed for extraction to storage was less than 30 min. The percentage of bilirubin conjugated was determined using reverse phase HPLC (21). Bilirubin and bilirubin conjugates were dissolved in 100  $\mu\text{l}$  chloroform/ethanol 1:1 (vol/vol), 20  $\mu\text{l}$  was injected onto a HPLC system equipped with a chromsep C18 column within 5 min after dissolving. Elution was monitored at 450 nm

using a diode array detector; to confirm the identity of the peaks spectra were examined.

The percentage of bilirubin conjugated was calculated by determining the integrated peak areas.

### Calculation of kinetic parameters

The contribution of diglucuronides to the total amount of glucuronides was highest at low bilirubin concentrations but never exceeded 10%. Since B-UGT has the same affinity for unconjugated bilirubin as for monoglucuronide and since both are competitors for the same binding site (22), diglucuronides were treated as two monoglucuronides.

Substrate concentration and the initial rates of glucuronide formation were fitted directly to the Michaelis-Menten equation using the 1 site ligand binding curve fitting option in slide write plus 5.0 to obtain the kinetic parameters.

### Protein electrophoresis and Western blotting

SDS PAGE was performed according to (23) on a Hoeffer Scientific instruments Mighty Small II system. Western blotting of the separated proteins on a nitrocellulose membrane was carried out in a electroblotting apparatus. UGT was visualized with mouse ascites containing the monoclonal antibody WP1 (18) or a polyclonal serum, 6215. Goat anti-rabbit or mouse immunoglobulins conjugated with alkaline phosphatase were used as secondary antibodies. The presence of the conjugate was detected with 0.33 mg/ml nitro blue tetrazolium and 0.17 mg/ml 5-bromo-4-chloro-3-indoyl phosphate in 100 mM Tris pH 9.5, 100 mM NaCl, and 5 mM MgCl.

Protein was determined with fluorescamine (24) using bovine serum albumin as a standard.

## Results

### Quantitation of recombinant B-UGT expressed in COS cells.

Wild type B-UGT1 and B-UGT2 cDNA and B-UGT1 cDNAs

Table 1. Summary of Clinical and Biochemical Data from CN Patients Described in This Study

Patient	CN type	Average serum bilirubin	Mutation DNA	Mutation Protein	B-UGT activity in liver	Activity of B-UGT1
		$\mu\text{M}$				
A	2	340	NT 625 T → C	AA 209 arg → tryp	ND	Yes
B	2	140	NT 524 T → A Del NT 973	AA 175 leu → glu frameshift	ND	Yes ND
C	1	410	NT 1069 C → T	AA 356 stop	No	ND
D	1	460	NT 1130 C → T	AA 375 ser → phe	No	No
E	1	430	Del exon 2		No	ND
F	1	430	Del NT 508-510	Del AA 170 phe	No	No
G	1	420	NT 826 G → T	AA 276 gly → arg	No	No
H	1	380	NT 529 T → C Del NT 879-892	AA 177 cys → arg Frameshift	No	No ND

Patients B and H are heterozygotes. Truncated proteins or proteins with a frameshift were not expressed. ND, not done; AA, amino acid residue; Del, deletion; NT, nucleotide.

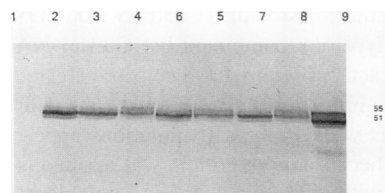


Figure 1. SDS PAGE and Western blotting of microsomes from transfected COS cells. Microsomes from COS cells transfected with various B-UGT1 cDNAs and human liver microsomes

were subjected to SDS PAGE, and Western blotting was performed with a polyclonal antiserum directed against human UGT. 35–100  $\mu\text{g}$  protein were applied to each lane. Lane 1, control COS cells; lane 2, B-UGT1; lane 3, patient F; lane 4, patient G; lane 5, patient H; lane 6, patient D; lane 7, patient B; lane 8, patient A; lane 9, human liver microsomes.

with the mutations described in Table I were expressed in COS cells. Microsomal fractions of the cells were subjected to SDS PAGE and Western blotting. UGT was detected using a polyclonal serum raised against human UGT. In Fig. 1 a Western blot is shown with the expressed proteins used for this study. The recombinant proteins appear identical, they have the same electrophoretic mobility and no signs of degradation are seen.

To investigate whether the UGT antibodies available to us were suitable to develop a quantitative sandwich ELISA, we subjected two identical gels to Western blotting with two different antibodies. This is shown in Fig. 2. B-UGT was visualized with the monoclonal antibody WP1, which is directed against the common region of all *UGT1* encoded enzymes and with a polyclonal antibody raised against immunopurified human UGT (18).

Human liver microsomes contain two immunoreactive

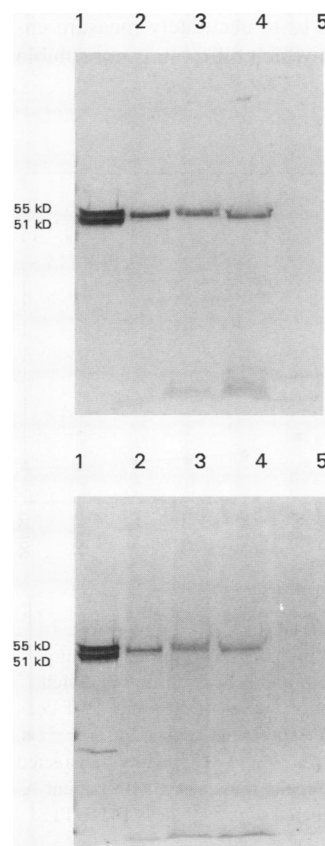


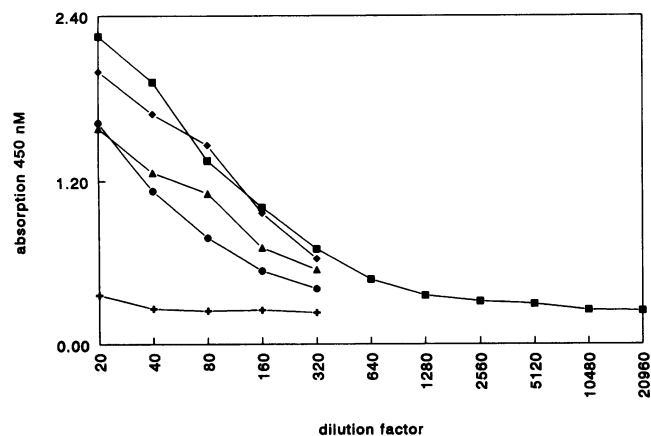
Figure 2. SDS PAGE and Western blotting of microsomes from transfected COS cells. Human liver microsomes and microsomes from COS cells transfected with B-UGT1 cDNAs from normal and CN type II patients, and from cells transfected with the lac Z gene, were subjected to SDS PAGE and Western blotting with the monoclonal antibody WP1 and a polyclonal antibody. Two identical gels are shown. (Upper panel) UGT detected with the monoclonal antibody, (Lower panel) UGT detected with the polyclonal antibody. Lane 1, 15  $\mu\text{g}$  human liver microsomes; lane 2, 18  $\mu\text{g}$ , B-UGT1; lane 3, 42  $\mu\text{g}$ , B-UGT1 patient A; lane 4, 51  $\mu\text{g}$ , B-UGT1 patient B; lane 5, 23  $\mu\text{g}$  lac Z gene.

UGTs with apparent molecular masses of 51 and 55 kD, COS cells transfected with wild type or recombinant B-UGT1 cDNA contain only one immunoreactive protein. COS cells transfected with the lac Z gene show no immunoreactivity at all. The bands of the recombinant proteins with the type II mutations appear somewhat fuzzy, probably because more protein was applied in these lanes. Western blots with the monoclonal and polyclonal antibody essentially show the same pattern. We concluded that both antibodies reacted with wild type and recombinant proteins and could be used to develop a quantitative ELISA.

The monoclonal antibody was used to coat microtiter plates. UGT is a membrane protein and had to be solubilized with 1% Triton X-100 before binding to the antibody-coated plate could take place. To prevent reconstitution during dilution of UGT 0.1% Triton X-100 was included in the dilution buffer. A preparation of immunopurified UGT (16) processed in the same way as the microsomal samples from the COS cells was used as a standard. The polyclonal antibody was used to detect UGT that bound to the immobilized monoclonal antibody.

Fig. 3 shows the titration of immunopurified standard UGT and microsomes from COS cells transfected with normal or recombinant UGT cDNA. As a control titration of COS cells transfected with the lac Z gene is shown. The titration curves have the same slope, indicating that the binding affinity of the antibodies is not affected by the mutations. This sandwich ELISA was used to quantitate the amount of UGT that was present in the transfected COS cells.

**Quantitation of B-UGT activity.** Bilirubin is poorly soluble in water but readily dissolves in membranes (25). It also associates rapidly with preformed liposomes (26, 27). The bilirubin liposome complex is a good substrate for B-UGT (28). Furthermore, dioleoyl phosphatidylcholine (DOPC) is a good activator of UGT (29), therefore a high concentration of DOPC liposomes was used in our assay system, this served not only to activate B-UGT but also enabled us to accurately measure enzyme activity at concentrations at which bilirubin is not soluble in water.



**Figure 3.** Sandwich ELISA for human UGT. Microsomes from transfected COS cells and a standard preparation of immunoaffinity-purified UGT were solubilized, diluted, and processed for sandwich ELISA as indicated in Methods. Titration curves of different UGT containing preparations in a representative experiment are shown. ■ — ■, immunoaffinity-purified human UGT; ♦ — ♦, COS cells transfected with normal B-UGT1; ● — ●, COS cells transfected with patient A B-UGT1; ▲ — ▲, COS cells transfected with patient B B-UGT1; + — +, COS cells transfected with lac Z gene.

**Table II.** Specific B-UGT Activities of Wild Type B-UGT1 and of B-UGT1 from CN Type II Patients Expressed in COS Cells

	B-UGT Activity
	pmol/min $\mu$ g B-UGT
Normal B-UGT1	25 $\pm$ 5
Patient A B-UGT1	1.1 $\pm$ 0.5
Patient B B-UGT1	9.6 $\pm$ 0.5

Specific activities of B-UGTs were obtained as follows: bilirubin conjugating activity in microsomal fractions from COS cells transfected with the indicated cDNAs was determined as described in Methods and the amount of expressed B-UGT1 in these fractions was measured by sandwich ELISA. Specific activities are expressed as pmol bilirubin glucuronide/min  $\mu$ g UGT. Bilirubin concentration in the assay mixture was 75  $\mu$ M. Values are the means $\pm$ SD from three separate experiments.

B-UGT activity was measured by HPLC and the amount of B-UGT polypeptide present in the fractions used for the B-UGT assay was measured by sandwich ELISA. In this way the specific activities of the expressed B-UGTs could be calculated.

The following control experiments were performed, prolonged incubations with microsomes from untreated COS cells and from COS cells transfected with the *Escherichia coli*  $\beta$  galactosidase gene and incubations with COS cell microsomes transfected with normal and mutant B-UGT without UDP-glucuronate. In all these cases no bilirubin mono- or diglucuronide peaks were seen in the HPLC chromatograms.

When B-UGT2 cDNA was expressed in COS cells, enzyme was produced which had less than 0.1% activity when compared to expressed B-UGT1.

All B-UGT1 cDNAs from type I patients expressed in COS cells yielded inactive enzyme. Even after prolonged incubation times, up to 4 h, no activity was detectable. We did not express cDNAs which would give rise to truncated proteins.

With B-UGT1 cDNA from type II patients, enzyme was produced with partial activity. At a substrate concentration of 75  $\mu$ M bilirubin, cDNA derived from patient A was found to encode for an enzyme with 4.4 $\pm$ 2% residual activity and cDNA from patient B for an enzyme with 38 $\pm$ 2% residual activity. These percentages are expressed relative to the specific activity of the wild type enzyme. In Table II the specific activities of wild type and mutated, type II B-UGT1s are summarized. Values for the specific activities are mean $\pm$ SD from three separate experiments.

Although patient B has only one functional allele, the high residual activity can not explain the elevated serum bilirubin levels observed in this patient. To investigate this further we determined the affinity of normal and mutant protein for bilirubin by measuring the activity at different substrate concentrations. The results are depicted in Table III. Values were obtained from three separate experiments. Double reciprocal plots of initial rates of glucuronide formation (1/V) versus bilirubin concentration (1/S) from a representative experiment are shown in Fig. 4. Kinetic parameters of expressed normal B-UGT1 and human liver microsomes are virtually identical. The observed  $K_m$  values agree with previously published data ( $K_m = 13.7 \mu$ M) although assay conditions and detection method employed were different (22). The mutation from patient B causes a 10-fold increase in the apparent  $K_m$  for bilirubin when compared to expressed B-UGT1 or human liver microsomes.

**Table III. Kinetic Parameters for Bilirubin Glucuronidation in Wild Type B-UGT1 and Recombinant B-UGT1 from Patient B Expressed in COS Cells and from Human Liver Microsomes**

	$K_m$	$V_{max}$
	$\mu M$	$pmol/min \mu g \text{ B-UGT}$
Human liver microsomes	$7.9 \pm 5.3$	ND
Normal B-UGT1	$5.1 \pm 0.9$	$65 \pm 22$
Patient B B-UGT1	$56 \pm 23$	$39 \pm 23$

Microsomal fractions were incubated with different bilirubin concentrations and the initial rates of bilirubin glucuronide formation were determined using HPLC. These data were fitted to the Michaelis–Menten equation and the  $V_{max}$  and  $K_m$  were calculated. Data represent means  $\pm$  SD from three separate experiments. ND, not done.

Interestingly this mutation causes only a small alteration in the  $V_{max}$ , an indication that only the binding of bilirubin is affected. Thus, at a physiological concentration of bilirubin, this type II patient will have a markedly reduced ability to glucuronidate bilirubin, whereas a significant level of residual activity is observed at the relatively high bilirubin concentration used in the in vitro assay.

## Discussion

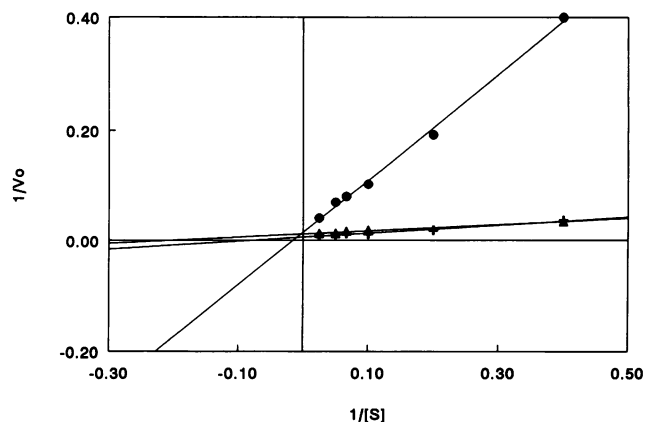
Crigler–Najjar disease can be divided in two subtypes, type I and II. The discrimination between these types is based upon the following clinical criteria: in type II, phenobarbital treatment lowers serum bilirubin levels with more than 30% and bilirubin glucuronides are present in bile. Type I patients do not respond to phenobarbital treatment and only traces of bilirubin glucuronides can be found in their bile (2). Analysis of liver material from biopsies or tissue removed during transplantation has revealed that in type I liver no B-UGT activity is present, whereas in type II liver sometimes a small residual activity can be observed (2–10). Until now it was not clear which enzyme was responsible for this activity.

Some heterogeneity in the clinical manifestation of CN type II is observed (2–10). Phenobarbital response varies between 27 and 78% and serum bilirubin levels can vary between 340 and 100  $\mu M$ . The similarity between all type II patients is that symptoms are less severe than in type I and that usually no treatment is needed. However, patients have been described who could not be classified by the existing criteria, CN type II patients with bilirubin-induced neurological damage have been reported (30, 31) and a patient has been described who could not be classified at all (32).

The cloning and characterization of the *UGT1* gene has led to the discovery of a rapidly accumulating array of mutations present in the B-UGT1 gene of patients with the CN syndrome (13–15, 33, 34). The role these different point mutations might play in the development of the subtypes of the CN syndrome has not been investigated so far.

In this study we examined several CN type I and II patients and identified the mutations present in the B-UGT1 gene of these patients. Subsequently the effect these mutations have on the specific B-UGT enzyme activity was determined.

When cDNAs from the CN type I patients described in this study were transfected in COS cells, inactive B-UGTs were produced. Patient A, diagnosed as CN type II, has a B-UGT1



**Figure 4. Lineweaver–Burk plots of bilirubin glucuronide formation.** Reciprocal plots of bilirubin glucuronide formation versus bilirubin concentration.  $S$  is in  $\mu M$  bilirubin and  $V_0$  is in  $pmol \text{ bilirubin glucuronide/min. } \mu g \text{ B-UGT}$ . Assay of B-UGT activity was as described in Methods.

Human liver microsomes (+ — +), COS cells transfected with normal B-UGT1 cDNAs ( $\blacktriangle$  —  $\blacktriangle$ ), and COS cells transfected with cDNA from patient B ( $\bullet$  —  $\bullet$ ). Data from three separate experiments were used to determine the kinetic parameters. A representative experiment is shown.

gene that gave rise to an enzyme with 4.4% residual activity, serum bilirubin levels are elevated to 340  $\mu M$ , but the patient is currently without any treatment. Patient B, also diagnosed as type II, has a B-UGT1 gene that gave rise to an enzyme with reduced activity as well. The reduction of B-UGT activity in this patient was caused by a 10-fold decreased affinity for bilirubin of B-UGT1. This will result in an approximately ten-fold reduction in the glucuronidation capacity at physiological bilirubin concentrations well below the  $K_m$ . At high concentrations of bilirubin, B-UGT activity will approach a normal value. This may explain why the serum bilirubin level is relatively low in this patient. With only limited treatment serum bilirubin levels are elevated to a value of only 100–130  $\mu M$ .

Several lines of evidence argue against a role of B-UGT2 in the development of CN syndrome. The common carboxy terminal regions of both B-UGT1 and B-UGT2 are derived from the four shared exons at the 3' region of the *UGT1* gene. As a consequence, mutations in any one of these exons will be present in both B-UGT isoforms. In this study such a mutation was found in patient D. When a mutation is present in the unique exon encoding the  $NH_2$  terminal region of B-UGT1 (*UGT1A*), B-UGT2 mRNA is not affected. This is the case in patients F, G, and H. Nevertheless B-UGT activity was absent in liver material of all these patients, suggesting that B-UGT2 does not contribute significantly to bilirubin conjugation in these patients. Furthermore we have shown that the specific activity of B-UGT2 is less than 0.1% of the activity of B-UGT1 (35). It is also known that B-UGT2 mRNA is expressed at a lower level than B-UGT1 mRNA (11). Together these arguments strongly suggest that B-UGT1 accounts for practically all bilirubin glucuronidating activity in human liver (35).

These data give rise to the following model: the development of CN disease is entirely governed by the presence of mutations in the B-UGT1 gene. Mutations in the constant or variable region that give rise to inactive enzymes result in CN type I. When the enzyme is only partially inactivated CN type II is developed.

The mechanism by which phenobarbital reduces serum bilirubin of CN type II patients is complex, probably more than one pathway is involved. In the Gunn rat, which has no B-UGT activity because it synthesizes a truncated enzyme (36) and which is an animal model for CN type I, phenobarbital treatment reduces serum bilirubin by 36% (37). In CN type I patients a small reduction of serum bilirubin upon phenobarbital treatment is sometimes also seen (4). The reduction of serum bilirubin after phenobarbital treatment in Gunn rats has been explained by a mobilization of bilirubin to the liver probably caused by the induction of cytosolic binding proteins such as ligandin (37). In type I patients similar mechanisms might play a role.

Although B-UGT1 (UGT1A) mRNA is not induced by phenobarbital in monkeys (38), studies with human liver (39, 40) and studies with the human hepatoma HEPG2 (41) suggest that both B-UGT transcription and B-UGT activity are increased after phenobarbital treatment. Other factors such as alteration of the membrane environment or a stabilization of microsomal protein (42) might also play a role in the phenobarbital induced induction of B-UGT activity. The reduction of serum bilirubin levels after phenobarbital treatment observed in type II patients may thus be attributed to the induction of the mutated B-UGT1, which has enzyme activity, albeit at a reduced level.

Patients with residual B-UGT1 activity, CN type II, will benefit from mechanisms which increase B-UGT activity, whereas reduction of serum bilirubin in type I patients may be caused by the aspecific processes described above. The response a given CN patient, either type I or II, will have to phenobarbital treatment will depend on the way the different pathways are already induced.

The discrimination between type I and II CN should, in our opinion, be based on the presence of residual B-UGT1 activity in type II patients and the absence of activity in type I patients. This differentiation is clinically important for prognostication and for prediction of the need for liver transplantation. Our studies demonstrate that this differentiation can now be made by the identification of the mutations in B-UGT1 and the in vitro expression of the mutagenized B-UGT1.

In other inherited syndromes, such as lysosomal storage diseases, variability in clinical symptoms can also be understood as the consequence of differences in the residual activity of the affected enzymes (43).

The criterion of > 30% reduction of serum bilirubin after phenobarbital treatment for type II seems somewhat arbitrary. For the initial clinical diagnosis, however, it will remain an important tool.

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