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Characterization of Mice Lacking the Multidrug Resistance Protein Mrp2 (Abcc2)

Xiao-Yan Chu, John R. Strauss, Michele A. Mariano, Jing Li, Deborah J. Newton, Xiaoxin Cai, Regina W. Wang, Jocelyn Yabut, Dylan P. Hartley, David C. Evans, and Raymond Evers

Department of Drug Metabolism (X.-Y.C., J.R.S., J.L., D.J.N., X.C., R.W.W., J.Y., D.P.H., D.C.E., R.E.) and Laboratory Animal Resources (M.A.M.), Merck & Co., Rahway, New Jersey

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ABSTRACT

The multidrug resistance protein Mrp2 is an ATP-binding cassette (ABC) transporter mainly expressed in liver, kidney, and intestine. One of the physiological roles of Mrp2 is to transport bilirubin glucuronides from the liver into the bile. Current in vivo models to study Mrp2 are the transporter-deficient and Eisai hyperbilirubinemic rat strains. Previous reports showed hyperbilirubinemia and induction of Mrp3 in the hepatocyte sinusoidal membrane in the mutant rats. In addition, differences in liver cytochrome P450 and UGT1a levels between wild-type and mutant rats were detected. To study whether these compensatory mechanisms were specific to rats, we characterized $Mrp2^{-/-}$ mice. Functional absence of Mrp2 in the knockout mice was demonstrated by showing increased levels of bilirubin and bilirubin glucuronides in serum and urine, a reduction in biliary excretion of bilirubin glucuronides and total glutathione, and a reduction in the biliary excretion of the Mrp2 substrate dibromosulfophthalein. To identify possible compensatory mechanisms in $Mrp2^{-/-}$ mice, the expression levels of 98 phase I, phase II, and transporter genes were compared in liver, kidney, and intestine of male and female $Mrp2^{-/-}$ and control mice. Unlike in Mrp2 mutant rats, no induction of Mrp3 in $Mrp2^{-/-}$ mice was detected. However, Mrp4 mRNA and protein in liver and kidney were increased ~6- and 2-fold, respectively. Phenotypic analysis of major cytochrome P450-mediated activities in liver microsomes did not show differences between wild-type and $Mrp2^{-/-}$ mice. In conclusion, $Mrp2^{-/-}$ mice are a new valuable tool to study the role of Mrp2 in drug disposition.

Several members of the ATP-binding cassette (ABC) superfamily of transporters are involved in the transport of both endogenous and xenobiotic compounds from major organs in the body (Borst and Oude Elferink, 2002). An example is the multidrug resistance protein 2 (MRP2 or ABCC2). MRP2 protein has mainly been detected in the apical plasma membrane of hepatocytes, kidney proximal tubules, and intestine (Paulusma et al., 1997; Schaub et al., 1999; Mottino et al., 2001). In addition, MRP2 is localized in lung, gallbladder, and placental trophoblasts (St-Pierre et al., 2000; Rost et al., 2001; Konig et al., 2003).

The substrate specificity of MRP2 is broad and includes glutathione, glucuronide, and sulfate conjugates of many drugs, some nonconjugated organic anions, and various neutral or positively charged drugs (Konig et al., 2003). Known high-affinity physiological substrates of MRP2 are glucuronides of the hemoglobin breakdown product bilirubin (Kamisako et al., 1999).

In humans, mutations in the *MRP2* gene can result in the autosomal recessive Dubin-Johnson syndrome (DJS). The primary defect in patients with DJS is a mild conjugated hyperbilirubinemia, caused by the impaired hepatobiliary transport system of nonbile salt organic anions across the canalicular membrane (Oude Elferink and Groen, 2002). Although, in general, DJS is an extremely rare disorder, it is relatively frequent in Iranian Jews, in whom the prevalence is 1:1300. Several mutations have been reported in patients with DJS, resulting in the absence of MRP2 or intracellular trafficking defects (Suzuki and Sugiyama, 2002).

The animal models for DJS are the Groningen Yellow/transporter-deficient rat (TR^-) and Eisai hyperbilirubinemic rat (EHBR) strains, spontaneous mutants from the Wistar and Sprague-Dawley strains, respectively (Jansen et al.,

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ABBREVIATIONS: ABC, ATP-binding cassette; MRP, multidrug resistance protein; DJS, Dubin-Johnson syndrome; TR^-, transporter-deficient rat(s); EHBR, Eisai hyperbilirubinemic rat(s); PCR, polymerase chain reaction; bp, base pairs; RT, reverse transcription; EROD, ethoxyresorufin *O*-dealkylation; PROD, pentoxyresorufin *O*-dealkylation; HPLC, high-performance liquid chromatography; DBSP, dibromosulfophthalein; HT, hydroxytestosterone; CAR, constitutive androstane receptor; GSH, glutathione.

1985; Hosokawa et al., 1992). The Mrp2 mutation in the TR$^-$ is a single nucleotide deletion leading to a frameshift and a stop codon (Paulusma et al., 1996). In EHBR, a one-nucleotide substitution results in a stop codon (Ito et al., 1997). Both mutant rat strains have been extensively characterized, and main differences between the mutant and wild-type rats are decreased bile flow, increased plasma levels of bilirubin glucuronides, a strong reduction in the biliary secretion of reduced glutathione, and a range of glutathione, glucuronide, and sulfate conjugates and unconjugated compounds (Oude Elferink et al., 1995; Konig et al., 2003).

The phenotype of patients with DJS and Mrp2-deficient rats is relatively mild, and life expectancy is not significantly affected (Oude Elferink et al., 1995). This is most likely explained by the strong induction of MRP3 (ABCC3) in hepatocytes under conditions in which the function of MRP2 is impaired (Hirohashi et al., 1998; Konig et al., 1999). MRP3 is localized in the sinusoidal membrane, and recently, it has been shown that bilirubin glucuronides are substrates for human MRP3 (Lee et al., 2004). In the absence of a functional MRP2, MRP3 compensates for the lack of MRP2-mediated transport by exporting at least part of the intrahepatically formed bilirubin glucuronides into the blood. In normal human liver, MRP3 protein was detected mainly in the bile ducts and not in hepatocytes (Scheffer et al., 2002). In addition, MRP3 protein was found in adrenal gland, kidney, and intestine.

Analysis of TR$^-$ and EHBR has resulted in a wealth of information regarding the function and substrate specificity of Mrp2 and has demonstrated the importance of this transporter in the biliary and renal elimination of many drugs, such as, for example, pravastatin and SN38 (an active metabolite of irinotecan) (Chu et al., 1997; Yamazaki et al., 1997). Interpretation of data obtained with TR$^-$ and EHBR was in some cases complicated by the induction of Mrp3, UGT1a, and significant differences in cytochrome P450 enzyme levels between wild-type and mutant strains (Jager et al., 1998; Johnson et al., 2005; Newton et al., 2005). The availability of additional in vivo models to study the role of Mrp2 in the pharmacokinetics of drugs would therefore be desirable. The goal of this report was to characterize mice with a homozygous disruption of the *Mrp2* gene. Differences between the knockout mice and TR$^-$ and EHBR strains are discussed.

Materials and Methods

Animals. Mrp2$^{-/-}$ mice were generated by Deltagen, Inc. (San Carlos, CA). In brief, the *Mrp2* gene was inactivated by homologous recombination in ES cells derived from the 129/OlaHsd mouse substrain by deletion of nucleotides 1886 to 1897 of the coding sequence of the *Mrp2* gene and introduction of a *LacZ-Neo* cassette. F1 mice were generated by breeding with C57BL/6 females. Heterozygous offspring of the F1 generation were used for rederivation by embryo transfer at Taconic Farms (Germantown, NY). Postderivation, mice were backcrossed for eight generations to a Taconic C57BL/6 background. Mice used in the experiments in this article were from heterozygote × heterozygote crosses of F2 mice. All mice were maintained at Taconic in a sterile isolator and monitored to verify their germ-free/defined-flora condition. Animals were kept in a temperature-controlled environment with a 12-h light/12-h dark cycle. Animals received food and water ad libitum. All animal handling was performed according to Animal Procedure Statements approved by the Merck Rahway Institutional Animal Care and Use Committee.

Mice were genotyped by PCR analysis of purified tail DNA. Wild-type Mrp2 was detected with oligonucleotides GS(E) (5′-gttcagcacctaacagaggtgattg-3′) and GS(E, T) (5′-tcctgaggaagagctggaaagcaag-3′), and the knockout allele was detected with oligonucleotides NEO(T) (5′-gggtgggattagataaatgcctgctct-3′) and GS(E, T), resulting in fragments of 237 and 502 bp, respectively (see Fig. 1A).

Analysis of Serum and Urine. Male and female Mrp2$^{-/-}$ and wild-type mice (between 20 and 24 weeks of age) were used in this study. The animals were fasted overnight with free access to water. Blood samples were taken by cardiac puncture. The serum samples obtained were used for analysis. Urine samples were collected overnight in metabolic cages with three mice per cage and three to four groups for each study. During urine collection, samples were kept on ice and in the dark. Serum and urine chemistry parameters listed in Tables 1 and 2 were determined using a Hitachi 911 clinical chemistry analyzer (Roche Diagnostics, Indianapolis, IN). Sodium, potassium, and chloride levels were determined using ion-specific electrodes. Other tests were performed by standard biochemical methods.

Analysis of Bilirubin Glucuronides and Total Glutathione. Male Mrp2$^{-/-}$ and wild-type mice (between 20 and 24 weeks of age) were used in this study. The animals were fasted overnight with free access to water. Mice were anesthetized via isoflurane inhalation, administered at a rate of 2% in oxygen at 0.5 l/min during the surgical procedure. The bile duct was catheterized, and the gallbladder was ligated and removed. After a recovery period of 1 to 3 h, the bile samples were collected on ice at 0 to 15, 15 to 30, 30 to 45, and 45 to 60 min. For measurement of bilirubin glucuronides, the bile samples were collected in the dark and immediately immersed in liquid nitrogen until analysis. Direct bilirubin (bilirubin glucuronides) in bile was analyzed with a Stanbio direct bilirubin assay kit (Stanbio Laboratory, Boerne, TX). Total glutathione (reduced plus oxidized) in bile was measured using a glutathione assay kit (Sigma Chemical, St. Louis, MO). Glutathione in liver extracts and plasma was determined using the same kit.

Western Blotting. Crude plasma membranes were prepared from mouse liver and kidney homogenates as described previously (Kobayashi et al., 1990). Protein was separated in a 7.5% denaturing polyacrylamide gel. Gels were immunoblotted onto nitrocellulose membranes. Blotting efficiency was assessed by staining blots with Ponceau S. Mouse Mrp2 was detected with monoclonal antibody M_2III-5 (Kamiya Biomedical Company, Seattle, WA), and mouse Mrp4 was detected with M_4I-10 (Alexis Corp., San Diego, CA). Anti-mouse and anti-rat horseradish peroxidase-labeled secondary antibodies were from Amersham Corp. (Arlington Heights, IL). Blots were developed with the enhanced chemiluminescence kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Enhanced chemiluminescence signals were detected with a GeneGnome System (Syngene, Frederick, MD), and signals were quantified using Gene Tools Software, version 3.04 (Syngene).

Quantitative RT-PCR. RNA was isolated from tissues of Mrp2$^{-/-}$ and wild-type male and female mice (20–24 weeks of age) with three animals per group. RNA was purified with a RNeasy Midi kit (QIAGEN Inc., Valencia, CA) according to instructions provided by the manufacturer. A two-step RT-PCR reaction was conducted by reverse-transcribing an aliquot of total RNA (~500 ng) to cDNA using a High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). cDNA reactions from three animals in each group were pooled. PCR reactions were then prepared by adding cDNA to a reaction mixture containing TaqMan 2x Universal PCR Master Mix (Applied Biosystems). Low-density microarrays (microfluidic cards) were custom-made by Applied Biosystems and contained probes in duplicate for the detection of 98 genes (see Table 4). Samples were applied to low-density microarrays by centrifugation twice for 1 min at 1200*g*. RT products from the pooled cDNAs of three animals were analyzed in three independent experiments. Real-time quantitative PCR was performed using an ABI PRISM 7900 Sequence Detector instrument and Sequence Detector 2.1 software (PerkinElmer In-

struments, Shelton, CT). Quantitation of the target cDNAs in all samples was normalized to 18S ribosomal RNA ($Ct_{target} - Ct_{18S} = \Delta Ct$), and the difference in expression for each target cDNA in the Mrp2$^{-/-}$ mice was expressed to the amount in the wild-type mice ($\Delta Ct_{wild\text{-}type} - \Delta Ct_{Mrp2^{-/-}} = \Delta\Delta Ct$). Fold changes in target gene expression were determined by taking 2 to the power of this number ($2^{-\Delta\Delta Ct}$).

Liver Microsomal Cytochrome P450 Enzyme Activities. Microsomes from mouse livers were prepared as described previously (Newton et al., 2005), resuspended in 10 mM potassium phosphate buffer (pH 7.4) containing 0.25 M sucrose, and stored at −80°C until use. Protein concentrations were measured using the bicinchoninic acid procedure. Ethoxyresorufin *O*-dealkylation (EROD) and pentoxyresorufin *O*-dealkylation (PROD) activities were measured as described with some modifications (Newton et al., 2005). Testosterone hydroxylation activity was determined by incubating microsomes (0.25 mg/ml) with substrate (250 μM) in potassium phosphate buffer (100 mM, pH 7.4) with $MgCl_2$ (3 mM) and an NADPH-generating system. Incubations were performed for 10 min at 37°C. Reactions were quenched by adding an equal volume of methanol. Samples were centrifuged for 10 min at 14,000*g*, and the supernatants were injected directly for reversed-phase HPLC analysis. The HPLC system consisted of a SCL 10A system controller (Shimadzu, Columbia, MD), two LC 10AT pumps, a SIL 10AD automatic sample injector, and a SPD-M10A UV-visible spectrophotometric detector. Aliquots of the supernatant (50 μl) were injected onto a Zorbax SB-C_8 column (4.6 × 75 mm, 3.5 μm; Agilent Technologies, Palo Alto, CA) and eluted at a flow rate of 2 ml/min. The mobile phase consisted of a mixture of buffer A (10 mM ammonium acetate) and buffer B (10 mM ammonium acetate in 90% acetonitrile and 10% methanol). A linear gradient elution with buffer B was run from 20 to 60% in 16 min, and chromatographic peaks of testosterone and its metabolites were monitored at 254 nm at the following retention times: 6β-hydroxytestosterone at 5.3 min, 7α-hydroxytestosterone at 4.5 min, 16α-hydroxytestosterone at 6.4 min, and 2α-hydroxytestosterone at 7.1 min.

Biliary Excretion and Pharmacokinetics of DBSP. Male Mrp2$^{-/-}$ and wild-type mice (between 20 and 24 weeks of age) were used. The animals were fasted overnight with free access to water. DBSP (Laboratoire SERB, Paris, France) was dissolved in saline. For biliary excretion studies, after intravenous administration of DBSP (5 mg/kg), bile samples were collected over a total of 90 min in preweighted tubes on ice. For the pharmacokinetic study, after intravenous dosing of DBSP (5 mg/kg), 20 μl of blood samples were taken from the tail vein at designated time points, and blood was added to 60 μl of sodium citrate. All samples were kept at −80°C until further analysis.

Quantification of DBSP in Bile and Blood. In brief, aliquots of bile (10 μl) were diluted with 50 mM Tris-HCl buffer (pH 7.4) and then made alkaline by the addition of 0.1 M NaOH (100 μl). Concentrations of DBSP in bile samples were determined in a spectrophotometer (Spectramax M2; Molecular Devices, Sunnyvale, CA) at a wavelength of 570 nm (Sathirakul et al., 1993).

Blood samples were thawed on ice. Two volumes of acetonitrile containing formic acid (0.1% v/v) were added to the samples and standard solutions. Labetolol (Sigma Co., St. Louis, MO) was added as an internal standard. After mixing, the contents were centrifuged at 1800*g* for 10 min. The supernatant was then transferred to a 96-well plate containing an equal volume of water and analyzed using liquid chromatography-tandem mass spectrometry. Chromatography was performed on a Beta Basic Phenyl Dash column (10 × 1 mm, 5 μm) and an HPLC system consisting of PerkinElmer series 200 micro pumps (PerkinElmer Instruments) and a LEAP HTS PAL Autosampler (LEAP Technologies, Carrboro, NC) using a gradient mobile phase of water (A) and acetonitrile (B). The HPLC flow rate was 0.6 ml/min. The gradient was started with 5% B, increased linearly to 95% B in 0.7 min, and held at 95% B for 0.5 min. Detection of the analyte was performed using a Sciex API 4000 mass spectrometer (MDS-Sciex, Toronto, ON, Canada) in the negative ion mode using the Turbo-Ion Spray source. Mass transition (*m/z*) monitoring for DBSP was 316.90 → 294.85. The concentration of DBSP in blood samples were determined by comparing the analyte to internal standard peak area ratios against a standard curve.

Determination of DBSP Pharmacokinetic Parameters. Pharmacokinetic parameters of DBSP were calculated using Watson software (version 6; Watson Software System) with noncompartmental models.

Statistical Analysis. Student's *t* tests were used to determine the significance of differences between groups of animals. Differences with *P* values <0.05 or <0.01 were considered significant.

Results

Generation of Mrp2$^{-/-}$ Mice. Constitutive Mrp2$^{-/-}$ mice were generated by Deltagen by inserting a *Lac-Neo* cassette into the *Mrp2* gene, resulting in deletion of bp 1886 to 1897. PCR analysis of purified tail DNA allowed the detection of the wild-type and mutant alleles (Fig. 1A). To confirm the absence of Mrp2 protein in the knockout animals, crude membrane fractions were isolated from livers from Mrp2$^{-/-}$ and control animals, and Mrp2 was detected by Western blotting using monoclonal antibody M_2III-5. No Mrp2 protein was detectable in membranes of knockout mice (Fig. 1B, lane 2), whereas a strong signal was observed in the membranes isolated from wild-type mice (lane 1). Overall, no gross abnormalities were observed in the Mrp2$^{-/-}$ mice, their life span was normal, and mice were born at the expected Mendelian ratio.

Serum Analysis of Mrp2$^{-/-}$ Mice. Table 1 shows the values for several clinical chemical parameters measured in serum. No significant increases were observed in alanine aminotransferase and aspartate aminotransferase between Mrp2$^{-/-}$ and wild-type mice, suggesting no hepatocellular damage in livers of knockout mice. Total bilirubin levels were 5-fold higher in knockout animals. In Mrp2$^{-/-}$ mice, 60% of total (conjugated plus unconjugated) bilirubin was conjugated (direct) bilirubin, whereas the level of conjugated bilirubin in serum of wild-type mice was below the limit of

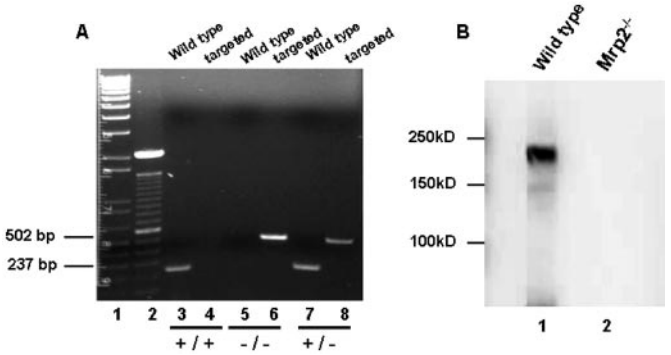

Fig. 1. Targeted disruption of the *Mrp2* gene in mice. A, PCR analysis of purified tail DNA isolated from wild-type (lanes 3 and 4), Mrp2$^{-/-}$ (lanes 5 and 6), and Mrp2$^{+/-}$ (lanes 7 and 8) mice. Two PCR reactions with different sets of primers were performed for each mouse tail DNA as described under *Materials and Methods*. The wild-type allele and targeted allele generate fragments of 237 and 502 bp, respectively. B, Western blot analysis of Mrp2 in liver of wild-type (lane 1) and Mrp2$^{-/-}$ (lane 2) mice. Ten micrograms of crude plasma membranes from wild-type and Mrp2$^{-/-}$ mouse liver were separated in a 7.5% denaturing polyacrylamide gel. Gels were immunoblotted onto nitrocellulose membranes. Mouse Mrp2 was detected with M_2III-5, an anti-MRP2 monoclonal antibody.

TABLE 1
Serum chemistry parameters of wild-type and Mrp2$^{-/-}$ mice
Data represent means ± S.E. determined from five to six animals.

Parameter	Males		Females	
	Wild Type	Mrp2$^{-/-}$	Wild Type	Mrp2$^{-/-}$
Glucose (mg/dl)	128 ± 10	145 ± 21	139 ± 6	119 ± 9
Urea nitrogen (mg/dl)	26.1 ± 1.5	30 ± 2.9	25.0 ± 2.8	19.8 ± 1.8
Total protein (g/dl)	6.2 ± 0.2	6.0 ± 0.1	5.7 ± 0.1	6.1 ± 0.04
Albumin (g/dl)	4.1 ± 0.2	4.1 ± 0.1	4.3 ± 0.1	4.5 ± 0.1
Total bilirubin (mg/dl)	0.1 ± 0.02	0.5** ± 0.02	0.1 ± 0.02	0.5** ± 0.1
Direct bilirubin (mg/dl)	<0.1[a]	0.3 ± 0.02	<0.1[a]	0.3 ± 0.03
Alkaline phosphatase (U/l)	89.2 ± 5.0	81.8 ± 8.7	183 ± 14	132** ± 15
Alanine aminotransferase (U/l)	40.3 ± 5.4	34.7 ± 3.8	56.6 ± 10.1	36.8 ± 5.4
Aspartate aminotransferase (U/l)	234 ± 41	164 ± 42	217 ± 45	142 ± 20
Lactate dehydrogenase (U/l)	543 ± 127	334 ± 67	403 ± 56	275 ± 43
Cholesterol (mg/dl)	83.5 ± 8.2	130** ± 9	80.8 ± 6.4	95.2 ± 4.6
Calcium (mg/dl)	10.1 ± 0.1	10.3 ± 0.2	10.0 ± 0.1	10.3 ± 0.1
Phosphorus (mg/dl)	7.5 ± 0.2	7.9 ± 0.4	7.6 ± 0.7	7.3 ± 0.4
Sodium (mEq/l)	154 ± 1	155 ± 1	154 ± 2	154 ± 1
Potassium (mEq/l)	7.6 ± 0.4	7.4 ± 0.2	7.5 ± 0.3	6.9 ± 0.5
Chloride (mEq/l)	114 ± 1	114 ± 1	113 ± 2	111 ± 1
Triglycerides (mg/dl)	62.8 ± 5.3	75.0 ± 9.2	62.2 ± 8.4	64.6 ± 3.8
Creatine kinase (U/l)	676 ± 101	661 ± 197	484 ± 63	430 ± 152
Magnesium (mg/dl)	2.7 ± 0.1	2.7 ± 0.2	2.8 ± 0.2	2.8 ± 0.2
Osmolality (mOsmol/kg)	304 ± 5	316 ± 1	309 ± 3	309 ± 2

** $P < 0.01$, significantly different from wild-type mice.
[a] Below the detection limit.

quantitation. Cholesterol levels were 1.5-fold higher in male knockout animals, but no significant change was observed between female Mrp2$^{-/-}$ and control mice. The increased cholesterol level in male Mrp2$^{-/-}$ mouse serum did not result in changes in the biliary excretion of cholesterol (data not shown). No significant differences were found in any of the other parameters measured, except for a slightly decreased alkaline phosphatase level in Mrp2$^{-/-}$ compared with control female mice.

Urine Analysis of Mrp2$^{-/-}$ Mice. Part of the conjugated bilirubin was excreted into the urine in TR$^-$ (Jansen et al., 1985). To determine whether this was also the case in mice, total and direct bilirubin amounts in urine were measured (Table 2). In urine collected over a 12-h time period, levels of direct bilirubin were 6.7-fold higher in Mrp2$^{-/-}$ mice. The other urine parameters measured were not changed significantly.

Glutathione Levels in Mrp2 $^{-/-}$ Mice. Paulusma et al. (1999) found previously that the biliary excretion of glutathione was reduced strongly in TR$^-$, suggesting that Mrp2 was involved in the transport of glutathione. Therefore, the biliary excretion of glutathione in mice was measured (Fig. 2A). The biliary excretion rate of glutathione was 16-fold lower in Mrp2$^{-/-}$ than in controls (25 nmol/kg/min in Mrp2$^{-/-}$ versus 400 nmol/kg/min in wild-type mice). Some very low-glutathione export was still detectable in knockout mice, suggesting the presence of other compensatory (unknown) canalicular glutathione transporters. The reduced excretion of glutathione in the knockout mice correlated with a 3-fold increased glutathione level in Mrp2$^{-/-}$ liver extracts (Fig. 2B). Differences in plasma levels of glutathione between Mrp2$^{-/-}$ and control mice were not statistically significant (Fig. 2C).

Bilirubin Glucuronide Levels in Mrp2$^{-/-}$ Mice. Biliary secretion of endogenous bilirubin glucuronides, physiologically important substrates of Mrp2, in Mrp2$^{-/-}$ mice was evaluated. As shown in Fig. 2D, the biliary excretion rate of bilirubin glucuronides was approximately 2-fold lower in Mrp2$^{-/-}$ mice than that in control mice. This finding is in agreement with reports that the biliary excretion of bilirubin and bilirubin glucuronides in EHBR and TR$^-$ is only decreased mildly (Jansen et al., 1985; Kurisu et al., 1991; Paulusma et al., 1999).

TABLE 2
Urine chemistry parameters of wild-type and Mrp2$^{-/-}$ mice
The parameters tested were normalized to urine volume (milliliters).
Data represent means ± S.E. determined from urine samples obtained from five groups of animals: each group included three animals as described under *Materials and Methods.*

Parameter	Males		Females	
	Wild Type	Mrp2$^{-/-}$	Wild Type	Mrp2$^{-/-}$
Glucose (mg/dl/ml)	3.6 ± 1.6	4.0 ± 1.1	2.1 ± 0.4	3.8 ± 1.0
Urea nitrogen (mg/dl/ml)	496 ± 223	508 ± 145	252 ± 49	415 ± 110
Creatinine (mg/dl/ml)	8.7 ± 4.0	9.4 ± 2.4	3.8 ± 0.6	6.5 ± 1.7
Total protein (g/dl/ml)	0.3 ± 0.1	0.3 ± 0.1	0.04 ± 0.01	0.1 ± 0.01
Total bilirubin (mg/dl/ml)	0.7 ± 0.3	0.7 ± 0.2	0.4 ± 0.1	0.7 ± 0.2
Direct bilirubin (mg/dl/ml)	0.06 ± 0.01	0.4* ± 0.1	0.03 ± 0.003	0.2* ± 0.04
Phosphorus (mg/dl/ml)	36.2 ± 14.2	39.1 ± 9.3	21.2 ± 3.4	29.8 ± 6.8
Sodium (mEq/l/ml)	19.4 ± 5.0	19.4 ± 4.8	11.2 ± 1.7	16.1 ± 4.1
Potassium (mEq/l/ml)	52.7 ± 25.8	52.4 ± 15.2	21.1 ± 5.2	36.4 ± 9.8
Chloride (mEq/l/ml)	17.0 ± 4.3	16.8 ± 4.3	10.9 ± 1.9	16.3 ± 4.5

* $P < 0.05$, significantly different from wild-type mice.

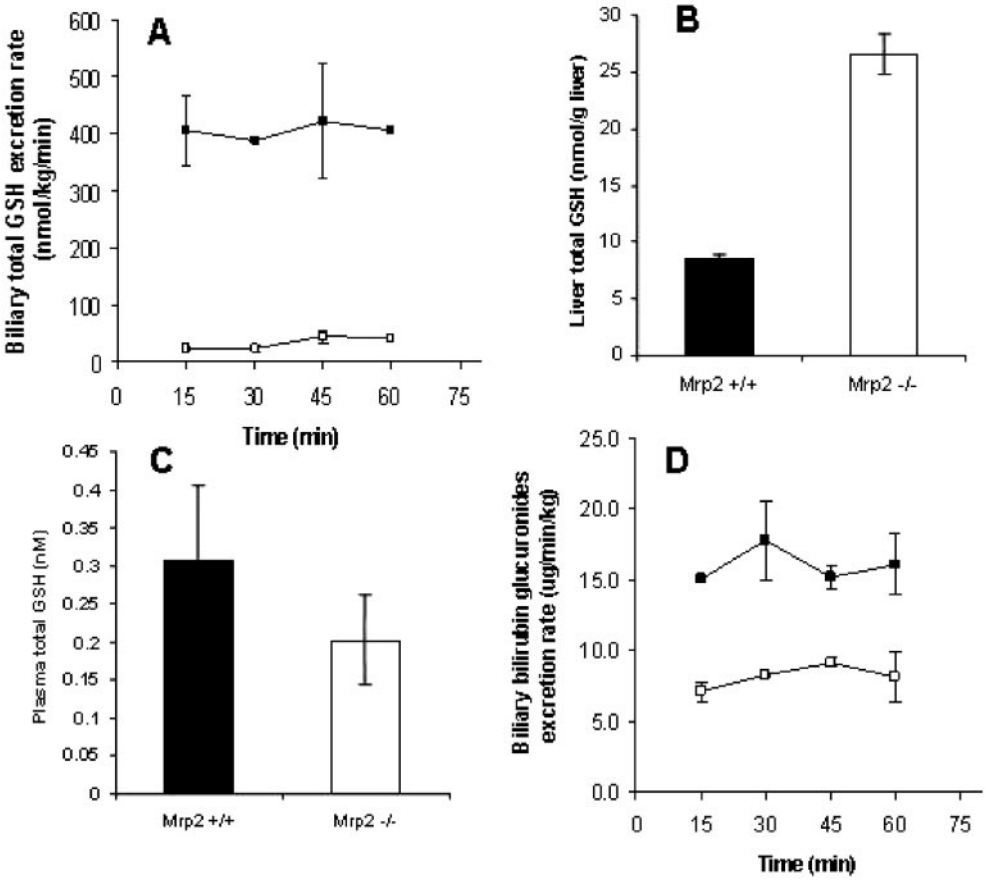

Fig. 2. Biliary excretion of glutathione and bilirubin glucuronides in wild-type and Mrp2$^{-/-}$ mice. A, biliary excretion rate of total glutathione in male Mrp2$^{-/-}$ (□) and wild-type (■) mice were measured using a glutathione assay kit as described under *Materials and Methods*. B and C, GSH levels in liver and plasma, respectively. D, biliary excretion rate of bilirubin glucuronides in male Mrp2$^{-/-}$ (□) and wild-type (■) mice. All data represent the means ± S.E. for three animals.

Decreased Biliary Secretion of Dibromosulfophthalein in Mrp2$^{-/-}$ Mice. To study the in vivo disposition of a nonmetabolized model Mrp2 substrate, DBSP (5 mg/kg) was administered intravenously to wild-type and Mrp2$^{-/-}$ mice. DBSP blood elimination was impaired in Mrp2$^{-/-}$ mice (Fig. 3D), resulting in a significant increase in the area under the curve and a decrease in blood clearance in the knockout mice (Table 3). In line with these findings, in bile duct-cathetered mice, the biliary excretion rate of DBSP (5 mg/kg i.v.) was slower in knockout than in wild-type mice (Fig. 3A). Cumulative excretion of DBSP in bile indicated that almost 100% of the dose was excreted at t = 50 min in wild-type animals, whereas only 25% was recovered in the Mrp2$^{-/-}$ mice at the same time point. This percentage increased to 70% at 90 min. Bile flow was somewhat variable between the different time points (Fig. 3C), but on average, it was ~25%

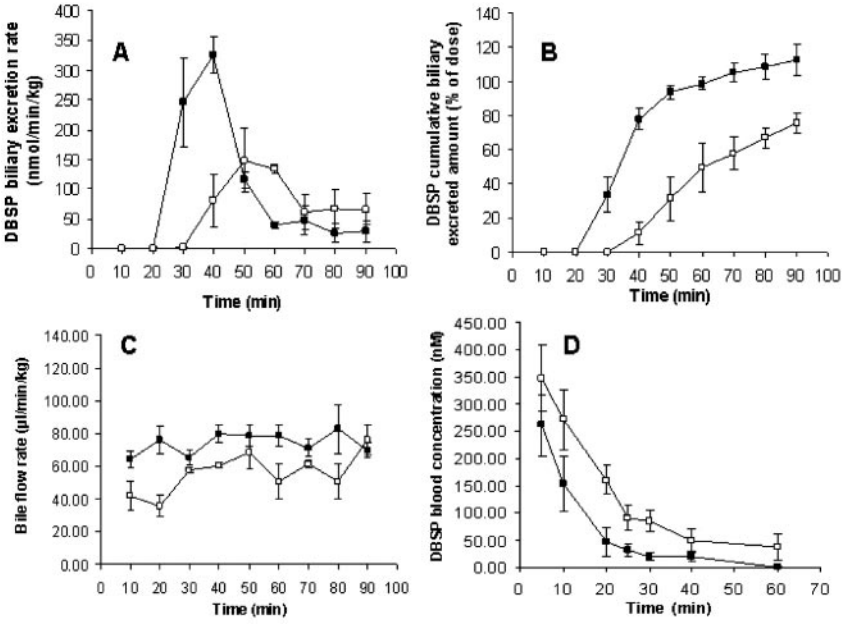

Fig. 3. In vivo disposition of DBSP in wild-type and Mrp2$^{-/-}$ mice. A and B, biliary excretion rate (A) and cumulative biliary excretion (B) of DBSP after i.v. administration of DBSP (5 mg/kg) in wild-type (■) and Mrp2$^{-/-}$ (□) mice. C, bile flow rate in wild-type (■) and Mrp2$^{-/-}$ (□) mice. D, blood DBSP concentration-time profile after i.v. administration of DBSP (5 mg/kg) in wild-type (■) and Mrp2$^{-/-}$ mice (□). All the data represent the means ± S.E. of three to four animals.

TABLE 3
Pharmacokinetic parameters of DBSP following intravenous administration (5 mg/kg) to wild-type and Mrp2$^{-/-}$ mice
Data represent means ± S.E. obtained from four animals.

Parameters	Wild Type	Mrp2$^{-/-}$
AUC (μM · min)	4.4 ± 1.0	8.2* ± 1.7
CL_b (l/kg/min)	1.9 ± 0.3	0.7* ± 0.3
$T_{1/2}$ (min)	6.6 ± 1.2	26.5 ± 14.2
V_{dss} (l/kg)	17.8 ± 5.2	21.5 ± 4.4

AUC, area under the curve; CL_b, blood clearance; $T_{1/2}$, terminal-phase half-life; V_{dss}, volume of distribution at steady-state.
* $P < 0.05$, significantly different from wild-type mice.

lower in Mrp2$^{-/-}$ than in wild-type mice. Taken together, these data demonstrated that Mrp2 was contributing significantly to the elimination of DBSP. Most likely, the delayed appearance of DBSP in Mrp2$^{-/-}$ mice relates to the fact that sinusoidal efflux is enhanced (see *Discussion*).

Gene Expression Profiling in Mrp2$^{-/-}$ Liver, Kidney, and Intestine. A general concern in the use of transporter knockout animals for pharmacokinetic studies is the potential for induction of compensatory mechanisms that may complicate the interpretation of data obtained with such animals. Using TaqMan low-density microarrays (microfluidic cards), we determined the expression level of a total of 98 phase I, phase II, transporter, and a number of other genes relevant to drug metabolism in liver, intestine, and kidney of wild-type and Mrp2$^{-/-}$ female and male mice (Table 4). Student's t tests were performed between $\Delta Ct_{\text{wild-type}}$ and $\Delta Ct_{\text{Mrp2}^{-/-}}$ from triplicate TaqMan analyses performed with the same reverse-transcription reaction products to determine whether the changes observed were statistically significant.

The Mrp2 mRNA level was 30- to 1000-fold lower in liver, kidney, and intestine from Mrp2$^{-/-}$ mice than in the corresponding organs from control mice, confirming that the *Mrp2* gene was inactivated in the knockout mice. The low RNA signal detected in tissues from Mrp2$^{-/-}$ mice was probably due to the presence of an unstable RNA transcript, which still may be formed as the Mrp2 promoter was not disrupted in these mice.

In humans and rats in which MRP2/Mrp2 activity is impaired or absent, MRP3/Mrp3 mRNA is induced in the liver (Hirohashi et al., 1998; Konig et al., 1999). In contrast, no induction of Abcc3 (Mrp3) mRNA was observed in the liver of either female or male Mrp2$^{-/-}$ mice (Table 4). Interestingly, Mrp4 (Abcc4) mRNA was induced 6- to 7-fold in livers of male and female Mrp2$^{-/-}$ mice. In females, a 2.5-fold lower level of Slc22a7 (Oat2) was found in livers of knockout versus wild-type mice, whereas no significant differences were detected for any of the other transporter genes analyzed.

In kidney, expression of Mrp4 was increased 2.5-fold in male and female Mrp2$^{-/-}$ mice. Expression of Abcb9, Slco1a1 (Oatp1), and Slco1a6 (Oatp5) was higher in males than in females. In female kidney, expression of Abcc3 was higher than that in male kidney.

Of the cytochrome P450 genes analyzed, *Cyp2b13* was expressed at a 10-fold lower level in livers of female Mrp2$^{-/-}$ than wild-type mice (Table 4). *Cyp2b9* was expressed at a 2-fold lower level in livers of the female knockout mice. The cholesterol-7α-hydroxylase *Cyp7a1* gene was expressed 2.2-fold higher in male Mrp2$^{-/-}$ liver, and *Cyp4a14* in male Mrp2$^{-/-}$ liver was expressed 2.5-fold lower than in liver from control mice. *Cyp4a14* in male Mrp2$^{-/-}$ kidney was expressed 2-fold lower than in wild-type kidney. Expression of *Cyp2d9* and *Cyp4a12* was higher in male than in female liver, whereas expression of *Cyp2b13*, *Cyp2b9*, *Cyp3a16*, and *Cyp3a41* was higher in female liver. In kidney, expression of *Cyp2d9* and *Cyp4a12* was higher in males than in females.

For the UDP-glucuronosyltransferase, glutathione *S*-transferase, and sulfotransferase genes analyzed, no significant differences were found between wild-type and knockout mice (Table 4). Expression of *Ugt1a1* was higher in female than in male kidney, and expression of *Ugt8* was higher in female then in male kidney.

Because nuclear receptors play an important role in the regulation of gene expression, the expression of several nuclear receptors, including the pregnane X receptor (Pxr, *Nr1i2*), the constitutive androstane receptor (Car, *Nr1i3*), the liver X receptor (Lxr, *Nr1h3*), and the farnesoid X receptor (Fxr, *Nr1h4*), was evaluated. No significant differences were detected for these genes in the liver, kidney, and intestine of knockout versus control mice (Table 4).

Mrp4 Protein Levels in Liver and Kidney. Because Mrp4 mRNA was increased in both liver and kidney of Mrp2$^{-/-}$ mice, protein levels were determined in crude membrane extracts of male mice by Western blotting. Mrp4 was detected with monoclonal antibody M_4I-10. By comparing the Western blot signal after loading various amounts of protein, Mrp4 protein was found to be increased 7- to 10-fold in livers of Mrp2$^{-/-}$ mice (Fig. 4A). In the kidney, the Mrp4 level was increased 2-fold in Mrp2$^{-/-}$ mice (Fig. 4B, lanes 3–6). The Mrp4 level in wild-type kidney was ~12-fold higher than that in wild-type liver (Fig. 4B, compare lanes 1 and 3).

Bile Salt Levels in Bile and Plasma. Because Mrp4 is localized to the basolateral membrane in hepatocytes and has been demonstrated to transport bile salts and conjugated bile acids in vitro (Rius et al., 2003), total bile salt levels were measured in the serum of male Mrp2$^{-/-}$ and control mice. Although bile salt levels were higher in the serum of some Mrp2$^{-/-}$ mice, variation was high and not statistically different from control mice (data not shown). Bile salt levels were also measured in bile. Over a 60-min sampling period, the excretion rate was somewhat higher in Mrp2$^{-/-}$ compared with control mice at all time points (average of 1.5 ± 0.1 μmol/kg/min in Mrp2$^{-/-}$ versus 0.9 ± 0.1 μmol/kg/min in control mice, $n = 3$).

Liver Microsomal Cytochrome P450 Enzyme Activities. To investigate whether the reduced expression of *Cyp2b13* in liver of female Mrp2$^{-/-}$ mice resulted in a phenotypic effect and to confirm the lack of clear induction or suppression in the level of expression of any of the other P450 isoforms tested (Table 4), we investigated P450-mediated enzyme activities in liver microsomes isolated from Mrp2$^{-/-}$ and wild-type male and female mice. Although the specificity of probe substrates for all of the mouse P450 enzymes have not been clearly identified, it has been inferred that, in mouse liver microsomes, ethoxyresorufin *O*-dealkylation and PROD activities are mediated by *Cyp1A* and *Cyp2B*, respectively, whereas testosterone 6β-hydroxylase (HT) activity is mediated by *Cyp3A*, 2α-HT activity is mediated by *Cyp2C*, 16α-HT activity is mediated by *Cyp2D9* and *Cyp2B*, 7α-HT-activity is mediated by *Cyp2A*, and 16β-HT activity is mediated by *Cyp2B* (Sapone et al., 2003). Table 5 shows that no significant differences were detected in liver microsomes

TABLE 4

Gene expression in Mrp2$^{-/-}$ mouse liver, kidney, and intestine

Data are expressed as means ± S.E. -fold change in Mrp2$^{-/-}$ mice compared with wild-type mice determined from three independent experiments.

Gene	Accession No.	Liver		Kidney		Intestine	
		Males	Females	Males	Females	Males	Females
Abca1	NM_013454	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.2	0.9 ± 0.1	1.0 ± 0.2
Abca13	NM_178259	—[b]	—[b]	0.7 ± 0.2	0.8 ± 0.2	—[b]	—[a]
Abca2	NM_007379	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.03	0.6 ± 0.2	0.6 ± 0.02	1.2 ± 0.2
Abca3	NM_013855	0.6 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.2	0.7 ± 0.1	0.9 ± 0.1
Abca4	NM_007378	—[a]	—[a]	1.0 ± 0.2	0.8 ± 0.2	—[b]	—[a]
Abca5	NM_147219	0.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.2	0.7 ± 0.3	—[a]	—[a]
Abca6	NM_147218	0.7 ± 0.1	0.8 ± 0.1	—[a]	—[a]	—[a]	—[a]
Abca7	NM_013850	0.5 ± 0.1	0.6 ± 0.1	0.9 ± 0.2	0.5 ± 0.2	0.8 ± 0.1	0.9 ± 0.04
Abca8a	NM_153145	0.7 ± 0.05	1.5 ± 0.1	0.7 ± 0.1	1.0 ± 0.2	—[a]	0.9 ± 0.2
Abca8b	NM_013851	0.8 ± 0.1	0.7 ± 0.1	—[a]	—[a]	—[a]	—[a]
Abca9	NM_147220	—[a]	—[a]	—[a]	—[a]	—[a]	1.0 ± 0.3
Abcb10	NM_019552	0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.6 ± 0.2	0.8 ± 0.1	0.8 ± 0.1
Abcb11	NM_021022	0.6 ± 0.1	0.6 ± 0.2	—[b]	—[b]	—[a]	—[a]
Abcb1a	NM_011076	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.2	0.7 ± 0.3	1.2 ± 0.2	1.3 ± 0.2
Abcb1b	NM_011075	1.0 ± 0.3	0.9 ± 0.4	0.5 ± 0.1	0.7 ± 0.2	—[a]	—[a]
Abcb4	NM_008830	0.5 ± 0.1	0.8 ± 0.1	—[a]	—[a]	—[a]	—[a]
Abcb6	NM_023732	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.6 ± 0.2	0.8 ± 0.1	1.0 ± 0.1
Abcb9	NM_019875	—[a]	—[a]	0.9[c] ± 0.1	0.6 ± 0.2	0.8 ± 0.1	0.8 ± 0.1
Abcc1	NM_008576	—[a]	—[a]	1.0 ± 0.3	0.7 ± 0.2	—[a]	0.8 ± 0.1
Abcc10	NM_170680	—[a]	—[a]	1.5 ± 0.5	0.6 ± 0.2	1.0 ± 0.2	0.7 ± 0.1
Abcc2	NM_013806	0.001 ± 0.0001*	0.004 ± 0.001*	0.003 ± 0.001*	0.004 ± 0.001*	0.03 ± 0.001*	0.03 ± 0.004*
Abcc3	NM_029600	0.9 ± 0.1	1.0 ± 0.1	0.6 ± 0.2	0.6[d] ± 0.2	1.0 ± 0.3	1.0 ± 0.1
Abcc4	GI:39771467	6.4 ± 0.9*	7.0 ± 1.7*	2.5 ± 0.2*	2.3 ± 0.3*	0.9 ± 0.2	1.3 ± 0.1*
Abcc5	NM_013790	—[a]	—[a]	0.7 ± 0.1	0.7 ± 0.2	—[a]	—[a]
Abcc6	NM_018795	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.2	0.5 ± 0.2	—[a]	—[a]
Abcc8	NM_011510	—[b]	—[b]	—[a]	—[a]	—[a]	—[a]
Abcc9	NM_011511	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.2	0.6 ± 0.2	0.9 ± 0.3
Abcg1	NM_009593	0.9 ± 0.1	0.8 ± 0.03	0.8 ± 0.2	0.5 ± 0.2	0.6 ± 0.2	0.6 ± 0.1
Abcg2	NM_011920	0.8 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	0.8 ± 0.2	0.7 ± 0.1	0.9 ± 0.1
Abcg3	NM_030239	—[a]	—[a]	—[a]	—[a]	—[a]	—[a]
Abcg4	NM_138955	—[b]	—[b]	—[b]	—[a]	—[a]	—[a]
Abcg5	NM_031884	0.8 ± 0.1	1.1 ± 0.1	—[a]	—[b]	1.1 ± 0.1	1.2 ± 0.1
Abcg8	NM_026180	0.8 ± 0.1	0.9 ± 0.1	—[a]	—[b]	1.0 ± 0.1	1.0 ± 0.1
Cftr	NM_021050	—[a]	—[a]	—[a]	—[a]	0.9 ± 0.1	0.8 ± 0.1
Slc10a1	NM_011387	0.7 ± 0.1	0.9 ± 0.2	—[a]	—[a]	—[a]	—[a]
Slc10a3	NM_145406	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.2	0.7 ± 0.1	0.8 ± 0.2
Slc15a2	NM_021301	—[a]	—[a]	0.9 ± 0.1	0.6 ± 0.2	—[a]	—[a]
Slc22a1	NM_009202	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.04	0.7 ± 0.2	0.6 ± 0.3	0.6 ± 0.3
Slc22a12	NM_009203	—[a]	—[b]	0.9 ± 0.2	0.9 ± 0.3	—[b]	—[b]
Slc22a13	NM_133980	—[b]	—[b]	0.7 ± 0.1	0.8 ± 0.2	—[b]	—[b]
Slc22a17	NM_021551	—[a]	—[a]	0.7 ± 0.1	0.8 ± 0.2	—[a]	—[a]
Slc22a18	NM_008767	0.7 ± 0.1	0.9 ± 0.1	0.7 ± 0.2	0.7 ± 0.2	0.9 ± 0.1	0.9 ± 0.1
Slc22a2	NM_013667	—[b]	—[b]	0.7 ± 0.1	0.7 ± 0.2	—[b]	—[b]
Slc22a3	NM_011395	0.6 ± 0.1	0.6 ± 0.1	—[a]	—[a]	—[a]	—[a]
Slc22a4	NM_019687	1.1 ± 0.2	0.8 ± 0.3	0.6 ± 0.2	0.8 ± 0.2	1.1 ± 0.2	0.9 ± 0.04
Slc22a5	NM_011396	0.6 ± 0.3	1.4 ± 0.6	0.7 ± 0.1	0.6 ± 0.2	0.8 ± 0.1	0.6 ± 0.2
Slc22a6	NM_008766	—[b]	—[b]	0.8 ± 0.3	0.7 ± 0.2	—[b]	—[b]
Slc22a7	NM_144856	1.3 ± 0.2	0.4 ± 0.02*	0.7 ± 0.1	0.7 ± 0.2	—[b]	—[b]
Slc22a8	NM_031194	—[b]	—[b]	0.7 ± 0.1	0.7 ± 0.2	—[a]	—[b]
Slc22a9	NM_019723	—[a]	—[a]	—[a]	—[a]	0.8 ± 0.1	1.1 ± 0.2
Slco1a1	NM_013797	0.6 ± 0.1	0.6 ± 0.1	0.8[c] ± 0.3	—[b]	—[a,b]	—[b]
Slco1a4	NM_030687	1.2 ± 0.1	1.2 ± 0.2	—[a]	—[a]	—[a]	—[b]
Slco1a5	NM_130861	—[b]	—[b]	—[b]	—[b]	—[b]	—[b]
Slco1a6	NM_023718	—[b]	—[b]	0.9[c] ± 0.2	0.8 ± 0.3	—[b]	—[b]
Slco1b2	NM_020495	0.7 ± 0.1	0.9 ± 0.2	—[b]	—[b]	—[b]	—[b]
Slco1c1	NM_021471	—[a]	—[a]	—[a]	—[a]	—[b]	—[b]
Slco2a1	NM_033314	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.2	0.8 ± 0.2	0.8 ± 0.2
Slco3a1	NM_023908	—[a]	—[a]	0.7 ± 0.2	0.9 ± 0.2	1.4 ± 0.4	0.9 ± 0.02
Slco4a1	NM_148933	—[b]	—[b]	—[a]	—[b]	—[b]	—[b]
Tap1	NM_013683	0.5 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.5 ± 0.2	0.7 ± 0.1	0.8 ± 0.1
Tap2	NM_011530	0.5 ± 0.04	0.5 ± 0.2	0.6 ± 0.1	0.7 ± 0.3	0.7 ± 0.1	1.1 ± 0.2
Acat2	NM_009338	0.6[e]	0.7[e]	0.9 ± 0.2	0.9 ± 0.2	1.0 ± 0.1	1.4 ± 0.2
Cyp1a1	Y00071,K02588	—[a]	—[a]	—[a]	—[a]	—[a]	—[a]
Cyp1a1	NM_009992	—[b]	—[b]	—[a]	—[b]	—[b]	—[a]
Cyp1a2	NM_009993	0.9 ± 0.1	1.1 ± 0.2	—[a]	—[b]	—[a]	—[b]
Cyp1b1	NM_009994	—[a]	—[a]	0.5 ± 0.1	0.6 ± 0.2	0.8 ± 0.1	—[a]
Cyp27a1	NM_024264	0.8 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	0.7 ± 0.3	1.1 ± 0.2	0.9 ± 0.1
Cyp2b10	NM_009998	—[a]	—[a]	—[a]	—[b]	—[a]	—[a]
Cyp2b13	NM_007813	—[a]	0.1[d] ± 0.02*	—[b]	—[b]	—[b]	—[b]
Cyp2b19	NM_007814	—[a]	—[a]	—[a]	—[a]	—[a]	—[b]
Cyp2b9	NM_010000	—[a]	0.5[d] ± 0.02*	—[b]	—[b]	—[b]	—[b]

TABLE 4
Continued

Gene	Accession No.	Liver		Kidney		Intestine	
		Males	Females	Males	Females	Males	Females
Cyp2d9	NM_010006	0.7[c] ± 0.2	0.6 ± 0.1	0.3[c] ± 0.1	0.5 ± 0.2	—[a]	—[a]
Cyp2e1	NM_021282	0.7 ± 0.2	0.6 ± 0.2	0.7 ± 0.1	0.7 ± 0.2	—[b]	1.1 ± 0.05
Cyp39a1	NM_018887	0.9 ± 0.1	0.9 ± 0.1	—[a]	—[a]	—[a]	—[a]
Cyp3a11	NM_007818	1.1 ± 0.1	1.3 ± 0.3	—[a]	—[a]	1.2 ± 0.04	1.3 ± 0.3
Cyp3a13	NM_007819	1.7 ± 0.2	1.1 ± 0.2	—[a]	—[a]	1.2 ± 0.2	1.0 ± 0.02
Cyp3a16	NM_007820	—[a]	1.0[d] ± 0.2	—[b]	—[b]	—[a]	—[b]
Cyp3a41	NM_017396	—[a]	1.2[d] ± 0.2	—[b]	—[b]	—[b]	—[b]
Cyp4a12	NM_172306	0.7[c] ± 0.1	—[a]	0.6[c] ± 0.1	0.6 ± 0.1	—[b]	—[b]
Cyp4a14	NM_007822	0.4 ± 0.1*	0.8 ± 0.1	0.5 ± 0.1*	0.8 ± 0.2	—[a]	—[a]
Cyp51	NM_020010	0.7 ± 0.1	0.8 ± 0.1	0.9 ± 0.2	0.7 ± 0.2	0.7 ± 0.1	0.7 ± 0.1
Cyp7a1	NM_007824	2.2 ± 0.2*	1.5 ± 0.1	—[b]	—[b]	—[a]	—[b]
Fasn	NM_007988	0.4[e]	0.5[e]	1.2 ± 0.3	0.7 ± 0.2	0.9 ± 0.1	0.9 ± 0.1
Gclc	NM_010295	0.5 ± 0.1	1.6 ± 0.3	0.7 ± 0.1	0.8 ± 0.2	1.65 ± 0.2	1.0 ± 0.1
Gss	NM_008180	0.9 ± 0.1	1.1 ± 0.1	0.9 ± 0.2	0.8 ± 0.3	0.8 ± 0.1	1.1 ± 0.04
Gsta2	NM_008182	—[b]	—[b]	—[b]	—[b]	—[b]	—[b]
Gstp1	NM_181796	0.8 ± 0.1	—[a]	0.9 ± 0.2	0.8 ± 0.2	1.1 ± 0.1	0.9 ± 0.03
Mvk	NM_023556	0.5[e]	0.6[e]	0.9 ± 0.02	0.6 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
Nr1i2	NM_010936	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.3	1.2 ± 0.1	1.5 ± 0.3	1.6 ± 0.5
Nr1i3	NM_009803	1.0 ± 0.2	1.5 ± 0.04	0.7 ± 0.1	1.5 ± 0.1	1.1 ± 0.2	1.4 ± 0.1
Nr1h3	NM_013839	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.2	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.2
Nr1h4	NM_009108	1.1 ± 0.1	1.3 ± 0.1	1.2 ± 0.2	1.6 ± 0.1	0.9 ± 0.1	1.4 ± 0.2
Pmvk	NM_026784	0.6[e]	0.7[e]	0.6 ± 0.2	0.8 ± 0.2	0.7 ± 0.1	0.8 ± 0.1
Sult1a1	NM_133670	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.2	1.1 ± 0.2	0.8 ± 0.1
Sult1d1	NM_016771	1.2 ± 0.2	0.9 ± 0.2	1.0 ± 0.1	0.8 ± 0.2	1.3 ± 0.1	1.1 ± 0.1
Ugt1a1	NM_013701	1.1 ± 0.2	1.2 ± 0.1	1.9 ± 0.3	1.0[d] ± 0.3	—[b]	—[b]
Ugt2a1	NM_053184	—[b]	—[a]	—[b]	—[b]	—[b]	—[b]
Ugt8	NM_011674	—[a]	—[a]	0.8[c] ± 0.1	0.6 ± 0.1	—[a]	—[b]

* $P < 0.05$, ΔC_t value significantly different from wild-type mice.
[a] Low gene expression C_t value >32).
[b] The gene was not amplified.
[c] Gene expression in male was higher than in female mice.
[d] Gene expression in female was higher than in male mice.
[e] Data were expressed as average value of duplicated experiments.

from wild-type versus knockout mice. Some gender-different activities were detected. 16α-HT activity was higher in male than in female mouse liver microsomes, and PROD activity was lower in male than in female liver microsomes. Taken together, these data suggest no significant differences in major P450 enzyme activities between the Mrp2$^{-/-}$ and wild-type mice.

Discussion

In this report, we describe the characterization of Mrp2 knockout mice. These mice are healthy and show no phenotypic abnormalities. Western blotting of liver membranes demonstrated that Mrp2 protein was not detectable in the liver of knockout mice. In serum and urine, significantly increased levels of conjugated and/or unconjugated bilirubin were found in the Mrp2$^{-/-}$ mice. The functional absence of Mrp2 was demonstrated by delayed excretion and incomplete recovery of the Mrp2 substrate DBSP in bile of the Mrp2$^{-/-}$ mice and a significant reduction in the excretion of glutathione and bilirubin glucuronides. In general, lack of Mrp2 may result in decreased biliary secretion, decreased renal elimination, and reduced intestinal secretion of Mrp2 substrates.

Unlike in TR$^-$/EHBR, Mrp3 mRNA was not induced in livers of Mrp2$^{-/-}$ mice (Table 4). This was unexpected because strong induction of Mrp3/MRP3 has been demonstrated in livers of TR$^-$/EHBR and in humans with DJS (Hirohashi et al., 1998; Konig et al., 1999). A plausible explanation for this discrepancy is that, under normal conditions, the level of Mrp3 in mouse hepatocytes is much higher than levels in rats and humans, and, consequently, mice have a constitutive mechanism to compensate for the inhibition or absence of Mrp2. This finding is in accord with a recent report by Belinsky et al. (2005), who demonstrated that Mrp3 protein was detectable at good levels in normal wild-type mouse liver. Our TaqMan data showed that Mrp2 was expressed at a level similar to that of Mrp3 in wild-type mouse liver. This result is in contrast to results in normal Wistar rats, in which the hepatic mRNA level of Mrp2 was approximately 60-fold higher than that of Mrp3 (data not shown). The above findings suggest that Mrp2$^{-/-}$ mice are not a good animal model to study human DJS.

The relatively strong induction of Mrp4 protein in liver (Fig. 4A) of Mrp2$^{-/-}$ mice was unexpected, because this has not been found in TR$^-$ (Chen et al., 2005). MRP4 is localized in the basolateral membrane in hepatocytes (Rius et al., 2003) and in the apical membrane of renal proximal tubule cells (van Aubel et al., 2002). Therefore, Mrp4 may act as a compensatory transporter for the lack of Mrp2 in mice. Although Mrp2 and Mrp4 show overlap in substrate specificity (Borst and Oude Elferink, 2002), it is not known whether Mrp4 transports bilirubin glucuronides. Because it has been demonstrated that human MRP4 transports bile salts in vitro (Ruis et al., 2003), we tested bile acid levels in serum of Mrp2$^{-/-}$ and control mice. Although bile salt levels were elevated in some Mrp2$^{-/-}$ mice, interanimal variation was high and differences from controls were not significant.

The mechanism explaining induction of Mrp4 in Mrp2$^{-/-}$ mice is not clear. Mrp4 is induced in mice lacking the nuclear receptor Fxr (*Nrih4*), whereas levels of Bsep (*Abcb11*) are reduced in these mice (Schuetz et al., 2001). Because the level

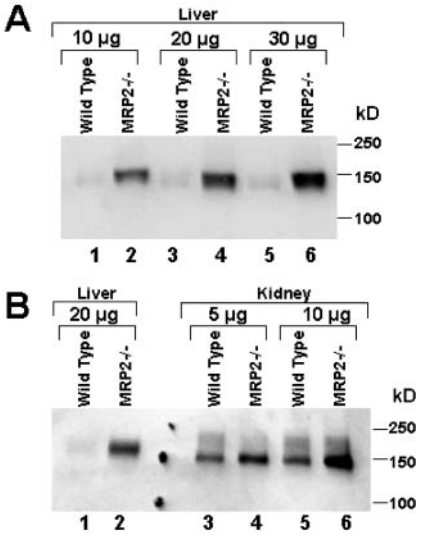

Fig. 4. Detection of Mrp4 protein in liver and kidney extracts. A, various amounts of crude membranes from liver of Mrp2$^{-/-}$ and control mice were separated in a 7.5% polyacrylamide gel. After Western blotting, Mrp4 was detected using monoclonal antibody M_4I-10. Protein amounts loaded are indicated above the lanes. B, same as A, but crude kidney membranes were analyzed (lanes 3–6). To compare relative protein levels, liver membranes were loaded onto the same gel (lanes 1 and 2).

of Fxr and *Abcb11* mRNA was not changed in Mrp2$^{-/-}$ mice (Table 4) and the excretion rate of total bile salts in the knockout was similar to that in control mice, down-regulation of Fxr or *Abcb11* is an unlikely explanation for the induction of Mrp4. A possible mechanism may be that absence of Mrp2 results in increased levels of bilirubin in hepatocytes and as a consequence activation of the constitutive androstane receptor (CAR) (Huang et al., 2003). Because *Mrp4* is a target gene of CAR, this may explain the induction of this transporter (Assem et al., 2004). However, we did not find activation of other CAR target genes as *Cyp2b10*, for instance (Table 4).

In the Mrp2$^{-/-}$ mice, total and conjugated bilirubin levels in serum were increased 5- and >3-fold, respectively (Table 1), and in urine, conjugated bilirubin was increased 6.7-fold (Table 2). In TR$^-$/EHBR, the levels of bilirubin conjugates in plasma were 50 to 100 times higher than those in Wistar or SD rats, respectively (Jansen et al., 1985; Hosokawa et al., 1992). Jansen et al. (1985) also showed that bilirubin and bilirubin conjugates are excreted via the urine in TR$^-$, whereas no excretion was detected in Wistar rats. However, it was concluded that the urinary excretion of total bilirubin was quantitatively low. At least four hypotheses could explain the relatively low increase in bilirubin plasma concentration in Mrp2$^{-/-}$ mice. 1) A mechanism for Mrp2-independent biliary excretion of bilirubin glucuronides may be present in mice, and this mechanism might be more potent than in rats. Quantitative comparison of bilirubin glucuronides secretion between Mrp2$^{-/-}$ mice and TR$^-$/EHBR would be needed to confirm this hypothesis. 2) The induction of Mrp3 in TR$^-$/EHBR might result in higher protein levels than the endogenous level detected in mice, causing more efficient export of bilirubin glucuronides from the liver into the circulation. 3) Johnson et al. (2005) indicated that expression of UGT1a protein was increased ~3.5-fold in TR$^-$ compared with controls. Therefore, increased bilirubin glucuronidation in the livers of TR$^-$ could contribute to the higher plasma bilirubin glucuronide levels in TR$^-$. 4) As in TR$^-$, urinary excretion of bilirubin glucuronides is increased in Mrp2$^{-/-}$ mice (Table 2). If the excretion is more efficient in mice than in rats (e.g., via Mrp4), this could result in lower plasma levels.

Differences in cytochrome P450 activities have been found between TR$^-$/EHBR and wild-type rats (Jager et al., 1998; Newton et al., 2005). One of the reasons to characterize the Mrp2$^{-/-}$ mice was to use these animals to study the role of Mrp2 in the pharmacokinetics of drugs. Therefore, it was important to investigate whether differences in results between knockout and control mice could be explained not only by the lack of Mrp2 but also potentially by differences in phase I enzymatic activities. Based on the phenotypic and mRNA expression analysis (Tables 4 and 5), no significant differences were found in P450 enzyme activities or mRNA levels in male mice. In female mice, Cyp2b13 was 10-fold lower in Mrp2$^{-/-}$ than in control livers. However, based on PROD and 16α-HT activities, this did not translate into a phenotypic effect.

Bile duct cannulation studies with the Mrp2 model substrate DBSP were performed to demonstrate that Mrp2 was functionally absent in the knockout mice (Fig. 3). DBSP was chosen because it is a nonmetabolized Mrp2 substrate (Klaassen, 1970), and it has been shown previously that the biliary excretion rate and plasma clearance of DBSP in both TR$^-$/EHBR were impaired (Jansen et al., 1987; Sathirakul et al., 1993). Residual transport was detected, however, in the mutant rats, which suggested that other transporters in the canalicular membrane transport DBSP. In Mrp2$^{-/-}$ mice, we observed a delayed and reduced biliary excretion rate of DBSP in bile (Fig. 3A). As in TR$^-$/EHBR, the residual excretion of DBSP in the knockout mice is probably explained by

TABLE 5

Comparison of Phase I metabolic enzyme activities between wild-type and Mrp2$^{-/-}$ mice

Data represent means ± S.E. determined from four liver microsome preparations, each preparation consisted of a pool of three to four livers. No statistically significant differences were found between wild-type and Mrp2$^{-/-}$ mice at $P < 0.05$.

Substrate	Males		Females	
	Wild Type	Mrp2$^{-/-}$	Wild Type	Mrp2$^{-/-}$
	nmol/min/mg protein			
EROD	81.56 ± 11.72	64.83 ± 6.86	96.39 ± 3.83	85.44 ± 8.28
PROD	18.49 ± 0.92**	17.31 ± 0.84**	33.11 ± 2.58	28.16 ± 0.78
6β-HT	1.25 ± 0.19	1.39 ± 0.19	1.42 ± 0.09	1.23 ± 0.15
2α-HT	0.24 ± 0.03	0.28 ± 0.03	0.26 ± 0.01	0.28 ± 0.04
16α-HT	0.50 ± 0.06**	0.41 ± 0.04**	0.15 ± 0.02	0.15 ± 0.02
7α-HT	0.30 ± 0.04	0.28 ± 0.05	0.28 ± 0.02	0.33 ± 0.05

** $P < 0.05$, significantly different from female mice.

another low-affinity/low-capacity transporter in the canalicular membrane. At present, the molecular identity of this transporter is not clear. A possible candidate is the breast cancer resistance protein (Bcrp or Abcg2), which is expressed in liver and is able to transport some negatively charged compounds (Doyle and Ross, 2003).

We found that, in the knockout mice, the biliary excretion of DBSP started to decrease after 50 min (Fig. 3A), although at that time point, only 30% of the dose had been excreted (Fig. 3B). As the bile flow was relatively constant over this time frame (Fig. 3C), this suggested that, after the intrahepatic DBSP concentration reached a certain threshold, DBSP was transported back into the blood. This transport may be via Mrp3, via Mrp4, or via an Oatp, because rat Oatps have been shown to act bidirectionally (Li et al., 2000).

In normal rats, excretion of glutathione into bile is high, and this transport is almost absent in TR$^-$ rats (Paulusma et al., 1999). In line with this finding, we also found that excretion of glutathione was reduced significantly in Mrp2$^{-/-}$ mice compared with control mice (Fig. 2A). In our measurements, we detected both reduced and oxidized glutathione, but levels of the latter are usually very low (Paulusma et al., 1999). Uptake experiments with membrane vesicles have suggested that GSH is a substrate for human and rat MRP2/Mrp2 with an apparent K_m in the millimolar range (Paulusma et al., 1999). GSH probably can be transported by MRP2/Mrp2 directly as a low-affinity substrate, or its transport can be associated with the transport of other compounds, such as, arsenite, vinblastine, etoposide, and sulfinpyrazone, for instance (Ballatori et al., 2005). We also found that biliary excretion of bilirubin glucuronides was only mildly decreased in Mrp2$^{-/-}$ mice (Fig. 2D), suggesting the involvement of other transporters on biliary excretion of bilirubin glucuronides.

In summary, we have characterized Mrp2$^{-/-}$ mice. These mice did not show differences in P450 enzyme levels in liver or induction of Mrp3. Mrp4 was increased in the Mrp2$^{-/-}$ mice, however, especially in liver. Because the Mrp2$^{-/-}$ mice show differences from TR$^-$/EHBR, we expect that these mice will be a valuable additional tool to study the role of Mrp2 in the absorption and disposition of drugs.

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Address correspondence to: Dr. Xiaoyan Chu, Merck & Co., RY80, 126 E. Lincoln Ave., Rahway, NJ 07065. E-mail: xiaoyan_chu@merck.com