

Characterization of inducible nature of MRP3 in rat liver

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Ogawa, Kotaro, Hiroshi Suzuki, Tomoko Hirohashi, Toshihisa Ishikawa, Peter J. Meier, Kenji Hirose, Toshifumi Akizawa, Masanori Yoshioka, and Yuichi Sugiyama. Characterization of inducible nature of MRP3 in rat liver. *Am. J. Physiol. Gastrointest. Liver Physiol.* 278: G438–G446, 2000.—We found previously that expression of multidrug resistance-associated protein (MRP) 3 is induced in a mutant rat strain (Eisai hyperbilirubinemic rats) whose canalicular multispecific organic anion transporter (cMOAT/MRP2) function is hereditarily defective and in normal Sprague-Dawley (SD) rats after ligation of the common bile duct. In the present study, the inducible nature of MRP3 was examined, using Northern and Western blot analyses, in comparison with that of other secondary active [Na^+ -taurocholic acid cotransporting polypeptide (Ntcp), organic anion transporting polypeptide 1 (oatp1), and organic cation transporter (OCT1)] and primary active [P-glycoprotein (P-gp), cMOAT/MRP2, and MRP6] transporters. α -Naphthylisothiocyanate treatment and common bile duct ligation induced expression of P-gp and MRP3, whereas expression of Ntcp, oatp1, and OCT1 was reduced by the same treatment. Although expression of MRP3 was also induced by administration of phenobarbital, that of cMOAT/MRP2, MRP1, and MRP6 was not affected by any of these treatments. Moreover, the mRNA level of MRP3, but not that of P-gp, was increased in SD rats after administration of bilirubin and in Gunn rats whose hepatic bilirubin concentration is elevated because of a defect in the expression of UDP-glucuronosyl transferase. However, the MRP3 protein level was not affected by bilirubin administration. Although the increased MRP3 mRNA level was associated with the increased concentration of bilirubin and/or its glucuronides in mutant rats and in SD rats that had undergone common bile duct ligation or α -naphthylisothiocyanate treatment, we must assume that factor(s) other than these physiological substances are also involved in the increased protein level of MRP3.

induction; multidrug resistance-associated protein; canalicular multispecific organic anion transporter; cholestasis; bilirubin glucuronide

THE LIVER IS ONE of the most important organs for the detoxification of xenobiotics. Many compounds are metabolized in hepatocytes after being taken up across the sinusoidal membrane and then excreted into the bile across the bile canalicular membrane. Thus transporters and metabolic enzymes have a synergistic action in the detoxification of xenobiotics. Recently, cDNA cloning of transporters for sinusoidal uptake and for canalicular export has been performed (29, 31). The former include several kinds of secondary active transporters such as Na^+ -taurocholic acid transporting polypeptide (Ntcp) (1) and organic anion transporting polypeptide (oatp) 1, which have been cloned as the transporters for the Na^+ -dependent uptake of taurocholic acid and Na^+ -independent uptake of organic anions, respectively (29, 31). As a homologue of oatp1, oatp2 has also been cloned and, indeed, it is capable of transporting organic anions (29, 31). Although OCT1, an organic cation transporter, was initially cloned from kidney (23), this transporter is also expressed on the sinusoidal membrane of hepatocytes (30). The function of these transporters has been studied by examining ligand uptake into cRNA-injected *Xenopus* oocytes and/or into cDNA-transfected mammalian cells (29).

In the same manner, the molecular mechanisms for ligand export across the canalicular membrane have also been identified. Several kinds of primary active transporters have been shown to act as export pumps for their ligands into bile; these belong to the ATP-binding cassette transmembrane transporters (ABC transporters) and include the multidrug resistance transporters mdr1 and -2, which are responsible for the biliary excretion of cationic and neutral amphipathic compounds and phospholipids, respectively (33, 36). In addition, certain bile acids are also excreted into the bile via the canalicular bile salt export pump, whose function is associated with the sister of P-glycoprotein (P-gp) (10). Moreover, canalicular multispecific organic anion transporter (cMOAT) has also been identified as a transporter involved in the biliary excretion of many organic anions including conjugated xenobiotics. The

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substrate for cMOAT has been determined by comparing transport across the bile canalicular membrane between normal rats and mutant rats whose cMOAT function is hereditarily defective; these mutant rats include Groningen yellow, transport-deficient, and Eisai hyperbilirubinemic rats (EHBR) (32, 38, 42). It has been shown that the substrate specificity of cMOAT resembles that of multidrug resistance-associated protein (MRP) 1; cMOAT is referred to as MRP2 (18, 31, 38). Although cMOAT/MRP2 is expressed on the bile canalicular membrane (18), the expression of MRP1 in the liver is minimal.

Previously, we amplified the partial cDNA sequences of novel ABC transporters that were initially referred to as MRP-like protein (MLP)-1 and -2 (11). Subsequent sequence alignment revealed that MLP-1 and -2 are homologues of human MRP6 and MRP3, respectively (11, 25). MRP6 is expressed in the liver of both SD rats and EHBR, whereas MRP3 is expressed only in EHBR liver (11). Moreover, hepatic expression of MRP3 in normal rats was induced by common bile duct ligation (11). In humans, it was also demonstrated that the hepatic expression of MRP3 is induced under cholestatic conditions (24). Recent immunohistochemical studies indicated that MRP3 is located on the basolateral membrane of hepatocytes and cholangiocytes (24, 26). Because MRP3 can transport organic anions including glucuronide conjugates (12), it is possible that MRP3 is induced to compensate for the impaired function of cMOAT/MRP2.

The present study has been undertaken to characterize the induction of MRP3 under *in vivo* experimental conditions in relation to that of other transporters responsible for hepatic uptake and biliary excretion. Several kinds of inducers were used in the present study. Effect of phenobarbital (PB) was examined, because we found that human MRP3 is induced by PB in Hep G2 cells *in vitro* (19), and in addition, PB has been reported to increase the biliary excretion of anionic compounds and to induce the uptake of sulfobromophthalein into rat hepatocytes (34). As an inducer, 3-methyl-cholanthrene (3-MC) was also used, because the induction of P-gp by this reagent has been reported in rat hepatocytes (6). Induction by 1-chloro-2,4-dinitrobenzene (CDNB) and zinc was examined, because the former is conjugated intracellularly with glutathione to yield dinitrophenyl-glutathione S conjugate, a typical substrate for cMOAT/MRP2 (22) and the latter induces MRP1 in the cisplatin-resistant human HL-60/R-CP cell line (13). The expression of transporters under cholestatic conditions induced by the administration of α -naphthylisothiocyanate (ANIT) was also studied (20).

METHODS

Animals. Male SD (220–260 g) and Wistar (220–240 g) rats were purchased from Nihon Ikagaku Doubutsu (Shizuoka, Japan). Male EHBR (200–250 g) were supplied by Eisai Laboratories (Gifu, Japan). Male Gunn rats (180–220 g), established from Wistar rats as mutants lacking UDP-

glucuronosyl transferase activity, were purchased from San-kyo Lab Service (Tokyo, Japan).

Drug treatment. Rats received PB (80 mg/kg in saline ip, daily for 4 days), 3-MC (40 mg/kg in corn oil ip, daily for 3 days), ANIT (250 mg/kg in polyethylene glycol, once po), CDNB (30 mg/kg in corn oil ip, daily for 3 days) or zinc acetate (6 mg/kg in saline iv, daily for 3 days). Control rats received the respective vehicle. Livers were dissected 24 h after the final administration. Bilirubin was intravenously injected into one group 3 times a day every 4 h for 2 days at a dose of 30 mg/kg, and livers were dissected 2 h after the final injection. All livers were immediately frozen in liquid nitrogen and then transferred to a freezer (-80°C) before preparation of mRNAs and membrane fractions. The serum concentrations of bilirubin and bilirubin-glucuronide were determined separately using a test kit (Wako Pure Chemical, Tokyo, Japan) according to the alkaline azobilirubin method.

Northern blot analysis. Specific probes for Ntcp, oatp1, OCT1, cMOAT/MRP2, and MRP1 for Northern blot analysis were prepared from the sequence between bases 256 and 648 (393 bp) of rat Ntcp cDNA, the full length (2,200 bp) of rat oatp1 cDNA, bases 549–1,090 (542 bp) of rat OCT1 cDNA, bases 3,972–4,392 (421 bp) of rat cMOAT/MRP2 cDNA, and a 421-bp fragment (carboxy terminal ABC region) of rat MRP1 cDNA, respectively. The *mdr* probe prepared from the sequence between bases 3,187 and 3,599 (413 bp) of rat *mdr1b* cDNA could not distinguish *mdr* isoforms (*mdr1a*, -1b, and -2). The MRP3 and -6 probes were prepared from the sequence between bases 3,937 and 4,359 (423 bp) and between bases 3,875 and 4,296 (422 bp), respectively (11). Northern blot analysis was performed as described previously (14). Two or five micrograms of poly(A)⁺ RNA prepared from rat liver was separated on 0.8% agarose gel containing 3.7% formaldehyde and transferred to a nylon membrane (Biodyne; Pall, Glen Cove, NY) before fixation by baking for 2 h at 80°C . Blots were prehybridized in hybridization buffer containing $4\times$ SSC ($1\times$ SSC consists of 0.15 M NaCl and 0.15 M sodium citrate, pH 7.0), $5\times$ Denhardt's solution, 0.2% SDS, 0.1 mg/ml sonicated salmon sperm DNA, and 50% formamide at 42°C for 2 h. Hybridization was performed overnight in the same buffer containing 10^6 cpm/ml ^{32}P -labeled cDNAs prepared by a random primed labeling method. As a control to ensure equal gel loading of mRNAs, ^{32}P -labeled cDNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Clontech Laboratories, Palo Alto, CA) was used. The hybridized membrane was washed in $2\times$ SSC-0.1% SDS at room temperature for 20 min, followed by washing in $2\times$ SSC-0.1% SDS at 55°C for 20 min and then in $0.1\times$ SSC-0.1% SDS at 55°C for 20 min. The membranes were exposed to an imaging plate (Fuji Photo Film, Tokyo, Japan) for a period ranging from 3 h to overnight. The relative induction ratio was defined as the intensity of specific bands of the treated group to that of the control group after normalization for the intensity of GAPDH mRNA. The detection limit of band intensity was assumed to be 20% of the background radioactivity detected on an imaging plate.

Antibodies for Western blot analysis. Polyclonal anti-rat Ntcp antibody and EAG15 polyclonal anti-rat cMOAT/MRP2 antibody described previously were used in the present study. C219 monoclonal anti-mouse P-gp antibody was purchased from Centcor (Malvern, PA). The polyclonal anti-rat MRP3 antibody was raised against a maltose-binding protein fusion protein containing the 136 amino acids corresponding to bases 838–973 of the deduced rat MRP3 amino acid sequence. The pMAL-c2 expression vector (New England Biolabs, Beverly, MA) was used for the expression of the fusion protein. After purification by amylose resin, rabbits were immunized

with 250 μg of the fusion protein mixed with Freund's complete adjuvant (Sigma).

Membrane preparation. Crude liver membranes were prepared by the method of Gant et al. (6). Liver was homogenized in 5 vols of 0.1 M Tris·HCl buffer (pH 7.4) containing 1 $\mu\text{g}/\text{ml}$ leupeptin and pepstatin A and 50 $\mu\text{g}/\text{ml}$ phenylmethylsulfonyl fluoride with 20 strokes of a Dounce homogenizer. After centrifugation (1,500 g for 10 min), the homogenate supernatant was centrifuged at 100,000 g for 30 min. The precipitate was suspended in Tris·HCl buffer and again centrifuged at 100,000 g for 30 min. The crude membrane fraction was resuspended in 0.1 M Tris·HCl buffer (pH 7.4) containing the proteinase inhibitors using five strokes of a Dounce homogenizer. Plasma membranes were prepared as described previously (22). Briefly, livers were homogenized in *buffer A* containing 250 mM sucrose, 1 mM EGTA, and 5 mM HEPES (pH 7.4) with a Dounce homogenizer. After centrifugation of the homogenate at 1,500 g for 15 min, the resulting pellet was suspended in *buffer A* and Percoll (Pharmacia Biotech, Uppsala, Sweden), and centrifuged at 30,000 g for 60 min. The turbid layer was suspended in *buffer B* containing 250 mM sucrose and 50 mM Tris·HCl (pH 7.4) and centrifuged at 8,000 g for 10 min. The resulting pellet was suspended in *buffer B*, homogenized with a Dounce homogenizer, and layered over 38% sucrose. After centrifugation at 16,000 g for 70 min, the interfaces were collected, washed by centrifugation at 75,000 g for 30 min in *buffer B*, and suspended in *buffer B* using a Teflon homogenizer. All procedures were performed at 0–4°C. The crude and plasma membranes were stored at –80°C before being used for Western blot analysis. The membrane protein concentrations were assayed according to the method of Bradford (3).

Western blot analysis. Crude membrane (25 μg) or plasma membrane (20 μg) was dissolved in 10 μl of 0.25 M Tris·HCl buffer containing 2% SDS, 30% glycerol, and 0.01% bromophenol blue (pH 6.8), without boiling, and loaded onto a 7.5% SDS-polyacrylamide electrophoresis gel with a 4.4% stacking gel. Proteins were transferred electrophoretically to nitrocellulose membranes (Immobilon; Millipore, Bedford, MA) using a blotter (Trans-blot; Bio-Rad, Richmond, CA) at 15 V for 1 h. The membranes were blocked with Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 5% BSA for 1–2 h at room temperature. After being washed with TBS-T (3 \times 5 min), the membranes were incubated with the following concentrations of primary antibodies in TBS-T containing 5% BSA overnight at 4°C and then washed with TBS-T (3 \times 5 min): polyclonal anti-rat Ntcp serum (dilution 1:5,000) (16), EAG15 (1:10,000) (4), C219 (1 $\mu\text{g}/\text{ml}$), or polyclonal anti-rat MRP3 antibody (1:200). The membranes were allowed to bind ¹²⁵I-labeled sheep anti-rabbit IgG antibody for Ntcp and cMOAT/MRP2 or sheep anti-mouse IgG antibody for P-gp diluted 1:200 in TBS-T containing 5% BSA for 1 h at room temperature and then were placed in contact with an imaging plate for a period ranging from 3 h to overnight after being washed with TBS-T (3 \times 5 min). The intensity of specific bands was quantified from a standard curve using a BAS 2000 system (Fuji Photo Film, Tokyo, Japan). The relative induction ratio was defined as the ratio of the intensity of a specific band of the treated group to that of the control group.

Statistical analysis. Statistical analysis of differences between control and treated groups was performed by paired *t*-test. Statistical significance was taken as a *P* value of <0.05. Unless otherwise stated, all data represent means \pm SE of three to five animals.

RESULTS

Expression of transporters in drug-treated SD rats. The expression levels of transcripts of transporters after different treatments were examined by Northern blot analysis (Fig. 1 and Table 1). Oatp1 mRNA was significantly reduced by ANIT treatment and bile duct ligation to 47% ($P < 0.01$) and 33% ($P < 0.01$) of the control, respectively (Fig. 1 and Table 1). Ntcp mRNA was also reduced by treatment with PB, ANIT, and bile duct ligation to 84% ($P < 0.05$), 69% ($P < 0.05$) and 36% ($P < 0.01$) of the control, respectively (Fig. 1 and Table 1). OCT1 mRNA was reduced to 64% ($P < 0.01$) only by bile duct ligation (Fig. 1 and Table 1).

No significant change was observed in the level of cMOAT/MRP2 mRNA after any of the treatments, except for a slight reduction to 80% ($P > 0.05$) of the control after bile duct ligation (Fig. 1 and Table 1). P-gp mRNA was induced 4.8-fold ($P < 0.01$) and 8.2-fold ($P < 0.01$) by ANIT treatment and bile duct ligation, respectively (Fig. 1 and Table 1). MRP6 mRNA was significantly reduced by treatment with PB and ANIT and by bile duct ligation to 51% ($P < 0.05$), 69% ($P < 0.05$), and 65% ($P < 0.05$) of the control, respectively (Fig. 1 and Table 1). In contrast, MRP3 mRNA was induced >4.6-fold ($P < 0.01$), 5.9-fold ($P < 0.01$), and 6.1-fold ($P < 0.01$) after PB and ANIT treatment and bile duct ligation, respectively, whereas MRP3 mRNA was at marginal levels in untreated SD rats (Figs. 1 and 2 and Table 1).

The expression level of transporter proteins was determined by Western blot using membrane fractions and specific antibodies (Fig. 2 and Table 1). Both Ntcp protein (43 kDa) and cMOAT/MRP2 protein (190 kDa) were significantly reduced to 60% ($P < 0.05$) and 59% ($P < 0.05$) of the control after bile duct ligation, respectively (Fig. 2 and Table 1). In contrast, P-gp protein (170 kDa) was markedly induced by 10-fold ($P < 0.01$) and 22-fold ($P < 0.01$) after ANIT treatment and bile duct ligation, respectively (Fig. 2 and Table 1). PB treatment did not affect the expression of Ntcp, cMOAT/MRP2, and P-gp at all protein levels (Fig. 2 and Table 1). MRP3 protein (~170 kDa) was markedly induced by 16-fold ($P < 0.01$), 4.8-fold ($P < 0.05$), and 3.8-fold ($P < 0.05$) by bile duct ligation and PB and ANIT treatment, respectively (Fig. 2 and Table 1). The increase in transporter proteins by these treatments was well correlated with the increased mRNA levels (Figs. 1 and 2 and Table 1).

Relationship between serum levels of bilirubin and/or its glucuronides and MRP3 induction. Because the expression of MRP3, but not that of P-gp, was induced in EHBR, whose bilirubin and glucuronide levels are elevated in serum (Fig. 3 and Table 2) (11), we examined the hypothesis that these endogenous compounds could be associated with the induction of MRP3. Indeed, the increased expression of MRP3 by ANIT treatment and bile duct ligation (Figs. 1 and 2) was associated with the elevated plasma levels of bilirubin and its glucuronide.

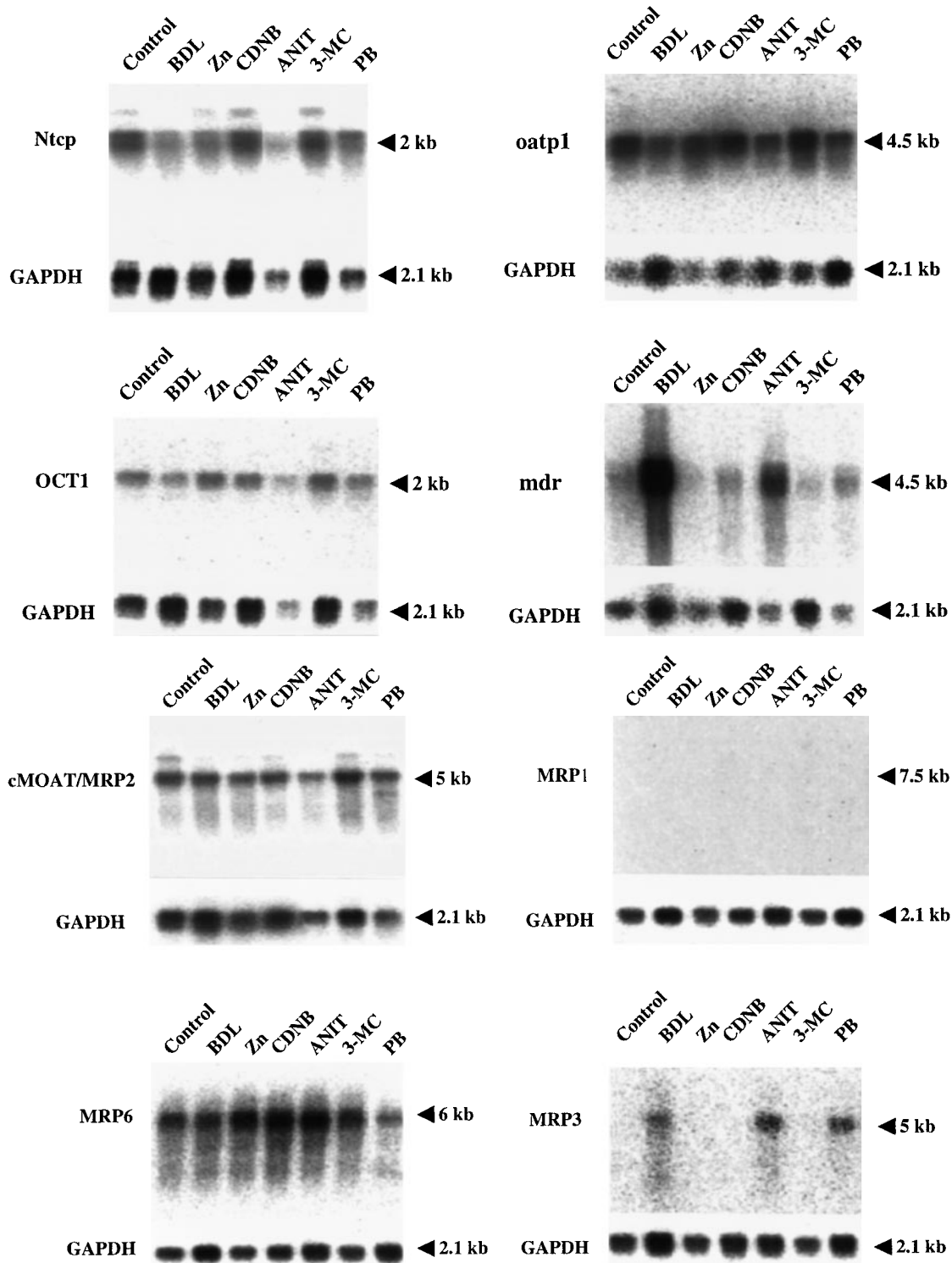


Fig. 1. Typical patterns of Northern blot analysis of mRNAs of transporters in male Sprague-Dawley (SD) rat liver after drug treatment. Hepatic expression of transporters was examined in male SD rats that received phenobarbital (PB; 80 mg/kg ip for 4 days), 3-methylcholanthrene (3-MC; 40 mg/kg ip for 3 days), α -naphthylisothiocyanate (ANIT; 250 mg/kg po, single dose), 1-chloro-2,4-dinitrobenzene (CDNB; 30 mg/kg ip for 3 days), zinc acetate (6 mg/kg iv for 3 days) and bile duct ligation (BDL; for 3 days). Two micrograms of mRNA were applied to each lane. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control for equal loading. Ntcp, Na⁺-taurocholic acid cotransporting polypeptide; oatp1, organic anion transporting polypeptide; OCT1, organic cation transporter; mdr, multidrug resistance transporter; cMOAT, canalicular multispecific organic anion transporter; MRP, multidrug resistance-associated protein.

Table 1. Effects of drug treatments on expression of mRNAs and proteins of transporters in SD rat liver

Treatment	Induction Ratio							
	oatp1	Ntcp	OCT1	MRP1	cMOAT/MRP2	MRP3	MRP6	P-gp (mdr)
Phenobarbital								
mRNA	0.93 ± 0.19	0.84 ± 0.06*	1.67 ± 0.28	ND	1.16 ± 0.15	>4.64 ± 0.85†	0.51 ± 0.12*	0.75 ± 0.18
protein		0.88 ± 0.10			1.40 ± 0.20	9.32 ± 1.23*		1.75 ± 0.36
3-MC								
mRNA	1.01 ± 0.24	1.15 ± 0.14	1.39 ± 0.19	ND	1.12 ± 0.03	ND	0.97 ± 0.23	1.35 ± 0.23
ANIT								
mRNA	0.47 ± 0.08†	0.69 ± 0.11*	1.11 ± 0.17	ND	1.03 ± 0.11	>5.92 ± 1.95†	0.69 ± 0.09*	4.83 ± 0.81†
protein		0.77 ± 0.12			0.99 ± 0.20	10.27 ± 1.15*		10.11 ± 0.81†
CDNB								
mRNA	1.07 ± 0.19	1.07 ± 0.06	1.11 ± 0.14	ND	1.17 ± 0.26	ND	0.76 ± 0.08	0.73 ± 0.20
Zn acetate								
mRNA	1.03 ± 0.30	0.97 ± 0.13	1.13 ± 0.27	ND	0.94 ± 0.23	ND	0.66 ± 0.12	1.05 ± 0.41
Bile duct ligation								
mRNA	0.33 ± 0.06†	0.36 ± 0.05†	0.64 ± 0.06†	ND	0.80 ± 0.15	>6.07 ± 1.89†	0.65 ± 0.08*	8.21 ± 1.01†
protein		0.60 ± 0.12*			0.59 ± 0.14*	30.73 ± 1.59†		22.03 ± 0.14†

Values are means ± SE for 3–5 rats. Liver samples were obtained from male Sprague-Dawley (SD) rats with phenobarbital (80 mg/kg ip for 4 days), 3-methylcholanthrene (3-MC; 40 mg/kg ip for 3 days), α -naphthylisothiocyanate (ANIT; 250 mg/kg po, single dose), 1-chloro-2,4-dinitrobenzene (CDNB; 30 mg/kg ip for 3 days), zinc acetate (6 mg/kg iv for 3 days), and bile duct ligation (for 3 days). Induction ratios of transporter gene expression were calculated by Northern blot analysis; induction ratios of transporter proteins were calculated by Western blot analysis. Typical patterns of Northern and Western blot analysis are shown in Figs. 1 and 2, respectively. ND, not detected. * $P < 0.05$, † $P < 0.01$ vs. control.

The expression of MRP3 mRNA in the liver of Gunn rats was also measured to examine whether unconjugated bilirubin was involved in the induction of this transporter. In Gunn rats, the serum concentration of

bilirubin, rather than its glucuronide, was markedly higher (5.5 mg/dl) than in normal rats (Table 2). MRP3 mRNA was found to be significantly increased to 3.4-fold ($P < 0.05$) of the control level (Fig. 4 and Table 2).

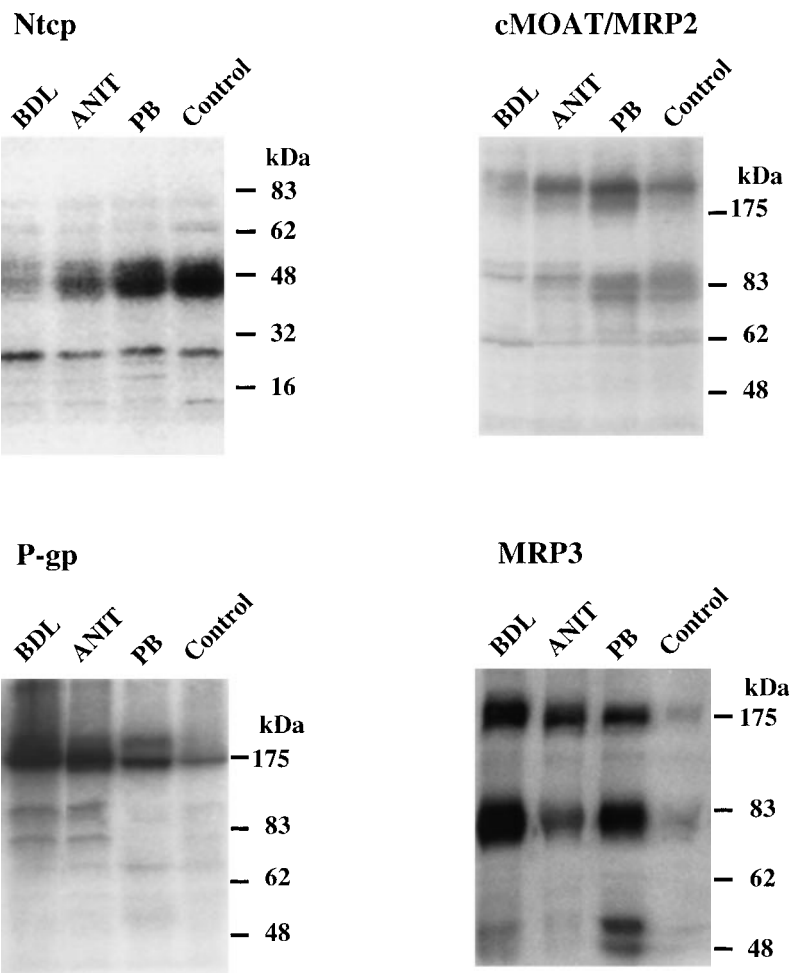


Fig. 2. Typical patterns of Western blot analysis of transporters in male SD rat liver after drug treatment. Hepatic expression of transporters was examined in male SD rats that received PB (80 mg/kg ip for 4 days), ANIT (250 mg/kg po, single dose) and BDL (for 3 days). Twenty-five micrograms of protein of crude membrane fraction prepared from liver were applied to each lane; P-gp, P-glycoprotein.

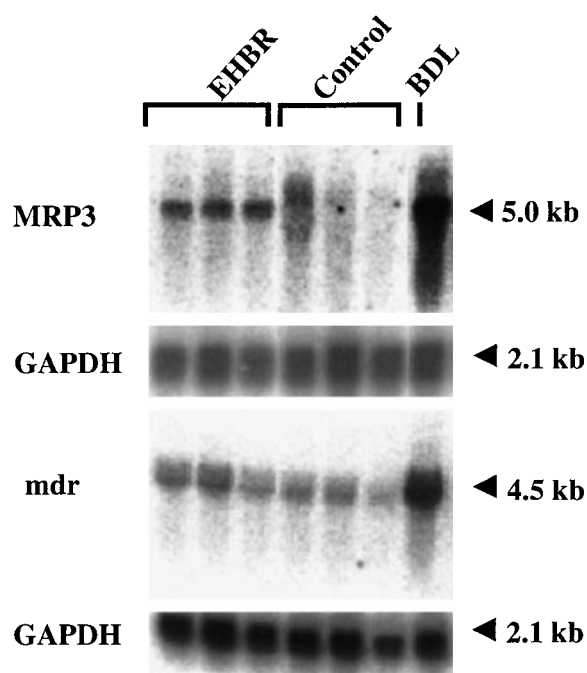


Fig. 3. Typical patterns of Northern blot analysis of mRNAs of P-gp and MRP3 in Eisai hyperbilirubinemic rat (EHBR) liver. Five micrograms of mRNA from normal rat (SD rat) liver (control), EHBR liver (EHBR), and liver from rats after BDL were hybridized with cDNA probes for rat P-gp, MRP3, and GAPDH as a control to ensure equal loading.

The expression of P-gp mRNA in Gunn rats was comparable to that in normal rats (Fig. 4 and Table 2).

The expression of MRP3 was then examined in SD rats after an intravenous injection of bilirubin. The serum bilirubin concentration rose markedly (~8 mg/dl) immediately after treatment and then declined rapidly to a normal level (~0.3 mg/dl) within 2 h after each treatment (Table 2). Serum bilirubin glucuronide was also elevated, ranging from 0.4 to 2 mg/dl. Hepatic MRP3 mRNA was induced 3.1-fold ($P < 0.05$) in liver from bilirubin-treated SD rats (Fig. 5 and Table 2). On the other hand, P-gp mRNA did not change significantly after bilirubin treatment, although marked induction was observed after bile duct ligation (Fig. 5 and Table 2). The protein level of MRP3, however, was not affected by bilirubin administration (Fig. 6).

DISCUSSION

In the present study, we determined the expression levels of hepatic transporters that are responsible for the uptake and export into the bile. In Northern blot analysis, the mRNA level of transporters was corrected by that of GAPDH. Because the expression level of GAPDH may also be affected by several kinds of treatments, the results may contain some bias. In the cholestatic rat liver, downregulation of Ntcp (7, 8) oatp1 (9), and cMOAT/MRP2 (39), along with induction of P-gp (35), have been reported. In the present study, the expression of Ntcp and oatp1 was significantly reduced by ANIT and bile duct ligation (Fig. 1 and Table 1). The reduced expression of Ntcp and oatp1 in the cholestatic liver is consistent with the previous observations (7–9,

Table 2. Relationship between serum bilirubin and bilirubin-glucuronide concentrations and expression of mRNAs and proteins of transporters in liver of EHBR, Gunn, and drug-treated SD rats

Treatment Group	Induction Ratio		Bilirubin, mg/dl	
	MRP3	P-gp (mdr)	Conjugated	Unconjugated
Control				
mRNA	1.00	1.00	0.20 ± 0.04	0.20 ± 0.04
protein	1.00			
Bilirubin treated				
mRNA	3.12 ± 0.55 ^d	0.82 ± 0.35	~0.41–2.25 ^a	~0.34–8.34 ^a
protein	1.60 ± 0.31			
Gunn rat				
mRNA	3.36 ± 0.62 ^d	1.09 ± 0.06	0.43 ± 0.04	5.46 ± 0.06
protein	11.84 ± 0.74 ^e			
EHBR				
mRNA	4.96 ± 0.83 ^d	0.98 ± 0.12	6.62 ^b	0.84 ^b
protein	10.57 ± 1.01 ^d			
ANIT treated				
mRNA	>5.92 ± 1.95 ^{c,e}	4.83 ± 0.81 ^{c,e}	6.48 ± 1.06	0.98 ± 0.07
protein	10.27 ± 1.15 ^{c,e}			
Bile duct ligation				
mRNA	>6.07 ± 1.89 ^{c,e}	8.21 ± 1.01 ^{c,e}	6.90 ± 0.69	1.20 ± 0.30
protein	30.73 ± 1.59 ^{c,e}			

Values are means ± SE for 3–5 rats. Induction ratios of P-glycoprotein (P-gp) and multidrug resistance-associated protein (MRP)3 gene expression were calculated by Northern blot and normalized with respect to glyceraldehyde-3-phosphate dehydrogenase expression. Typical patterns of Northern and Western blot are shown in Figs. 3–5 and Fig. 6, respectively. EHBR, Eisai hyperbilirubinemic rat. ^aFrom 1 rat; ^bfrom 2 rats; ^cfrom Table 1; ^d $P < 0.05$ vs. control; ^e $P < 0.01$ vs. control.

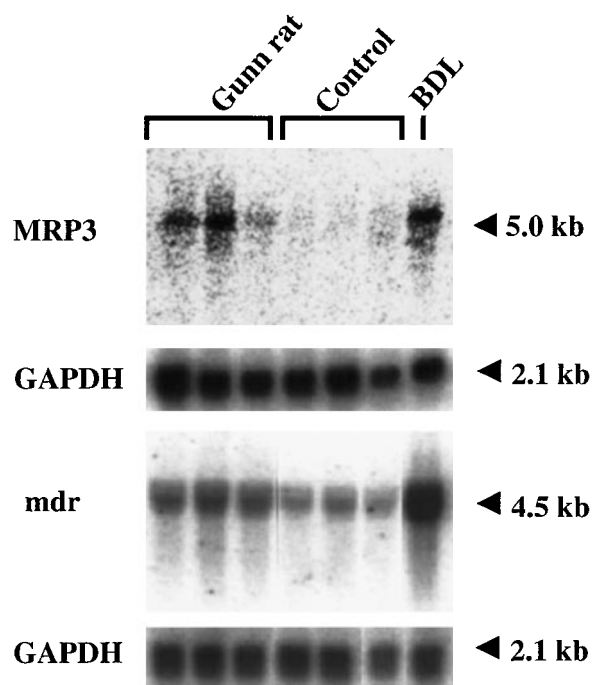


Fig. 4. Typical patterns of Northern blot analysis of mRNA of P-gp and MRP3 in Gunn rat liver. Five micrograms of mRNA from normal rat (Wistar rat) liver (control), Gunn rat liver, and liver of rats subjected to BDL were hybridized with cDNA probes for rat P-gp, MRP3, and GAPDH as a control to ensure equal loading.

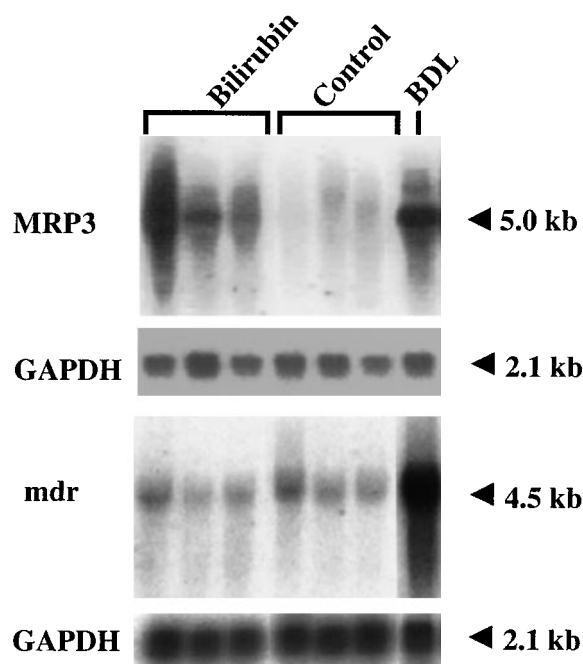


Fig. 5. Typical patterns of Northern blot analysis of mRNAs of P-gp and MRP3 in bilirubin-treated rat liver. Bilirubin (30 mg/kg iv) was administered 3 times/day for 2 days to SD rats. Five micrograms of mRNA from SD rat liver (control), liver of bilirubin-treated animals, and liver of animals subjected to BDL were hybridized with cDNA probes for rat P-gp, MRP3, and GAPDH as a control to ensure equal loading.

40). The molecular mechanism reported by Trauner et al. (40) may be related to the downregulation of Ntcp. At the present time, the mechanism for the reduced expression of oatp1 still remains to be clarified; although the sequence of the promoter region of human OATP was reported, the element controlling the transcription of oatp1 is different from that for OATP (27).

In contrast to these transporters, the expression of P-gp and MRP3 was markedly induced by ANIT treatment and by bile duct ligation (Fig. 1 and Table 1). The upregulation of P-gp under cholestatic conditions is consistent with previous findings (35). Moreover, MRP3 was also markedly induced by PB treatment (Fig. 1 and

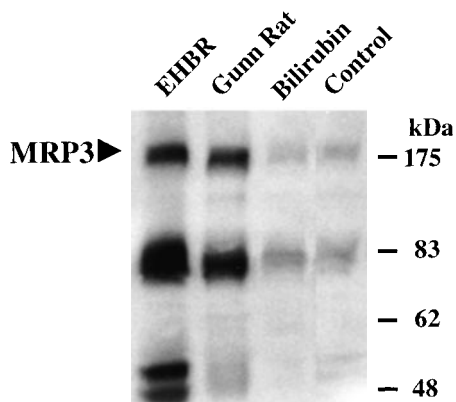


Fig. 6. Typical patterns of Western blot analysis of MRP3 in bilirubin-treated rat liver. Bilirubin (30 mg/kg iv) was administered 3 times/day for 2 days to SD rats. Twenty-five micrograms of protein of crude membrane fraction prepared from liver were applied to each lane.

Table 1). These observations contrast with the fact that neither of these treatments affected the mRNA levels of cMOAT/MRP2 and MRP6 (Fig. 1 and Table 1). The reduced cMOAT/MRP2 protein after the bile duct ligation is consistent with previous observations (39, 41). Although rat and human cMOAT/MRP2 is induced by PB in cultured cells in vitro (17, 19), PB was not effective in in vivo studies (Fig. 1 and Table 1). The same discrepancy was observed for 3-MC; 3-MC induced rat mdr1 in vitro (6) but not in vivo (Fig. 1 and Table 1). The difference between in vitro and in vivo inducibility may be ascribed to the difference in drug exposure, although the administered doses were sufficient to induce the hepatic expression of metabolic enzymes (2).

We could not distinguish the expression levels of mdr isoforms by using the present cDNA fragment and antibody. Using a much more sophisticated method, Schrenk et al. (35) indicated that both mdr1a and -1b, but not mdr2, were markedly induced by ANIT treatment and by bile duct ligation and that the expression level of mdr1b in the treated liver was much higher than that of mdr1a (35). Moreover, Vos et al. (41) reported the downregulated expression of sister of P-gp, the increased expression of mdr1b, and unchanged expression of mdr1a and mdr2 in endotoxin-treated rats. These results are consistent with the hypothesis that the marked induction of P-gp observed in the present study (Fig. 1 and Table 1) may represent the induction of mdr1b.

The present study clearly demonstrates that hepatic MRP3 expression is induced in normal rat liver after treatment with PB, ANIT, or bile duct ligation (Figs. 1 and 2), as with untreated EHBR. Because hyperbilirubinemia was associated with ANIT treatment and bile duct ligation, as with untreated EHBR (Table 2), it was hypothesized that bilirubin and/or its glucuronide may participate in the induction of MRP3. Although PB induced MRP3, the plasma concentration of bilirubin and its glucuronide remained normal after administration of PB (data not shown), suggesting that PB activates the transcription of MRP3 by another mechanism(s). It is possible that some nuclear receptors (such as the pregnane X receptor) may be involved in this induction, as reported for the cytochrome *P*-450 3A enzymes (21, 28). To examine the role of bilirubin and/or bilirubin glucuronides in the induction, we determined the expression of MRP3 in Gunn rats with higher serum bilirubin concentrations caused by a hereditary defect in the expression of enzymes responsible for the glucuronide conjugation of bilirubin (UDP-glucuronosyltransferase) (15). The expression of MRP3 was enhanced in Gunn rats, in which markedly increased bilirubin, with an increase in bilirubin glucuronide to a lesser extent, was observed (Fig. 4 and Table 2). In the same manner, after administration of bilirubin, marked increase in bilirubin, with a marginal increase in its glucuronides, was also observed (Table 2), which was associated with the increase in the mRNA level of MRP3. These results are consistent with the hypothesis that bilirubin and/or its glucuronide

may be related to the transcriptional control of MRP3. The previous finding that bilirubin is an endogenous substrate for aryl hydrocarbon receptor (37) is consistent with the hypothesis that bilirubin and/or bilirubin glucuronide may be related to the regulation of transcription. Although MRP3 is also induced in human liver under cholestatic conditions (24), the mechanism may be different from that for rat MRP3, because the xenobiotic responsible element is not located in the 5'-flanking region of human MRP3 (5). The protein level of MRP3, however, was not significantly affected by the administration of bilirubin (Fig. 6). To account for these data, we have to assume that factor(s) other than bilirubin and/or its glucuronide are also involved in the increased protein level of MRP3. The precise mechanism for the posttranscriptional regulation of MRP3 still remains to be clarified. In contrast to MRP3, P-gp was not induced in either Gunn rats or EHBR (Figs. 4 and 5 and Table 2), irrespective of the fact that the highest induction of P-gp was observed after ANIT treatment and bile duct ligation. The mechanism for P-gp induction under cholestatic conditions may be different from that of MRP3, because P-gp was not induced in either Gunn rats or EHBR (Figs. 4 and 5 and Table 2). These results agree with the report that *mdr1a* and *-1b* are not induced by bilirubin in primary cultured rat hepatocytes (35).

In conclusion, the results of the present study suggest that MRP3 expression is induced under cholestatic conditions. Together with the recent finding that MRP3 transports organic anions including glucuronide conjugates, it is possible that MRP3 can compensate for the impaired expression of cMOAT/MRP2 in EHBR. Although it was found that the increased mRNA level of MRP3 was associated with the increased concentration of bilirubin and/or its glucuronides in mutant rats and in SD rats that had undergone common bile duct ligation or ANIT treatment, administration of bilirubin resulted in the increase in the level of mRNA, but not the protein level, of MRP3. It was suggested that we must assume that factor(s) other than these physiological substances are also involved in the increased protein level of MRP3. Identification of this factor(s) still remains to be clarified.

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