THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 21, Issue of May 21, pp. 22250–22257, 2004
Printed in U.S.A.

Interactions between Hepatic *Mrp4* and *Sult2a* as Revealed by the Constitutive Androstane Receptor and *Mrp4* Knockout Mice*

Received for publication, December 23, 2003, and in revised form, March 3, 2004
Published, JBC Papers in Press, March 5, 2004, DOI 10.1074/jbc.M314111200

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The ABC transporter, Mrp4, transports the sulfated steroid DHEA-s, and sulfated bile acids interact with Mrp4 with high affinity. Hepatic Mrp4 levels are low, but increase under cholestatic conditions. We therefore inferred that up-regulation of Mrp4 during cholestasis is a compensatory mechanism to protect the liver from accumulation of hydrophobic bile acids. We determined that the nuclear receptor CAR is required to coordinately up-regulate hepatic expression of Mrp4 and an enzyme known to sulfate hydroxy-bile acids and steroids, Sult2a1. CAR activators increased Mrp4 and Sult2a1 expression in primary human hepatocytes and HepG2, a human liver cell line. Sult2a1 was down-regulated in *Mrp4*-null mice, further indicating an inter-relation between *Mrp4* and *Sult2a1* gene expression. Based on the hydrophilic nature of sulfated bile acids and the Mrp4 capability to transport sulfated steroids, our findings suggest that Mrp4 and Sult2a1 participate in an integrated pathway mediating elimination of sulfated steroid and bile acid metabolites from the liver.

Bile acid homeostasis is the result of a balance between hepatic uptake of secondary bile acids, efflux of conjugated bile acids, and bile acid biosynthesis. The farnesoid X receptor (FXR)[1] contributes to bile acid homeostasis by regulating bile acid biosynthesis and controlling the expression of the major bile salt efflux transporter, BSEP/SPGP, located at the canalicular membrane (1–5). FXR is activated by a number of bile acids, including cholic acid, chenodeoxycholic acid, and lithocholic acid (6–8). Under normal conditions, there is a balance between secondary bile acid uptake and canalicular efflux by BSEP and Mrp2 of monoanionic and dianionic conjugated bile acids, respectively, and bile acid synthesis (9, 10). In the absence of liver pathology, little or no bile acid is detected in the serum; however, in cases of liver failure, bile duct obstruction, or production of excess bile acid from dietary sources, there is a dramatic increase in the serum concentration of bile acids, many of which exist as sulfated conjugates. These dianionic bile acids are potentially cholestatic. They are not substrates for BSEP (9, 10) and can be removed by basolateral transporters from the liver. In the Fxr knockout mice, absence of Fxr mimics intrahepatic cholestasis because serum bile acid levels increase. This condition is exacerbated when mice are challenged with bile acids or when the common bile duct is ligated (1). Under these conditions, the Mrp4 level is dramatically induced in the livers of $Fxr^{-/-}$ mice, whereas other ABC transporters (*e.g.* Mrp3) are only modestly affected (11). Indeed, $Mrp3^{-/-}$ mice are not detectably affected in transport of bile salts from the liver under cholestatic conditions (12). Collectively, these findings suggested that, under cholestatic conditions, Mrp4 mediates bile acid transport from the liver. Notably, during cholestasis, sulfated bile acids increase in the serum (9, 13). Our demonstration that sulfated bile acids (taurolithocholate, glycolithocholate, and lithocholate (LCA)) interact with Mrp4 and the fact that serum bile acids are sulfated under cholestatic conditions (9, 13, 14) led us to hypothesize that a bile acid/steroid-preferring sulfotransferase would also participate in this process of removing bile acids from the liver. To test this hypothesis we specifically examined the relation between Mrp4 and Sult2a1, the sulfotransferase most highly expressed in the liver (15).

Sulfation of steroids, xenobiotics, and bile acids occurs by a phase II conjugation reaction catalyzed by sulfotransferases. It is known that rhesus monkeys defective in lithocholate sulfation activity accumulate LCA and this causes hepatotoxicity (16). Sult2a1 is a cytosolic enzyme that preferentially transfers a sulfate moiety to hydroxysteroid substrates such as LCA (17). Sult2a1 is highly expressed in the liver and readily adds a sulfate to cholestatic bile acids (*e.g.* LCA) and steroids (*e.g.* DHEA, Ref. 15). This suggests that Sult2a1 could play an important role in the elimination of steroids and bile acids.

It has recently been observed that CAR is up-regulated in the

* This work was supported by National Institutes of Health Research Grants CA77545, GM60904, ES058571, GM60346, U01-GM61393, P30 CA21745, a Cancer Center support grant, the American Lebanese Syrian Associated Charities (ALSAC), and Dutch Cancer Society Grants NKI 2001-2473 and NKI 1998-1764. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: FXR, farnesoid X receptor; ABC, ATP-binding cassette; CAR, constitutive androstane receptor; DR, direct repeat; ER, everted repeat; IR, inverted repeat; CYP, cytochrome P450; DHEA, dehydroepiandrosterone; LCA, lithocholate; MRP, multidrug resistance protein; PB, phenobarbital; PXR, pregnane X receptor; SULT, cytosolic sulfotransferase; TCPOBOP, 1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene; RT-PCR, reverse transcriptase PCR; GFP, green fluorescent protein; Gpdh, glyceraldehyde-3-phosphate dehydrogenase; BCRP, breast cancer-resistance protein; BSEP, bile salt export protein; SPGP, sister of P-glycoprotein; PMEA, 9-(2-phosphonylmethoxyethyl)adenine.

TABLE I
DNA sequence of the oligonucleotides used in these studies

Gene[a]	Primer sense	Primer antisense
Cyp3a11	GCCTGGATTCTAAGCAGAAGC	CAGAGCCTCATCGATCTCATC
Cyp2b10	AGGAGAAGTCCAACCAGAACG	GGATGACTGCATCTGAGTATG
Cyp2B6	TCCTTTCTGAGGTTCCGAGA	TCCCGAAGTCCCTCATAGTG_
Cyp3A4	CCAAGCTATGCTCTTCACCG	TCAGGCTCCACTTACGGTGC_
Mrp2	GTCATCACTATCGCACACAG	TTCTACAGGGTGGTTGAGAC
Mrp3	CGCTCTCAGCTCACCATCAT	GGTCATCCGTCTCCAAGTCA_
Mrp4	GGTTGGAATTGTGGGCAGAA	TCGTCCGTGTGCTCATTGAA_
MRP4	CAGTACCTCAAAGCTGCAAGTC	CCCAGTATGAAAGCCACCAA
Mrp5	CTGGCTGGAGGACCTGTTGTT	GTCACCACACTGGCGATCACT
Bcrp	CCATAGCCACAGGCCAAAGT	GGGCCACATGATTCTTCCAC_
Sult2a1	CTGGCTGTCCATGAGAGAAT	GGCTTGGAAAGAGCTGTACT_
SULT2A1	GATCACGAGGTCAGGAGACT	CTATAGGCGTGCACCACCAT
Sult1a2	CTCCACCTCGATGAACAGAAC	TAGCCAGATCCAGACCTGAGT
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA

[a] The gene names in all capital letters indicate nucleotide sequences derived from their human genes.

livers of $Fxr^{-/-}$ animals (18).[2] The increased expression of this nuclear receptor might account for the up-regulation of the ABC transporter, Mrp4 in the $Fxr^{-/-}$ animals (11). Here we show that the nuclear receptor CAR orchestrates the coordinate up-regulation of Mrp4 (a transporter of sulfated steroids and bile acids) and Sult2a1 (a transferase that preferentially sulfates, steroids and bile acids). This process of coordinate Mrp4 and Sult2a1 up-regulation is conserved from mouse to man. Furthermore, we show that deletion of the *Mrp4* gene results in a decreased expression of hepatic Sult2a1, a finding compatible with the idea that sulfation and transport of sulfated compounds are inter-related to form a pathway for excretion of sulfated steroids and bile acids.

EXPERIMENTAL PROCEDURES

Materials—All compounds were purchased from Sigma unless otherwise indicated. Bis-pivaloyloxymethyl-PMEA and [^{3}H]Bis-pivaloyloxymethyl-PMEA were purchased from Moraveck Biochemicals (Brea, CA) and TCPOBOP (1,4-bis[2-(3,5-dichloropyridyloxy)]benzene) was a gift from Dr. Stephen Safe. pMSCV-Neo and pGL2 basic were purchased respectively from Clontech (Palo Alto, CA) and Promega (Madison, WI); pEQPAM3-E and pRD118 are gifts from Dr. Elio Vanin (St Jude Children's Research Hospital). The anti-Cyp3A and Cyp2b antibodies have been reported previously (11). The anti-BCRP and BSEP/SPGP antibody have been previously described (5, 11, 19).

Animals and Cell Lines—C57BL/6 mice (8–10 weeks old) (Charles River Laboratory) were administered phenobarbital (PB, 100 mg/kg) or TCPOBOP (0.3 mg/kg) by intraperitoneal injection with corn oil as vehicle (3 mice per group). Animals were sacrificed 48 h after injection with CO_2. Livers were removed and snap-frozen in liquid nitrogen for molecular biology studies or were fixed in 10% buffered formalin and embedded in paraffin for immunohistochemistry studies. The derivation of the Mrp4 knockout mice will be described elsewhere.[3] HepG2 and Hepa1c1c7 cell lines were maintained in MEM-α supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% glutamine. 293T and NIH3T3 cell lines were maintained in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin, and 1% glutamine. Primary human hepatocytes were obtained and cultured as described elsewhere (11).

Retroviral Transduction—We used an MSCV-VP16-IRES-GFP that was constructed by the insertion of a synthetic polylinker with appropriate restriction sites, a VP16 transactivator domain, IRES and GFP instead of the neomycin resistance gene in the original plasmid (pMSCV-Neo). A recombinant retrovirus vector carrying human CAR (MSCV-VP16-CAR-IRES-GFP) was constructed according to a previously reported procedure (15). In brief, CAR was excised from pcDNA3-hCAR by using NotI and SalI and inserted into the NotI and XhoI sites of the MSCV-VP16-IRES-GFP vector. The constructs were co-transfected with pEQPAM3-E helper and pRDII8 envelope plasmids into the 293T cell line. The recombinant retrovirus was grown in 293T cells, purified, and titered in NIH3T3 cells by conventional protocols.

Hepa1c1c7 and HepG2 cells were transduced with either MSCV-VP16-CAR-IRES-GFP or MSCV-VP16-IRES-GFP. Briefly, 10^5 cells were plated on 60-mm tissue culture dishes, the medium was replaced with the retroviral supernatant supplemented with 10 μg/ml polybrene, and the cells were incubated overnight at 37 °C in a 5% CO_2 humidified atmosphere. This transduction was repeated two more times every 24 h. The GFP-positive-transduced cells were identified by fluorescence-activated cell sorting, isolated by sterile sorting, and the culture was expanded. For both cell types, the retention of the insert was monitored periodically by assessing GFP by immunofluorescence and expression of CAR targets by Western blot.

Isolation of RNA, Semiquantitative RT-PCR, and Real-time RT-PCR—Total RNA was extracted by using TRIzol reagent (Invitrogen). Specific mRNAs were analyzed by semiquantitative RT-PCR and real-time PCR using the Abi Prism 7900HT (Applied Biosystems, Foster City, CA). The following genes were amplified as described previously: *Cyp3a11*, *Cyp2b10*, *Mrp2*, *Mrp3*, *Mrp4*, *Mrp5*, *Bcrp*, *Sult2a1*, *Sult1a2*, *Sult2b*, and *Gapdh* (11). The sequence of the primers is summarized in Table I.

Western Blot—The preparation of plasma membrane fractions and Western blot analysis were described elsewhere (5, 11). Protein quantity was estimated by using the Bio-Rad protein assay with bovine serum albumin as standard. Crude membrane proteins or total homogenate (100 μg) were separated on 7.5 or 10% polyacrylamide gels and immunoblotted with primary antibody (SPGP/BSEP Mrp4, CYP2B, CYP3A, and actin) and appropriate secondary antibody coupled with peroxidase. The blot was developed with the ECL detection system (Amersham Biosciences). The mouse Mrp4 antibody was developed to a peptide derived from the predicted sequence of the C terminus of mouse Mrp4 (20). This antibody did not cross-react with cells expressing Mrp1, Mrp2, BCRP, MDR1, Mdr1a, Mdr1b, and SPGP.

Immunohistochemistry—The expression of Mrp4 and Bcrp were studied in formalin-fixed paraffin-embedded tissue sections with the anti-MRP4 and anti-BCRP antibodies described above. Deparaffinized slides were heated in a pressure cooker for 2 min at maximum pressure in 0.1 M citrate buffer, pH 6 (conditions for optimal antigen detection). Slides were treated with 3% hydrogen peroxide in water for 5 min and then with avidin and biotin blocking solutions for 15 min each (Dako, Carpinteria, CA). Sections were labeled by the immunoperoxidase technique as follows. Slides were incubated for 30 min at room temperature with rabbit anti-Mrp4 antibody diluted in 5% goat serum, washed three times with phosphate-buffered saline/Tween-20 (0.05%), and then incubated with a biotin-labeled anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA). A tertiary complex of streptavidin horseradish peroxidase (Dako) was used to develop the AEC chromogen or DAB diaminobenzidine chromogen (Dako). The protocol was similar for BCRP detection. Optimum antigen recovery for BCRP was obtained as described above but with 0.1 M Tris-HCl, pH 9.5 buffer. All slides were counterstained with hematoxylin.

Transient Transfection and Reporter Gene Assay—HepG2 cells were maintained in MEMα medium supplemented with 10% fetal bovine serum. Cells were plated in a 24-well plate (3×10^5 cells per well). After 24 h, they were transfected overnight with 1 μg of mCAR or MSCV VP16-CAR plasmids and 1 μg of *Mrp4* promoter-pGL2 Basic (Promega) by using FuGENE-6 (Roche Applied Science). The next day, the cells

[2] J. Schuetz and M. Assem, unpublished observations.
[3] M. Leggas, M. Adachi, G. Scheffer, D. Sun, P. Wielinga, G. Du, K. Mercer, Y. Zhuang, J. Panetta, B. Johnston, R. Scheper, C. Stewart, and J. Schuetz, manuscript in preparation.

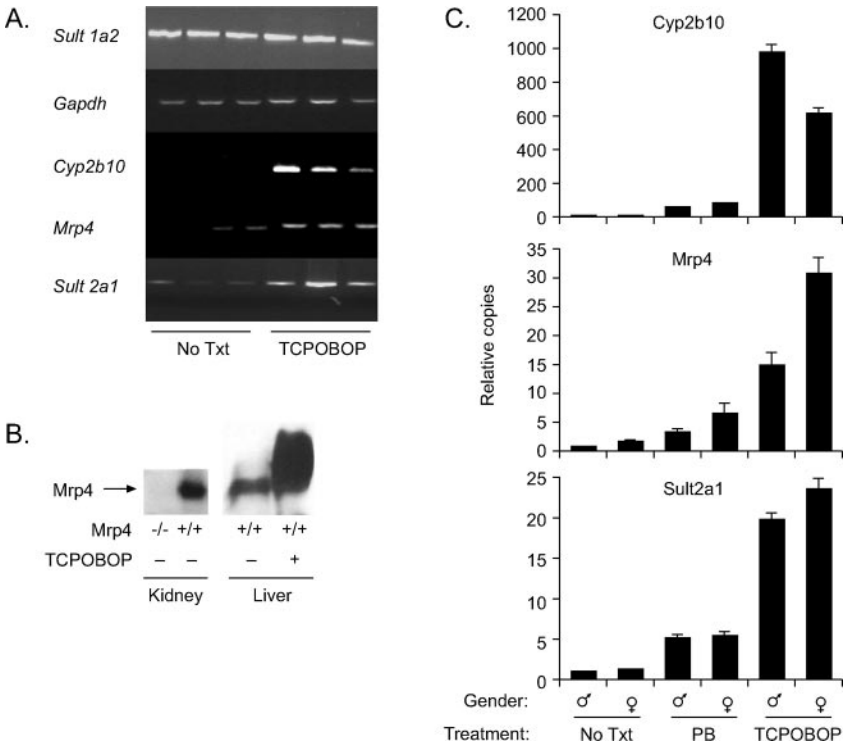

FIG. 1. **Hepatic Mrp4 and Sult2a1 are up-regulated by the CAR ligand, TCPOBOP.** Total liver RNA was extracted from male and females mice treated with TCPOBOP 0.3 mg/kg or phenobarbital 100 mg/kg. *A*, semiquantitative RT-PCR analysis of the indicated mRNAs. Gapdh was used as an internal control. Three animals were used per treatment group. *B*, Western blot analysis of crude membrane homogenates for kidneys from Mrp4$^{+/+}$ and Mrp4$^{-/-}$ animals and livers from Mrp4$^{+/+}$ animals treated with TCPOBOP or vehicle. Blots were developed using a rabbit anti-mouse Mrp4 as described under "Experimental Procedures." *C*, real time RT-PCR analysis of Mrp4, Sult2a1, and Cyp2b10 expressions (normalized to Gapdh level) in mouse liver after treatment with either with PB or TCPOBOP. *Txt*, treatment

were washed twice and harvested, lysed, and centrifuged at 10,000 × *g*. Luciferase activity was assayed in an aliquot of supernatant as directed by the manufacturer of the automated luminometer (Opticomp Luminoter). In most cases the results were expressed as luciferase activity per microgram of protein; however, in some cases an additional promoter driving β-galactosidase was included as a control. Regardless, the results of these assays were qualitatively similar.

In Vivo Transcription Assays—C57/B6 mice matched for age and body weight were rapidly injected via the tail vein with linearized plasmid DNA in a total volume that did not exceed 10% of body weight. Seven hours later, animals were anesthetized with a 0.2-ml mixture of ketamine and xylene and given an intraperitoneal injection of firefly D-luciferin as described elsewhere (21). Optical images were obtained with a Xenogen Imaging System (www.xenogen.com/demo4.html). The images were quantitatively analyzed by IGOR Pro 4.0 image analysis software.

PMEA Uptake—To measure Mrp4 function we determined the uptake of bis (pivaloyloxymethyl) PMEA, a PMEA pro-drug, as previously described (22, 23). Briefly, PMEA uptake was measured in cells incubated with various concentrations of ^{3}H-labeled bis(pivaloyloxymethyl) PMEA for 24 h at 37 °C. Monolayers were washed three times with ice-cold phosphate-buffered saline to remove any free [^{3}H]PMEA. Cells were lysed on an orbital shaker using 1.0 ml of 0.5 N NaOH in each well. The intracellular [^{3}H]PMEA was assayed by liquid scintillation counting and normalized to the total protein in each well. Protein quantity was estimated with the Bio-Rad protein assay. Bovine serum albumin was used to prepare standard curves (Bio-Rad).

Affymetrix Oligonucleotide Array Analysis—Total RNA was isolated with TRIzol. Briefly, first-strand synthesis was accomplished by using Superscript II reverse transcriptase (Invitrogen) and T7-oligo(dT) primer. Second strand was synthesized by using DNA polymerase I and RNase H. The double-stranded complementary DNA was used as a template for *in vitro* transcription with T7 RNA polymerase in the presence of biotin-labeled UTP and CTP (Enzo Diagnostics Inc., Farmingdale, NY). Labeled cRNA (10.0 μg) was fragmented by heat and ion-mediated hydrolysis and hybridized to the U133 oligonucleotide array (Affymetrix) containing ~32,000 full-length annotated and EST probe sets. After several washes, the arrays were stained with phycoerythrin-conjugated streptavidin (Molecular Probes, Eugene, OR) and the fluorescence intensity was determined using a laser confocal scanner (Hewlett-Packard, Palo Alto, CA). Data were analyzed by using Affymetrix GeneChip software, Affymetrix Microarray Suite Version 5.0 and Affymetrix Microarray Suite 4.1 (Affymetrix).

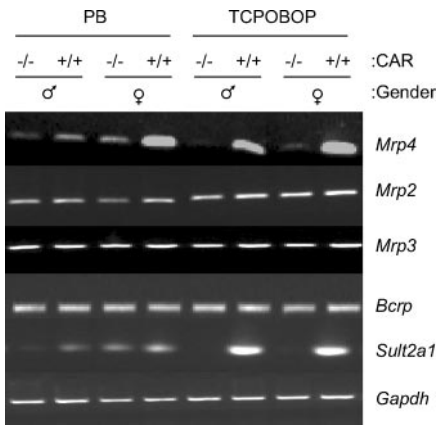

FIG. 2. **Up-regulation of hepatic Mrp4 and Sult2a1 mRNA is dependent upon CAR expression.** Total RNA was extracted from the livers of CAR$^{-/-}$ and CAR$^{+/+}$ mice treated with TCPOBOP or PB. Genes targets analyzed by semiquantitative RT-PCR using primers indicated in Table I under conditions described previously (11).

RESULTS

Hepatic MRP4 and Sult2a1 Are Up-regulated by CAR Activators—We previously demonstrated that hepatic Mrp4 is up-regulated as a compensatory response when *FXR*-null mice are challenged with cholic acid and also after bile duct ligation (11). Mrp4 up-regulation was attributed to hepatic bile acid accumulation, a consequence of strongly reduced expression of the major hepatic bile acid transporter, Spgp/Bsep (1–6). We evaluated whether CAR mediated Mrp4 up-regulation, because preliminary studies indicated that CAR expression increased in the *FXR*-null mice challenged with cholic acid,[2] a finding that is consistent with a recent report suggesting that CAR is a bile acid sensor (18). We treated mice with the prototypical CAR ligand, TCPOBOP (Fig. 1). Semiquantitative RT-PCR (Fig. 1*A*) found increased Mrp4 mRNA levels in their livers. Because we recently demonstrated that DHEA-s is a high affinity Mp4 substrate (14), we also tested Sult2a1. Indeed, hepatic Sult2a1 expression was increased by TCPOBOP treat-

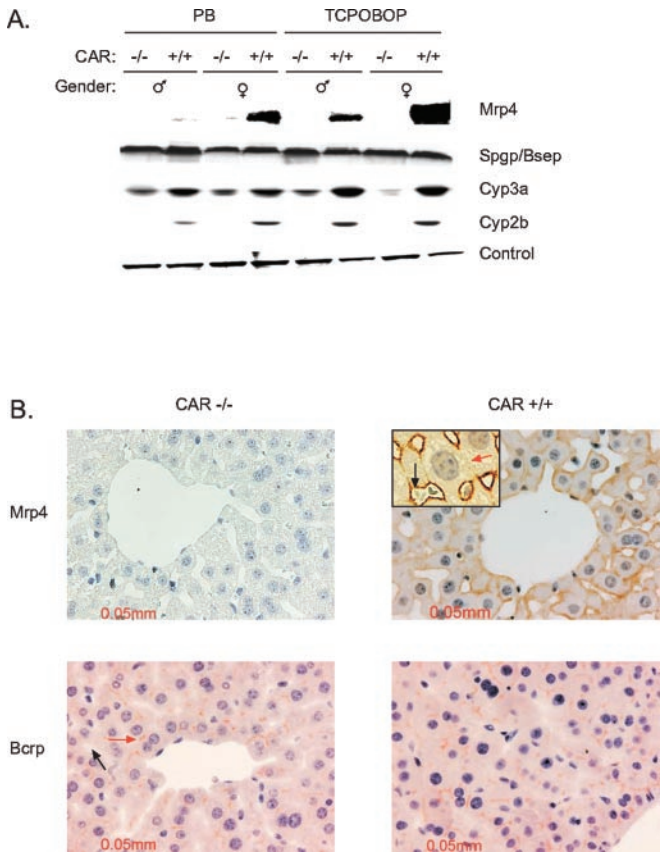

FIG. 3. **Increased hepatic Mrp4 expression by TCPOBOP requires CAR expression.** $CAR^{-/-}$ and $CAR^{+/+}$ mice were treated with a single dose of TCPOBOP or PB and livers harvested 48 h later. *A*, immunoblot analysis of crude liver membrane or microsomes with antibodies for Mrp4, Spgp/Bsep, Cyp3a, and Cyp2b (11). *B*, immunohistochemical analysis of hepatic Mrp4 and Bcrp expression in TCPOBOP-treated $CAR^{+/+}$ and $CAR^{-/-}$ mice. The *black arrows* indicate the predominant sinusoidal staining of Mrp4, whereas the *red arrow* shows the canaliculus and that Bcrp primarily stains the canalicular membrane.

ment whereas expression of the phenol-preferring sulfotransferase, Sult1a2 (24, 25) was not; a finding indicating that CAR activation does not generally up-regulate sulfotransferases (Fig. 1*A*). The increase in Mrp4 mRNA following exposure to TCPOBOP was accompanied by an increase in Mrp4 protein (Fig. 1*B*) detected with a new Mrp4 antibody (see "Experimental Procedures"). Fig. 1*C* shows that the CAR activator, phenobarbital (PB) also results in elevated levels of Mrp4 and Sult2a1 RNA, although induction by PB was less (4–6-fold) than by TCPOBOP (15–30-fold). Mrp4 and Sult2a1 were induced to a similar degree as the known CAR target Cyp2b10 (Fig. 1*C*) and Cyp3A11 (results not shown), supporting a role for CAR in activating Mrp4 and Sult2a1 expression.

CAR Is Required for Mrp4 and Sult2a1 Up-regulation—To directly test the role of CAR in Mrp4 and Sult2a1 regulation *in vivo*, we assessed their level of expression in wild-type and *CAR* nullizygous mice after treatment with either PB or TCPOBOP (26, 27). As shown in Fig. 2, both Mrp4 RNA and Sult2a1 RNA are unchanged by PB and TCPOBOP in the $CAR^{-/-}$ mice at concentrations that substantially increased their levels in the $CAR^{+/+}$ animals; notably, the Mrp4 induction was more pronounced in the female mice. No increase was observed in the expression of other ABC transporters that are capable of transporting sulfated steroids (Fig. 2), *i.e.* Bcrp, Mrp2, and Mrp3 (28, 29, 30, 31). In addition, no obvious changes in the level of Mdr2, the canalicular phospholipid flippase or Mdr1a at Mdr1b were detected (not shown). A previous study suggested Mrp2 was a

TABLE II
Nubiscan identification of CAR response elements in Mrp4 enhancer

The search program at www.nubiscan.unibas.ch was used. The plus indicates the + strand, and the − indicates the opposite strand. The numbers indicate the position relative to the transcription start site.

Element	Sequence	Position	Score
ER6	TGGCCTGCAGGTGGAACA	−464(+)	0.72
DR3	AGGTCACTTGGTCCA	−4962(−)	0.79
DR4	GGGCCAGAAAAGGCCA	−5470(−)	0.74
DR2	AGAACAGAAGTTCA	−6003(+)	0.86
ER6	TGCTCTGCTTTCAGAACA	−6273(+)	0.76
ER6	TGTCCTCAGGGCAGAGCA	−6732(+)	0.81
DR4	AGAGCAGCCAAGGTCA	−7286(−)	0.86
DR4	AGGTCAATAAAGCACA	−7296(−)	0.87
DR5	GGTTCAGGCAGAGGTCA	−8441(+)	0.89
DR5	AGGCCAACTATAGTACA	−9005(+)	0.84
DR2	GGGGCATGAGATCA	−9084(+)	0.78
DR5	AGCACAGTCTAGGATCA	−9774(−)	0.74
DR5	GGTGCACATGGAGGTCA	−9927(−)	0.81
DR1	AGGTCAGAGGACA	−9938(−)	0.95

CAR target. It is possible that our inability to detect CAR mediated up-regulation of Mrp2 reflects either the concentration of CAR activator or the fact that our studies were conducted in the whole animal, whereas Mrp2 activation was previously observed in hepatocyte cultures (32). The induction of Mrp3 by CAR appears controversial. We find no induction after a 48-h treatment whereas one previous study has reported rodent hepatic Mrp3 to be a CAR target (33) whereas, two other studies did not find a major role for CAR in activation of Mrp3 (34, 35). This discrepancy might be due to the duration of treatment. We conclude that both Mrp4 and Sult2a1 require CAR for up-regulation by CAR ligands and that Mrp4 is the only one affected by CAR ligands among the hepatic ABC transporters capable of transporting sulfated steroids or bile acids.

Hepatic Mrp4 Is Expressed in a Zonal Fashion and Localized to the Sinusoidal Membrane—Next we evaluated if Mrp4 protein was up-regulated by PB or TCPOBOP in CAR knockout and wild-type mice. We also determined if Spgp/Bsep expression changes, because PB-like inducers are known to enhance bile acid-dependent biliary excretion (36). Mrp4 protein was induced by both PB and TCPOBOP, with a greater induction in females (Fig. 3*A*). We confirmed that these agents activated CAR because both Cyp3a11 and Cyp2b10 (both CAR targets) required CAR for up-regulation by these agents. It is notable that Spgp/Bsep expression is unaltered by CAR activation as neither PB nor TCPOBOP increased its expression, a finding suggesting Bsep/Spgp up-regulation is not necessary for PB-like agents to increase biliary excretion. These findings are consistent with the reported inability of PB to up-regulate BSEP/SPGP in primary human hepatocytes (11).

Having demonstrated that the Mrp4 protein increases after treatment with TCPOBOP, we determined whether the additional Mrp4 is also routed to the sinusoidal membrane, the reported location of Mrp4 in the human liver (37). We prepared liver sections from *CAR*-null and *CAR* wild-type mice that were treated with TCPOBOP. Mrp4 was prominently labeled in the sinusoidal membranes only in animals that retained wild-type CAR (Fig. 3*B*). Lower magnification revealed that in $CAR^{+/+}$ animals, the level of Mrp4 expression increased dramatically from zone 1 (negligible) to intense staining in zone 3 (not shown). Analysis of Bcrp expression in a serial liver sections from the same animals revealed that the level of the ABC transporter, Bcrp (38) is unchanged by TCPOBOP treatment (consistent with our RT-PCR results, Fig. 2) and that its localization is still predominantly canalicular (Fig. 3*B*). Thus, the basolateral localization of Mrp4 in humans and mice allows it to transport substrates such as conjugated bile acids into the blood.

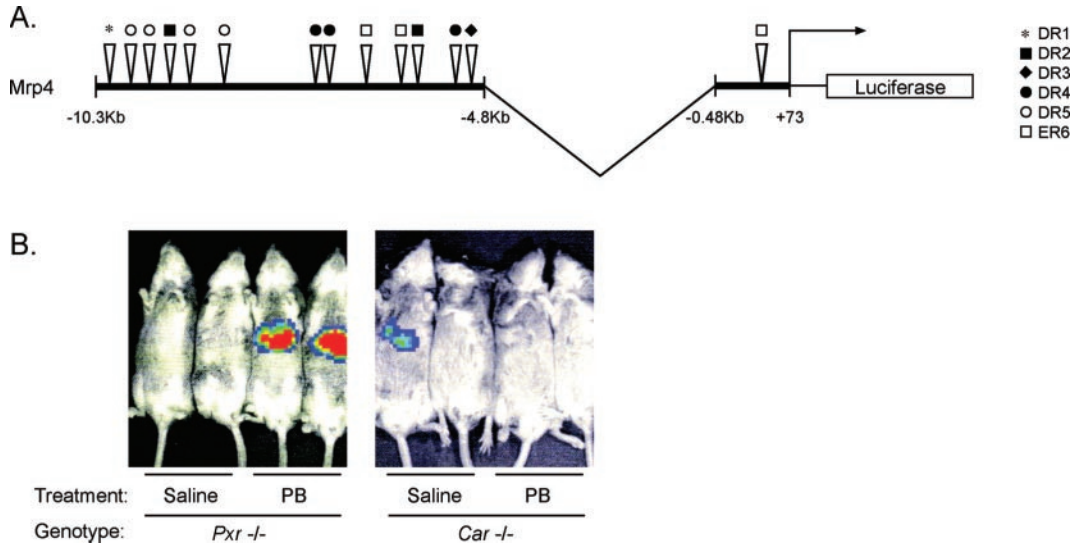

FIG. 4. **CAR is required for activation of the *Mrp4* promoter *in vivo*.** *A*, diagram of the *Mrp4* promoter-luciferase construct showing nuclear receptor sites that were scored as potential response elements using Nubiscan (www.nubiscan.unibas.ch). The Mrp4 sequence was obtained from a BAC clone we previously described (20). *B*, CAR and PXR knockout mice were injected via the tail vein with the linearized *Mrp4* promoter-pGL3 basic construct and analyzed using a Xenogen imaging system after 8 h. The photographs show a representative experiment with two animals per treatment that is representative of an experiment that was repeated three times. The minimum luminescent values representing were ~200 counts (*no visible color*) and the maximum value (*intense red*) was a value of >100,000. The *blue color* represents a luminescent value that is less than 1000. The average fold activation was 560 ± 73.

The Mouse Mrp4 Gene Is Flanked by Multiple NRE Binding Sites—An evaluation of the *Mrp4* 5′-flanking region revealed very few nuclear receptor response elements (DR1–5, ER6) within the first 5-kb upstream from the Mrp4 transcription start site. Other genes that are CAR targets (*Cyp2B6*, *Cyp3A4*, and *MDR1*) have distal (>7 kb) upstream nuclear receptor binding sites (39). Therefore, we used a nuclear receptor site computer scanning algorithm (Nubiscan, www.nubiscan.unibas.ch) to probe for putative upstream nuclear receptor binding sites further upstream than 5 kb of the *Mrp4* gene. We identified a region with several nuclear receptor sites preferred by CAR (*i.e.* DR3, DR4, and ER6) (Table II). The region between −5 and −10 kb upstream was amplified by PCR using a *Mrp4* BAC clone as template (20) and was appended to the minimal promoter of *Mrp4* driving a luciferase reporter gene (Fig. 4*A*). To measure the role of CAR in activation of the *Mrp4* promoter by PB *in vivo*, we introduced this luciferase reporter plasmid into the liver by hydrodynamic infusion, and analyzed Mrp4 transcription in real time using biophotonic imaging as described (21). Because PB only activates mouse CAR, not mouse pregnane X receptor (PXR) (40), we evaluated the hepatic activation of the *Mrp4* promoter by using *CAR*-null (27) and *PXR*-null (41) mice. The *Mrp4* promoter was strongly activated by PB in the *PXR*-null mice but not in the *CAR*-null mice (Fig. 4*B*). These findings indicate that CAR transcriptionally activates the *Mrp4* promoter, which is consistent with Mrp4 requiring CAR for up-regulation by PB in liver (Fig. 2).

Constitutively Active CAR Activates Endogenous MRP4 and Up-regulates Functional MRP4 in the Murine Hepa1c1c7 and Human HepG2 Hepatoma Cell Lines—To determine whether murine and human MRP4 and SULT2A1 are direct targets of CAR, we created cell lines stably expressing human CAR (hCAR) and enhanced its transcriptional activity by appending the VP-16 (VP) transactivation domain (41). We demonstrated in a reporter assay that both constitutively active mouse CAR and VPhCAR transcriptionally activated the *Cyp2B6* promoter reporter, which contains PXR/CAR response elements independent of ligand (not shown). Murine liver cells (Hepa1c1c7) were then transduced with the MSCV-VP16-hCAR-GFP or the empty virus (MSCV-VP16-GFP), and a homogeneous population of cells was isolated by cell sorting on the basis of fluorescence. The RNA and proteins were extracted from these cell lines, and Mrp4, Cyb2b10, Cyp3a11, and Gapdh were assayed by semiquantitative RT-PCR, real time RT-PCR, and Western blot analysis (Fig 5, *A–C*). The up-regulation of Sult2a1 and Mrp4 mRNA in cells transduced with VP16-hCAR was 5-fold. This finding is consistent with Mrp4 and Sult2a1 as downstream targets of CAR.

Fig. 5*D* shows that the coordinate activation of MRP4 and SULT2A1 is phylogenetically conserved and also present in human liver cells. PB induced SULT2A1 and MRP4 in the hepatoma cell line HepG2 (Fig. 5*D*, *left panel*) and in primary hepatocytes (Table III), which presents a microarray analysis. The activated CAR retrovirus resulted in similar induction of MRP4, SULT2A1, and CYP2B6 in HepG2 cells (Fig. 5*D*, *right panel*) as in the murine cells (Fig. 5*A*), but had no effect on Sult1a2 levels.

To determine if the induced Mrp4 is functional, we tested transport of PMEA, a known substrate of MRP4 (22, 23). The Hepa1c1c7 cells were loaded with Bis-Pom-PMEA, a pro-drug of PMEA (Fig. 5*E*), and the intracellular accumulation of PMEA was significantly less in the cells overexpressing Mrp4 than in the empty vector cells, thus confirming that Mrp4 was functional. Because PMEA is also a Mrp5 substrate, we assessed Mrp5 expression in these same cells by RT-PCR. The presence of activated CAR made no difference in the level of Mrp5 expressed (Fig. 5*A*). This finding indicates that Mrp5 is not a target of CAR and that the difference in PMEA accumulation is due to increased Mrp4.

Mrp4-null Mice Show Reduced Sult2a1 Expression—The parallel induction of Mrp4 and Sult2a1 by CAR suggests these genes might be coordinately regulated to remove sulfated steroids and bile acids. We wondered what would happen to Sult2a1 RNA levels in livers of mice in which sulfated steroids and bile acids cannot be removed. We therefore tested this idea by performing RT-PCR analysis of Sult2a1 and Sult2b1 (the murine homologue of cholesterol sulfotransferase) on RNA from the livers of $Mrp4^{-/-}$ or $Mrp4^{+/+}$ mice. We found almost no difference in Sult2b1 expression (Fig. 6*A*). In contrast, Sult2a1 expression in *Mrp4*-null liver was 6-fold lower than in

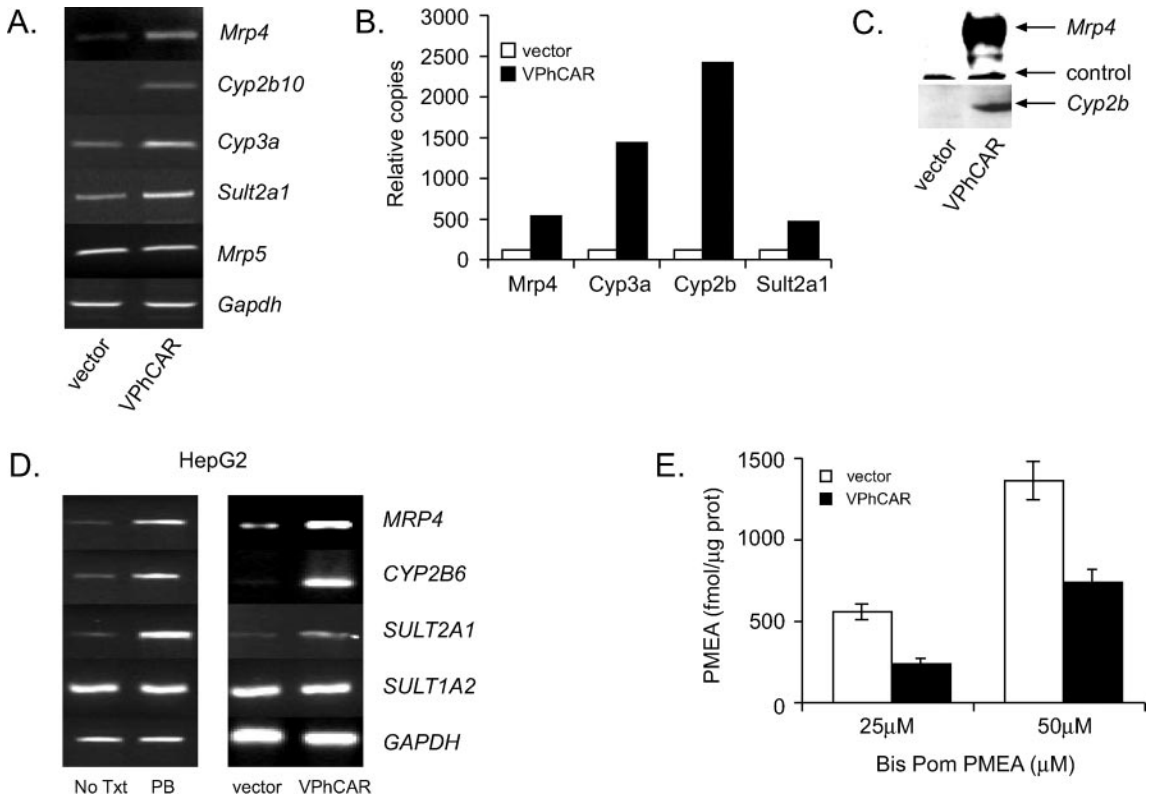

FIG. 5. **CAR up-regulates Mrp4 and Sult2a1 in murine and human liver cells.** The Hepa1c1c7 mouse hepatoma cell line was stably transduced with either the GFP retrovirus or the VPhCAR-GFP retrovirus as described under "Experimental Procedures." Expression of the indicated genes was analyzed by semiquantitative RT-PCR (*A*), real-time PCR (*B*), and immunoblot analysis of Hepa1c1c7 vector or Hepa1c1c7 MRP4 GFP cells (*C*). *D*, RNA extracted from the HepG2 cells that were either untreated or treated with PB (*left panel*). The HepG2 cells were either transduced with either the GFP retrovirus or VPhCAR-GFP and GFP-positive cells were selected. Subsequently, RNA was isolated and selected target genes were analyzed by semiquantitative PCR (*right panel*). *E*, Hepa1c1c7 vector or Hepa1c1c7 VPhCAR GFP cells (2×10^6) were incubated for 24 h with [^{3}H]Bis-Pom-PMEA. After removing extracellular radiolabel, intracellular radiolabel was evaluated by liquid scintillation counting. *Txt*, no treatment

TABLE III
Analysis of the change in expression of some genes following PB treatment in human hepatocytes

Total RNA was extracted from primary human hepatocytes treated with 1 mM PB for 48 hours. RNA was hybridized to the Affymetrix microarray U133. Results show fold increase in expression compared to vehicle-treated primary hepatocytes.

Gene	PB
	fold increase
CYP3A4	4.5
CYP2B6	9.8
SULT21	2.1
MRP4	2.7

wild-type liver (Fig. 6, *A* and *B*). These results demonstrate that in the absence of Mrp4 the level of the major hepatic bile and steroid-preferring sulfotransferase (Sult2a1) decreases. We present a model in the "Discussion" that integrates our previous findings with the Fxr$^{-/-}$ mice (11) and our current understanding of CAR regulation of Mrp4 and Sult2a1 (Fig. 6*C*).

DISCUSSION

In this report, we demonstrate that the ABC transporter, Mrp4, is located at the basolateral membrane of the murine hepatocyte and is regulated by the nuclear receptor CAR as is Sult2a1, a bile acid sulfotransferase (24). Previous studies have indicated that Sult2a1 (STD) is a target of the PXR (42). The up-regulation of Sult2a1 by CAR implicates it as *bona fide* member of a hepatic bile acid detoxification cascade. Moreover, since bile acid sulfates are less toxic than unconjugated bile acids (*e.g.* LCA *versus* LCA-sulfate), Mrp4 basolateral localization in humans and rodents (this article and Ref. 37) and its capacity to transport bile acid sulfate conjugates along with its up-regulation under conditions of bile acid overload strongly support a role for Mrp4 in the removal of toxic bile acid conjugates.

We had previously proposed that Mrp4 is up-regulated to compensate for the accumulation of hepatic bile acids due to impaired bile acid excretion caused by the absence of the nuclear receptor FXR. In the absence of FXR, intrahepatic bile acids increase due to the reduced expression of the major bile salt transporter, Bsep. In addition, Cyp3A, an enzyme that hydroxylates some bile acids (*e.g.* lithocholate) at the 6 position, is up-regulated in Fxr$^{-/-}$ animals (11). Thus, we envisioned a pathway in which bile acids activated the nuclear receptor PXR, and increased Cyp3A expression (11). Elevated Cyp3A would be predicted to convert lithocholic acid to 6-hydroxy lithocholic acid. We then proposed that the 6-hydroxylithocholate, after enzymatic glucuronidation, would be a candidate Mrp4 substrate. However, our recent studies evaluating Mrp4-mediated transport suggests that Mrp4 prefers sulfated steroid conjugates instead of glucuronides (14). This and the low affinity of Cyp3A for lithocholate (43) suggest an alternate pathway would be favored.

Therefore, based upon Mrp4 substrate preference for sulfate conjugates of steroids and bile acids we proposed that Mrp4 might be regulated along with a bile acid preferring sulfotransferase. This proposition was based on our finding that the bile acids and steroids that have a sulfate added to the 3′-hydroxyl group are high affinity inhibitors and presumed Mrp4 substrates (14). We tested the idea that the nuclear receptor, CAR,

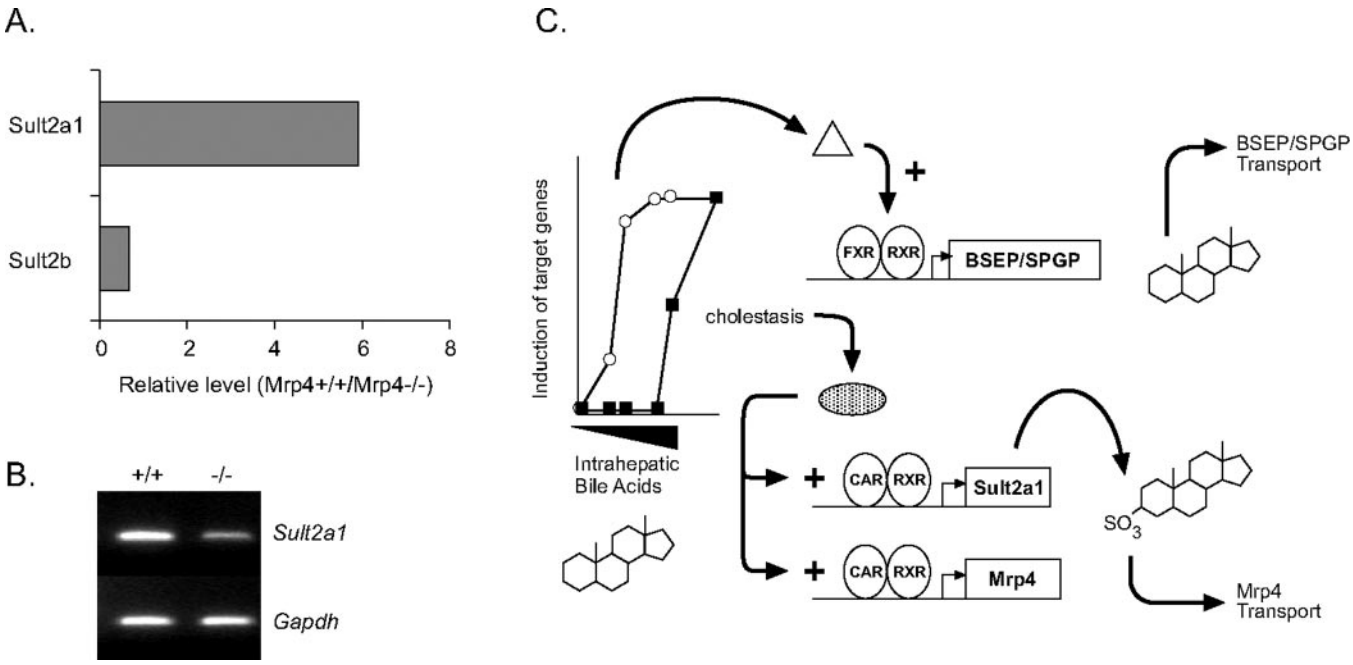

FIG. 6. **Hepatic expression of Sult2a1 is reduced in Mrp4 knockout animals.** *A*, real-time PCR analysis of Sult2a1 and Sult2b1 expression in liver, where ratio of values between $Mrp4^{+/+}$ and $Mrp4^{-/-}$ animals is shown. *B*, semiquantitative RT-PCR analysis of liver expression of Sult2a1 and Sult2b1 expression in $Mrp4^{+/+}$ and $Mrp4^{-/-}$ animals. *C*, in the schematic under bile acids (*triangle*) activate FXR, which increases expression of the major bile acid transporter, BSEP/SPGP. Under cholestatic conditions the level of CAR increases and an unknown inducer (*shaded ellipse*) is produced to activate CAR. This leads to an activation of both Sult2a1 and Mrp4, which sequentially act to remove sulfated bile acids from the liver.

was part of this pathway because CAR expression increased in the $Fxr^{-/-}$ animals fed cholic acid, *i.e.* the exact conditions that led to increased Mrp4 expression (11, 18).[2] Our results showed that activation of CAR increased Mrp4 expression and Sult2a1. This up-regulation of Mrp4 by CAR did not represent a general up-regulation of genes that transport sulfated steroids and/or bile acids because expression of *Bcrp*, *Mrp2*, and *Mrp3* were unaffected by CAR activation. Further, the minimal effect of CAR activation on Mrp3 expression is consistent with our previous findings demonstrating only small changes in the expression of Mrp3 after bile acid overload in the $FXR^{-/-}$, and a more recent study that demonstrated modest elevation in Mrp3 mRNA expression after bile duct ligation to induce cholestasis (44). Furthermore, because Mrp3 activation is not directly linked to CAR activation *in vivo* (34), our observed lack of Mrp3 up-regulation by CAR activation is likely to indicate that Mrp3 is preferentially activated by its close relative, the nuclear receptor PXR *in vivo* (33).

The co-regulation of Mrp4 and Sult2a1 by CAR in murine and human liver cells reveals a conserved excretion route for steroid and bile acid conjugate removal. This pathway is likely a protective pathway because NTCP, a transporter mediating the uptake of conjugated bile acids, is unchanged by CAR activation (not shown). Thus, we speculate that increased Mrp4 expression changes the hepatic bile acid flux toward removal of sulfated bile acid conjugates. The inter-relationship between Mrp4 and Sult2a1 is further confirmed by our finding that genetic deletion of Mrp4 results in the down-regulation of Sult2a1 expression. The co-regulation of Mrp4 and Sult2a1 by CAR is intriguing from the perspective of the control of cellular homeostasis of sulfated bile acids (and steroids), because it suggests regulated expression of these genes might be part of a concerted process to remove these compounds, especially under bile acid overload (Fig. 6*C*, *scheme*). The activation of Mrp4 and Sult2a1 by CAR might circumvent the arylsulfatases that catalyze the removal of sulfate residues from steroids or bile acids (24). This sulfation and vectorial Mrp4 mediated excretion would be predicted to prevent a futile cycle of sulfate addition to bile acids and then their subsequent enzymatic removal by arylsulfatases. It is notable that hepatic arylsulfatase expression is decreased by TCPOBOP treatment of $CAR^{+/+}$ animals but not of $CAR^{-/-}$ animals,[2] a finding suggesting CAR activation primes the liver to remove some bile acids and steroids.

Our results are consistent with the idea that increased Mrp4 expression occurs by enhanced transcription of Mrp4. We infer that murine Sult2a1 is also transcriptionally activated by CAR because previous studies indicated that the rodent Sult2a1 has nuclear receptor response elements that are activated by PXR through an IR0 (inverted repeat with no spaces) cis-element (42). Notably, in the absence of Mrp4, Sult2a1 levels decrease. This finding suggests that Sult2a1 is decreased because of the accumulation of an endogenous Mrp4 substrate. It has been reported that the rodent *Sult2a1* promoter is repressed by androgens (45). Thus, a likely explanation for the decreased Sult2a1 in $Mrp4^{-/-}$ animals is that an androgen (*e.g.* the Mrp4 substrate, DHEA-s) accumulates in the *Mrp4*-null animals and represses Sult2a1 expression. Alternately, the androgen, androstenol, might accumulate in $Mrp4^{-/-}$ animals, and because of its potent inhibitory effects on CAR activity (47) it would lead to decreased expression of Sult2a1. Based on the decreased Sult2a1 expression in the *Mrp4*-null mice, we propose that these mice will be more susceptible to bile acid overload due to both the loss of Mrp4-mediated bile acid transport and the concurrent down-regulation of Sult2a1. Cumulatively, these results support a model in which Mrp4 serves as a transporter that is not only directly activated by CAR to remove sulfated bile acids but that also regulates Sult2a1 expression.

We previously speculated that hepatic Mrp4 was elevated in the *FXR*-null animal because bile acids activated PXR (11). However, two additional explanations are possible: bile acids activate CAR directly or that an additional endogenous substance activates CAR. First, we have shown that the major bile acids do not ligand activate CAR,[2] but instead, modestly repress the constitutive CAR activity, a result which agrees with

a recent report (48). A second possibility is that an endogenous substrate activates hepatic CAR under cholestatic conditions. This endogenous substance, by activating CAR, could increase Mrp4 and Sult2a1 expression. A good candidate as an endogenous activator of CAR is bilirubin (49). Bilirubin is not a direct activator of CAR, but instead, appears to act like PB in inducing nuclear translocation (49). Hepatic bilirubin levels increase during cholestasis, because of increased heme degradation (46, 50). Thus, based upon our current findings demonstrating CAR activates Mrp4 and Sult2a1, it is possible that Mrp4 and Sult2a1 expression increase during cholestasis as occurs when FXR signaling or BSEP/SPGP function is impaired or ablated (Fig. 6*C*, *model*). The results from the Mrp4$^{-/-}$ mice also reveal a feedback portion of this model. In the absence of Mrp4, Sult2a1 is down-regulated. We speculate that this is due to the buildup of an endogenous repressor of CAR (*e.g.* androsterol) that is normally removed by Mrp4. The elucidation of this mechanism is currently being investigated.

Acknowledgment—We thank J. Mital for assistance in the preparation of this article.

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