

Expression of the *MRP* Gene-encoded Conjugate Export Pump in Liver and Its Selective Absence from the Canalicular Membrane in Transport-deficient Mutant Hepatocytes

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Abstract. We have previously shown that the multidrug resistance protein (MRP) mediates the ATP-dependent membrane transport of glutathione *S*-conjugates and additional amphiphilic organic anions. In the present study we demonstrate the expression of MRP in hepatocytes where it functions in hepatobiliary excretion. Analysis by reverse transcription-PCR of human and normal rat liver mRNA resulted in two expected cDNA fragments of *MRP*. Four different antibodies against MRP reacted on immunoblots with the glycoprotein of about 190 kD from human canalicular as well as basolateral hepatocyte membrane preparations. A polyclonal antibody directed against the carboxy-terminal sequence of MRP detected the rat homolog of MRP in liver. Double immunofluorescence microscopy and confocal laser scanning microscopy showed the presence of human MRP and rat Mrp in the canalicular as well as in the lateral membrane

domains of hepatocytes.

The transport function of the *mrp* gene-encoded conjugate export pump was assayed in plasma membrane vesicles with leukotriene C₄ as a high-affinity glutathione *S*-conjugate substrate. The deficient ATP-dependent conjugate transport in canalicular membranes from TR⁻ mutant rat hepatocytes was associated with a lack of amplification of one of the *mrp* cDNA fragments and with a selective loss of Mrp on immunoblots of canalicular membranes. Double immunofluorescence microscopy of livers from transport-deficient TR⁻ mutant rats localized Mrp only to the lateral but not to the canalicular membrane. Our results indicate that the absence of Mrp or an isoform of Mrp from the canalicular membrane is the basis for the hereditary defect of the hepatobiliary excretion of anionic conjugates by the transport-deficient hepatocyte.

EXCRETION into bile is a major pathway for the elimination of endogenous and xenobiotic lipophilic compounds from the mammalian organism. Drug-metabolizing transferases in the hepatocyte convert many of these compounds into amphiphilic anionic conjugates with glutathione, glucuronate, or sulfate. Excretion of these conjugates across the hepatocyte canalicular membrane into bile is mediated by a primary-active ATP-dependent export pump which has been characterized functionally and termed multispecific organic anion transporter (MOAT;¹ Oude Elferink and Jansen, 1994), non-bile acid organic anion transporter (Arias et al., 1993), glutathione *S*-conju-

gate export pump (Ishikawa, 1992), or leukotriene export pump (Keppler, 1992). The latter term refers to the endogenous glutathione *S*-conjugate leukotriene C₄ (LTC₄) which is the substrate with the highest affinity for this transporter (Ishikawa et al., 1990; Keppler, 1992; Leier et al., 1994b). Neither the protein nor the gene encoding this ATP-dependent conjugate export pump in the hepatocyte canalicular membrane were previously identified.

Our recent work in human leukemia cells overexpressing the multidrug resistance protein (MRP) and in HeLa cells transfected with an *MRP* expression vector has demonstrated that the *MRP* gene encodes the ATP-dependent export pump for LTC₄ and structurally related conjugates

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1. *Abbreviations used in this paper:* ABC, ATP-binding cassette; BLM, basolateral membrane preparation; CM, canalicular membrane preparation; Dg, desmoglein; DPPIV, dipeptidyl-peptidase IV (CD26; EC 3.4.14.5); GY, Groningen Yellow; Hu, human; LM, liver membrane preparation;

LTC₄, leukotriene C₄; MOAT, multispecific organic anion transporter; MRP, human multidrug resistance protein or multidrug resistance-associated protein; *MRP*, gene encoding the human multidrug resistance protein; Mrp, rat multidrug resistance protein; *mrp*, gene encoding the rat multidrug resistance protein; TR⁺, normal Wistar rat; TR⁻ transport-deficient Wistar rat; TTBS, TBS containing 0.05% Tween 20.

of lipophilic compounds with glutathione and several other anionic residues (Jedlitschky et al., 1994b; Leier et al., 1994b). This high-affinity LTC₄ export pump has functional properties similar to the ATP-dependent conjugate export pump in the hepatocyte canalicular membrane (Kobayashi et al., 1988; Ishikawa et al., 1990; Kitamura et al., 1990; Akerboom et al., 1991; Fernández-Checa et al., 1992; Keppler, 1992; Oude Elferink and Jansen, 1994). The functional characterization of the hepatocyte conjugate export pump has been promoted and facilitated by the detection of mutant rat strains with a defect in the canalicular excretion of amphiphilic organic anions (Jansen et al., 1985; Kuipers et al., 1988; Takikawa et al., 1991). In these transport-deficient (TR⁻) Wistar rats we observed that excretion of the labeled glutathione *S*-conjugate LTC₄ into bile was selectively reduced to less than 2% of normal and labeled leukotrienes accumulated in liver (Huber et al., 1987). Accordingly, ATP-dependent LTC₄ transport was below detectability in hepatocyte canalicular membrane vesicles from transport-deficient TR⁻ rats (Ishikawa et al., 1990; Fernández-Checa et al., 1992). It has been suggested, however, that the sinusoidal efflux of glutathione *S*-conjugates is unaffected in the TR⁻ rat hepatocytes (Oude Elferink et al., 1989). Furthermore, the distinct ATP-dependent canalicular export pumps for bile salts and amphiphilic cations functioned normally in the mutant TR⁻ rats (Kitamura et al., 1990; Müller et al., 1991). The selective defect of the ATP-dependent canalicular conjugate export pump in mutant TR⁻ rats is comparable to the hereditary defect in the human Dubin-Johnson syndrome (for reviews see Oude Elferink and Jansen, 1994; Roy Chowdhury et al., 1994). The Dubin-Johnson-syndrome is characterized by a selective abnormality in the excretion of conjugated anions into the bile canaliculus with chronic conjugated hyperbilirubinemia and a grossly black liver due to pigment accumulation. In contrast to the findings in the polarized hepatocyte, the ATP-dependent conjugate export pump was fully active in erythrocyte membranes from both patients with Dubin-Johnson syndrome and transport-deficient TR⁻ rats (Board et al., 1992). At present, the genetic defect in Dubin-Johnson syndrome has not been elucidated.

The *MRP* gene encodes a protein of 1,531 amino acids belonging to the ATP-binding cassette (ABC) superfamily of membrane transport proteins (Cole et al., 1992; Cole and Deeley, 1993). Overexpression of this membrane glycoprophosphoprotein with a molecular mass ranging from 170 to 195 kD results in the resistance of human tumor cells to a number of cytotoxic drugs (Grant et al., 1994; Zaman et al., 1994; Krishnamachary et al., 1994; Cole et al., 1994). High levels of *MRP* mRNA have been detected in various tumor cells and in several normal tissues. In liver, however, *MRP* mRNA was not detected in significant amounts (Cole et al., 1992; Zaman et al., 1993). In this study we demonstrate the expression of the *MRP* gene in human liver and of its homolog in the liver of normal and transport-deficient TR⁻ rats by reversed transcription-PCR and sequencing of cDNA fragments. Immunodetection by Western blotting and immunofluorescence microscopy show the *MRP* transporter in the canalicular as well as in the lateral hepatocyte plasma membrane domain. In the canalicular, but not in the lateral membrane of transport-

deficient TR⁻ rat hepatocytes, Mrp, or possibly an isoform of Mrp, is selectively absent. This altered localization of Mrp explains the hereditary defect of the hepatobiliary excretion of amphiphilic anionic conjugates in the mutants.

Materials and Methods

Materials

[14,15,19,20-³H]LTC₄ (4.7 TBq/mmol) was obtained from Du Pont-New England Nuclear (Boston, MA). Unlabeled LTC₄ was from Amersham-Buchler (Braunschweig, FRG). ATP, 5'-AMP, aprotinin, leupeptin, pepstatin, 1,10-phenanthroline, and potassium creatine phosphate were purchased from Sigma Chemicals (Deisenhofen, FRG). Creatine kinase, *N*-[*N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine (E64), glycopeptide *N*-glycosidase (EC 3.5.1.52), and DNase I (EC 3.1.21.1) were from Boehringer Mannheim (Mannheim, FRG). The mRNA isolation kit, the RNase inhibitor (RNasin), StrataScriptTM reverse transcriptase, Taq DNA polymerase, as well as random and β -actin primers were from Stratagene (Heidelberg, FRG). Reinforced cellulose nitrate membranes and nitrocellulose filters (pore size 0.2 μ m) were from Schleicher and Schuell (Dassel, FRG). Protein standard mixture (*M*_r 43,000–200,000) was from Merck (Darmstadt, FRG). Ultrafree-MC (30,000 NMWL) filter units were from Millipore (Eschborn, FRG).

Antibodies

Polyclonal rabbit anti-MRP antisera, kindly provided by Dr. M. Center (Kansas State University, Manhattan, KS), were raised against three different synthetic peptides near the carboxyl terminus of the deduced amino acid sequence of MRP (Krishnamachary et al., 1994): 6KQ (QRGLFYS-MAKDAGLV; amino acids 1517–1531), 7QE (QERFIHQSDLKVDEN; amino acids 1171–1185), and 8KR (RMNLDPFQSYSDEEV; amino acids 1385–1399).

The affinity purification of the 6KQ antiserum was performed as follows: membrane preparations of MRP-overexpressing HL60/ADR cells were subjected to 7.5% SDS-PAGE and subsequently transferred to nitrocellulose membranes. Blots on nitrocellulose membranes were stained with 0.2% Ponceau-S in 3% TCA and the MRP region was excised, washed, and blocked for 1 h at room temperature. Subsequently excised pieces were incubated for 1 h at room temperature with a 1:25 dilution of the 6KQ antiserum in TBS containing 5% BSA and 0.2% Na₃. The 6KQ solution was removed and stored on ice prior to the next incubation cycle. The excised blot pieces were washed with PBS and bound antibodies were eluted by incubation of the blot pieces for 20 min at 52°C in PBS containing 0.2% Na₃. The blot pieces were incubated again with the 6KQ solution. This procedure was repeated 10 times. The eluted antibody fractions were pooled and concentrated (Ultrafree-MC filter units; 30,000 NMWL).

The monoclonal antibody QCRL-1, recognizing MRP in multidrug-resistant H69AR cells and *MRP*-transfected HeLa T5 cells (Hipfner et al., 1994), was kindly provided by Drs. R. G. Deeley and S. P. C. Cole (Queen's University, Kingston, Ontario, Canada). Monoclonal anti-rat dipeptidyl-peptidase IV (DPPIV) antibody De 13.4 (Becker et al., 1986) was kindly provided by Dr. W. Reutter (Freie Universität, Berlin, FRG). Purified mouse anti-human dipeptidyl-peptidase IV (CD 26) antibody was purchased from Dianova (Hamburg, FRG). The monoclonal anti-desmoglein antibody (Dg 3.10) has been described (Schmelz et al., 1986). The mAb antidesmoglein I and II (2.15, 2.17, 2.19) was prepared as described (Cowin et al., 1985). The P-glycoprotein-reactive mAb C219 was from Isotopen Diagnostik CIS (Dreieich, FRG).

Goat anti-mouse and goat anti-rabbit secondary antibodies coupled to FITC or Texas red were purchased from Dianova (Hamburg, FRG). Conjugates of alkaline phosphatase with monoclonal anti-rabbit IgG and goat anti-mouse antibodies were from Sigma Chemicals. The conjugate of horseradish peroxidase with a goat anti-mouse antibody was from Bio-Rad (München, FRG).

Tissues and Cells

Rat Liver. Male Wistar rats (250–350 g) were obtained from Charles River Wiga (Sulzfeld, FRG). Male Groningen Yellow (GY) rats (Kuipers et al., 1988), derived from the same original Wistar colony as the alternatively named TR⁻ mutant rats (Jansen et al., 1985), with hepatobiliary transport defect for glutathione *S*-conjugates and structurally related amphiphilic

anions, were kindly provided by Dr. F. Kuipers (University of Groningen, Groningen, The Netherlands). Animals were maintained on a standard diet with free access to food and water.

Human Liver and Cells. Liver samples were obtained perioperatively from excised hepatic tissue from three patients, 30–60 yr old, who had primary hepatocellular carcinoma or primary biliary cirrhosis. Pathological tissue was removed, as judged by macroscopic inspection, and only healthy liver tissue was further processed.

The MRP- (p190)-overexpressing doxorubicin (Adriamycin)-resistant cell line HL60/ADR has been characterized (McGrath et al., 1987; Marquardt et al., 1990; Krishnamachary et al., 1993) and was kindly provided by Dr. M. Center. Cells were grown in RPMI-1640 medium with 10% fetal calf serum in a humidified incubator with 5% CO₂ at 37°C.

mRNA Analysis by Reverse Transcription-PCR, Subcloning, and Sequencing

Poly(A)⁺ RNA was isolated from rapidly frozen liver tissue using the Stratagene mRNA isolation kit according to the manufacturers instructions. To prevent DNA contamination the mRNA samples (5 µg) were pretreated with 10 U of DNase I in 50 µl digestion buffer (100 mM sodium acetate, pH 5.0, 5 mM MgSO₄, and 40 U of the RNase inhibitor RNasin) at 37°C for 1 h. After phenol/chloroform treatment and ethanol precipitation the mRNA (200 ng) was reverse transcribed in a 50 µl transcription buffer (50 mM Tris, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 1 mM dNTPs, 40 U RNasin, 300 ng of random primers) with 50 U of StrataScript™ reverse transcriptase at 40°C for 1 h, followed by heating to 95°C for 5 min, and immediate cooling on ice. Reverse transcription without RNA template served as a negative control.

The reverse transcription mixture (5 µl) was amplified by PCR in 100 µl of buffer (10× reaction buffer as provided by the manufacturer) with 0.25 µM of sense and anti-sense primers, and 2.5 U of Taq DNA polymerase. The PCR was run at 94°C for 1 min, at 56°C for 1 min, and at 72°C for 2 min, for a total of 33 cycles. Amplification was terminated by a final incubation at 72°C for 10 min. The PCR products (9 µl) were separated on 1.2% agarose gels and visualized by ethidium bromide staining. PCR experiments using mRNA not reverse transcribed were run as negative controls to exclude amplification of genomic DNA. cDNA derived from mRNA of the MRP-overexpressing HL60/ADR cells served as positive control.

Primers for MRP gene sequences were as follows: 182-bp region sense primer (ForIII), TATGTGCCACAGCAGGCCTGGAT (bases 2,324–2,346), and anti-sense primer (RevIII), CCAGACAGGTTACGCCCTTC-TCGCCAA (bases 2,505–2,478); 347-bp region sense primer (ForI), AAGTCGGCATIGTGGCGGA (bases 4,160–4,181), and anti-sense primer (RevI), AGCTGICGCTGCCCIACICT (bases 4,506–4,487). As an internal control a sample of each first-strand cDNA was treated with primers amplifying a 661-bp fragment of human (and rat) β-actin. This reverse transcription reaction (1 µl) was amplified in 50 µl of PCR initiated with 2 U of Taq DNA polymerase. Amplification proceeded through 28 cycles at 94°C for 45 s, at 60°C for 45 s, and at 72°C for 1.5 min.

PCR fragments were cloned into the pCRII vector (Invitrogen BV, NV Leek, The Netherlands) and sequenced according to the Sanger method using the sequencing kit from Pharmacia (Freiburg, FRG).

Plasma Membrane Vesicle Preparation and ATP-dependent LTC₄ Transport

Plasma Membrane Preparation. Membrane fractions enriched in canalicular (CM) or basolateral (BLM) membrane domains from human and rat liver were prepared as described (Meier and Boyer, 1990; Kadmon et al., 1993; Böhme et al., 1994). The marker enzyme activities in our CM and BLM have been reported recently (Kadmon et al., 1993; Böhme et al., 1994). The contamination of rat liver CM with BLM was below 10% as judged from Na⁺.K⁺-ATPase activities. The relative amounts of DPPIV and desmoglein in CM and BLM are shown under Results (see Fig. 5).

Preparation of HL60/ADR plasma membrane vesicles was described recently (Jedlitschky et al., 1994b). All membranes were prepared in the presence of proteinase inhibitors (0.3 µM aprotinin, 1 µM leupeptin, 0.1 mM PMSF, 1 µM pepstatin, 2.8 µM E64, and 5 mM 1,10-phenanthroline).

ATP-dependent [³H]LTC₄ Transport into Membrane Vesicles. [³H]LTC₄ transport into membrane vesicles was determined by rapid filtration (Böhme et al., 1994; Leier et al., 1994a). In brief, vesicles (30 µg of protein) from liver plasma membranes were incubated at 37°C with 50 nM [³H]LTC₄ in an incubation buffer containing 250 mM sucrose and 10 mM

Tris-HCl, pH 7.4, in the presence of 4 mM ATP and an ATP-regenerating system. In control experiments ATP was replaced by 5'-AMP. The respective vesicle-associated [³H]LTC₄ in the blanks with 5'-AMP represented base-line values corresponding to 2.9 ± 0.6 pmol/mg protein in CM and 6.1 ± 1.3 pmol/mg protein in BLM. Reduced glutathione (5 mM) was added to prevent binding of LTC₄ to the membrane-bound glutathione S-transferase as well as the degradation of LTC₄ by the canalicular γ-glutamyltransferase. The final incubation volume was 110 µl. Aliquots (20 µl) were taken at the indicated time points and the vesicle associated radioactivity was determined.

Immunodetection by Western Blotting and Fluorescence Microscopy

Immunoblot Analysis. Membrane fractions (50 µg of protein, unless indicated otherwise) were subjected to 7.5% SDS-PAGE (Laemmli, 1970), without boiling, and transferred to nitrocellulose membrane (Towbin et al., 1979). Protein blots were blocked for 1 h at room temperature and incubated overnight at room temperature with polyclonal anti-MRP antibody diluted in TBS, containing 0.05% Tween 20 (TTBS) and 5% BSA. As the antibodies differed in their sensitivity, they were used at the following dilutions: 6KQ at 1:300, 7QE and 8KR at 1:100. After incubation with a 1:1,000 dilution of alkaline phosphatase conjugated to monoclonal anti-rabbit IgG, the blots were developed with an alkaline phosphatase color development kit (Bio-Rad, München, FRG).

For immunodetection with the mAb QCRL-1 (Hipfner et al., 1994) blots were incubated overnight with a 1:40 dilution of QCRL-1 in TTBS containing 5% BSA. The horseradish peroxidase-conjugated goat anti-mouse antibody was diluted 1:1,000. Antibody binding was determined by enhanced chemiluminescence detection (Amersham-Buchler, Braunschweig, FRG) with exposure on Hyperfilm-MP.

Immunoblots probed for P-glycoproteins were incubated overnight at room temperature with a 1:200 dilution of mAb C219 in TTBS, containing 5% BSA, washed, probed with a 1:1,000 dilution of an alkaline phosphatase conjugate to goat anti-mouse antibody, and developed as described above.

For immunodetection of desmoglein, protein blots of human and rat membrane preparations were incubated for 1 h at room temperature with mAb Dg 3.10. A preparation of desmosomes from bovine muzzle (Gorbisky et al., 1981) and a cytoskeleton preparation (Achtstätter et al., 1986) of A431 cells were analyzed for comparison. Immunodetection of DPPIV was performed by incubation of protein blots from rat membrane preparations with mAb De 13.4 and protein blots from human membrane preparations were incubated with the anti-human DPPIV mAb at a 1:50 dilution. Goat anti-mouse antibodies coupled to alkaline phosphatase served as secondary antibodies in a 1:1,000 dilution.

Deglycosylation of Membrane Glycoproteins for Immunoblot Analysis. Membrane preparations (300 µg of protein) were pelleted for 20 min at 100,000 g at 4°C and resuspended in 40 µl of 10 mM Tris-HCl, pH 7.4, with 1% SDS by ultrasonication for 1 min and incubation for 30 min at 37°C. The samples were diluted 1:10 with a buffer consisting of 20 mM sodium phosphate, pH 7.4, 5 mM EDTA, 0.5% Nonidet P-40, 0.2% sodium azide, proteinase inhibitors, and again incubated for 30 min at 37°C. Samples were divided in two 200-µl aliquots, and one of them was supplemented with glycopeptide N-glycosidase (0.5 U). Deglycosylation was performed for 15 h at 37°C. Subsequently probes were diluted three times with 400 µl of 10 mM Tris-HCl, pH 7.4, concentrated with Ultrafree-MC filter units (30,000 NMWL) to a final volume of 50 µl, lyophilized, and subjected to immunoblot analysis.

Immunofluorescence Microscopy. Small pieces of rat liver were removed from anaesthetized animals and directly snap-frozen in isopentane precooled in liquid nitrogen. Samples from human liver were obtained as described above and snap-frozen within 20 min after surgical removal. For single- and double-label immunofluorescence microscopy tissue sections (4–5 µm) were prepared with a cryotome (Leica; Jung CM 3000, Nussloch, FRG), air-dried for 1–2 h and fixed for 10 min with ice-cold acetone (–20°C). For double-label immunofluorescence the two primary antibodies, one from mouse and one from rabbit, as well as the secondary antibodies, one goat anti-rabbit and one goat anti-mouse, were applied simultaneously. Application of the primary or secondary antibodies was for 45–60 min. For removal of unbound antibodies the sections were washed several times with PBS. Before mounting the sections were finally washed with distilled water and 100% ethanol at room temperature. Air-dried sections were mounted with Moviol (Hoechst, Frankfurt, FRG). Micrographs were taken with a Zeiss Axiophot (Carl Zeiss, Jena, FRG).

For confocal laser scanning fluorescence microscopy a LSM 410 appa-

ratus (Carl Zeiss, Jena, FRG) was used. The microscope operated with an argon ion (488 nm) and a helium/neon laser (543 nm) and was equipped with appropriate filter combinations. Prints were taken from optical sections of 0.8- μ m thickness.

Results

MRP Gene Expression in Liver

Expression of the *MRP* gene in human liver and of the *mrp* gene in rat liver was analyzed by PCR amplification of cDNA reverse transcribed from mRNA. PCR amplification was performed by use of primers corresponding to the sequence of bases 2,324–2,505 and 4,160–4,506 of the human *MRP* cDNA (Cole et al., 1992) in two highly conserved regions, as indicated by the amino acid similarity of 62 and 66% with the corresponding region (bases 2,267–2,448 and 4,115–4,461) in the *Leishmania* *ltpgpA* gene (Ouellette et al., 1990). This resulted in the expected 182- (primers ForIII and RevIII) and 347-bp (primers ForI and RevI) PCR fragments when separated on agarose gels as shown in Fig. 1. Both fragments were amplified and visible in the preparations from HL60/ADR cells, human liver, and liver from normal Wistar (TR⁺) rat. However, no 347-bp fragment was detectable in the preparations from TR⁻ mutant rat liver. Subcloning and sequencing of the 182- and the 347-bp fragment (*seq1*; Fig. 2) from normal TR⁺ rat liver indicated an amino acid sequence identity of 83.3 and 74.8%, respectively, when compared to the human *MRP* cDNA.

Additional PCR experiments using the 182-bp fragment

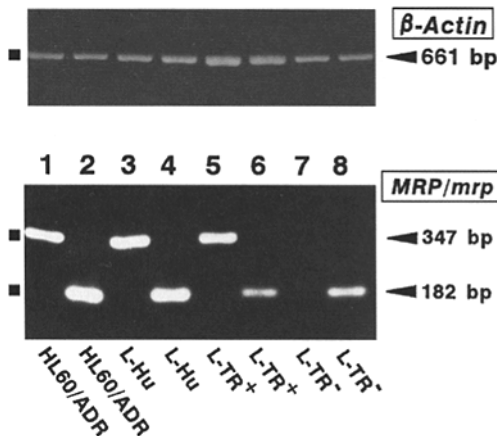


Figure 1. Analysis of *MRP* gene expression in human liver and liver from normal (TR⁺) and transport-deficient (TR⁻) Wistar rats. Reverse transcription was performed on mRNA with random primers. For PCR analysis two pairs of primers were used (347- and 182-bp fragments derived from the human *MRP* sequence (Cole et al., 1992; for details see Materials and Methods). Lanes 1 and 2 show, as a control, the expression in the *MRP*-overexpressing HL60/ADR cells. Both fragments of the *MRP* mRNA were detected in human liver (lanes 3 and 4), and in the homologous *mrp* mRNA in liver of TR⁺ (lanes 5 and 6), in liver from TR⁻ rats only the 182-bp fragment was detectable (lanes 7 and 8). Control experiments assaying the amplification of genomic DNA were negative (see Materials and Methods). A 661-bp fragment of β -actin cDNA, using primers for the human sequence, was run as an internal control to indicate the integrity of the isolated mRNA.

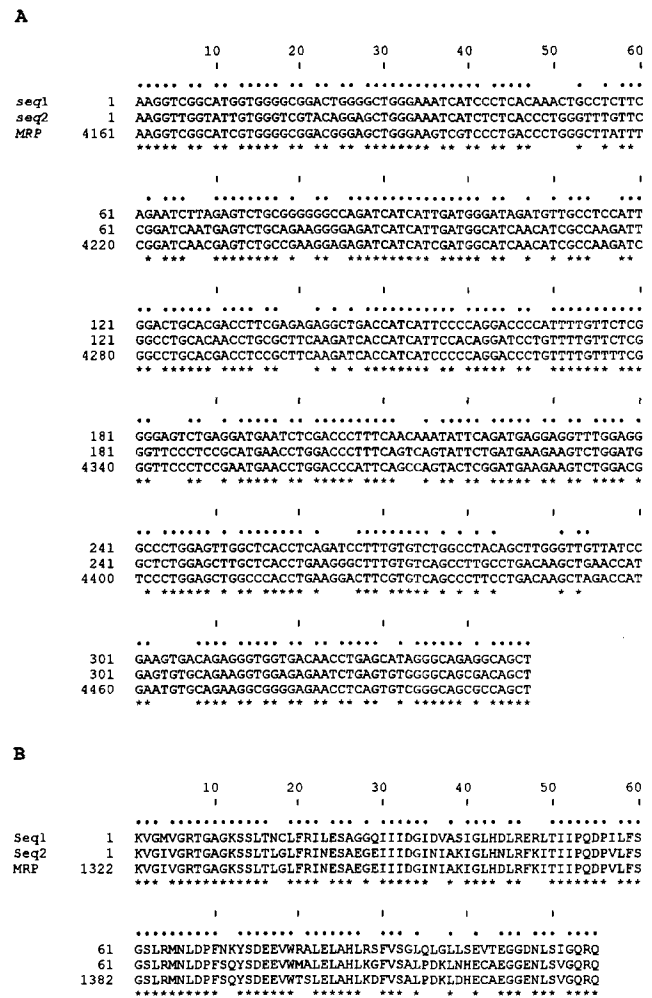


Figure 2. (A) Nucleotide sequence alignment of the two different 347-bp PCR fragments from normal TR⁺ rat liver and the human *MRP* sequence (Cole et al., 1992; these sequence data are available from EMBL/Genbank/DDBS under accession numbers L05628 (*MRP*), x90642 (*seq1*), and x90643 (*seq2*). *seq1* represents the sequence of a PCR fragment obtained with the ForI and RevI primers, whereas *seq2* exhibits the 347-bp part at the 3'-end of a 2.2-kb PCR fragment obtained with ForIII and RevI primers. Sequence identities of the *seq1* and *seq2* fragments are indicated by closed circles on the top line. Identities of all three sequences are indicated by asterisks on the bottom line. (B) Lineup of the corresponding amino acid sequences. Alignment of Seq1 and Seq2 resulted in an amino acid identity of 74.8%. The amino acid identities for the Seq1 and Seq2 sequences are 74.8 and 95.6%, respectively, when compared to the human *MRP* sequence. Alignments were generated with the HUSAR program.

sense primer (ForIII) and the 347-bp fragment antisense primer (RevI) resulted in an expected 2.2-kb fragment both in the liver of normal TR⁺ and TR⁻ mutant rats (data not shown). Cloning and sequencing of the 2.2-kb fragment showed a 5'-end sequence which was identical with the one of the 182-bp fragment in TR⁺ and TR⁻ liver. Interestingly, the 347-bp 3'-region (*seq2*; Fig. 2) of the 2.2-kb fragment in TR⁺ liver contained a somewhat different sequence information (95.6% amino acid sequence identity to the human sequence; Seq2) than the one determined above for the 347-bp fragment (74.8% amino acid se-

quence identity to the human sequence; Seq1; Fig. 2). These data provide evidence for the presence of at least two different *mrp* mRNA species in the liver of normal Wistar (TR⁺) rats. In TR⁻ mutant liver the results are consistent either with a loss of one mRNA species or with a mutation preventing amplification of the 347-bp cDNA fragment. Furthermore, the results are consistent with, but do not prove, an alternative splicing of *mrp* pre-mRNA.

Immunoblot Analysis of MRP in Liver Membranes

Western blots were performed with human liver CM and BLM. Protein blots were probed with three polyclonal antibodies (6KQ, 7QE, 8KR; see Materials and Methods) raised against three different sequences of MRP (Krishnamachary et al., 1994) and with the mAb QCRL-1 directed against MRP (Hipfner et al., 1994). Each of these antibodies detected a protein of about 190 kD in human CM and BLM (Fig. 3). The 6KQ antibody detected, in addition, a minor band of about 170 kD in the human BLM (Fig. 3). This band may correspond to an isoform differing in the length of the polypeptide or in glycosylation. The

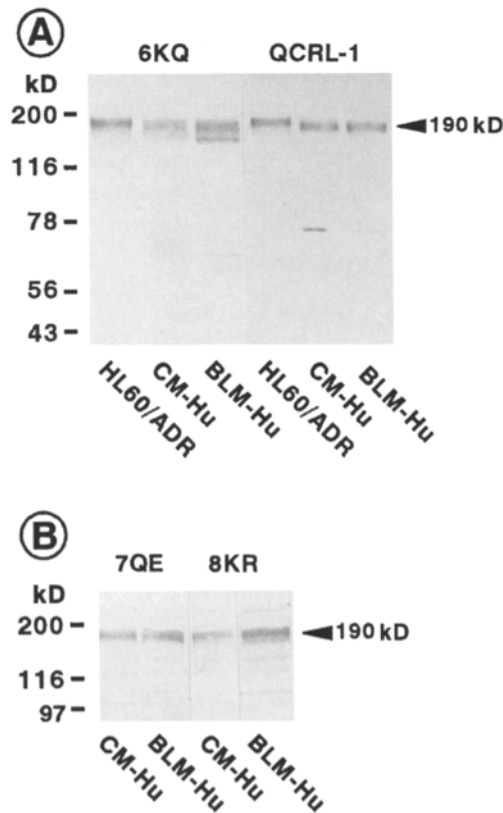


Figure 3. Immunoblot analysis of human liver MRP in bile canalicular (CM) and basolateral (BLM) membrane preparations by three antibodies raised against different peptide sequences of MRP and the mAb QCRL-1 directed against MRP. Membrane fractions (50 μ g of protein) were subjected to 7.5% SDS-PAGE and subsequently transferred to nitrocellulose membranes as described in Materials and Methods. Protein blots were probed with the purified polyclonal 6KQ and the monoclonal QCRL-1. For comparison, membranes (1 μ g of protein) from MRP-overexpressing HL60/ADR cells were probed with both antibodies (A). Protein blots developed with the polyclonal antibodies 7QE and 8KR (B) (see Materials and Methods).

major protein band comigrated with the MRP from HL60/ADR membranes indicating that the protein of about 190 kD is MRP expressed in human liver.

Immunoblot analysis of CM and BLM from normal Wistar rat liver, using the 6KQ antibody, detected a protein of about 170-kD in both membrane fractions (Fig. 4). Only the 6KQ antibody detected Mrp in rat liver. This 170-kD protein was different from P-glycoproteins expressed in rat liver CM (Schrenk et al., 1993) as demonstrated by Western blots already probed with the 6KQ antibody and subsequently developed with the P-glycoprotein-reactive mAb C219 which detected a protein of about 150 kD (Fig. 4). Vice versa it has been shown that the mAb C219 does not react with MRP (Zaman et al., 1994) and we did not detect a 170-kD protein in rat liver membranes with mAb C219. The amount of the 190-kD protein in human and of the 170-kD protein in rat liver BLM was at least as high as in CM, whereas the amount of the 150-kD P-glycoproteins in rat BLM was relatively small.

In addition, we studied the presence of domain-specific proteins in hepatocyte plasma membrane fractions from rat and human. mAbs against desmoglein (Dg), a transmembrane desmosomal glycoprotein of the cadherin family, localized only in the lateral membrane domain (Kartenbeck et al., 1993), and DPPIV, a canalicular (apical) membrane protein (Becker et al., 1986), were used for immunoblot analysis. A significant amount of Dg was detected in BLM from human and rat liver. Only a faint band was detected in CM from rat and human liver (Fig. 5 A). Immunodetection of DPPIV revealed a significant amount of the protein in CM from human and rat liver and only a weak signal in BLM (Fig. 5 B). These data confirm the relative purity of our membrane preparations and support the marker enzyme measurements (see Materials and Methods).

The 170-kD protein recognized by the polyclonal 6KQ antibody in rat liver membranes was considered a candidate for the rat homolog of the human MRP gene-encoded conjugate export pump. To further investigate this possibility, membranes were prepared from TR⁻ mutant rats with a hereditary defect in the hepatobiliary excretion of amphiphilic anionic conjugates. A comparison of liver plasma membrane fractions from TR⁺ and TR⁻ rats by immunoblot analysis with the 6KQ antibody revealed that there was only a very small amount of the 170-kD protein in CM from TR⁻ liver when compared to CM from TR⁺ liver (Fig. 6). In contrast, there was no significant quantitative difference between the proteins in TR⁺ and TR⁻ mutant BLM. An additional protein band migrating at about

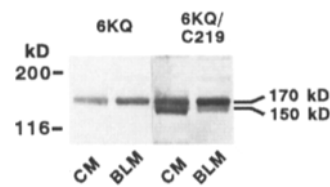


Figure 4. Immunodetection in canalicular (CM) and basolateral (BLM) hepatocyte membranes of rat Mrp and *mdr*-encoded P-glycoproteins with apparent masses of about 170 and 150 kD, respectively. Blots were developed with the 6KQ antibody, directed against the carboxyl terminus of MRP, and subsequently stained with the P-glycoprotein-reactive mAb C219. Membrane protein amounted to 50 μ g of protein (see Materials and Methods).

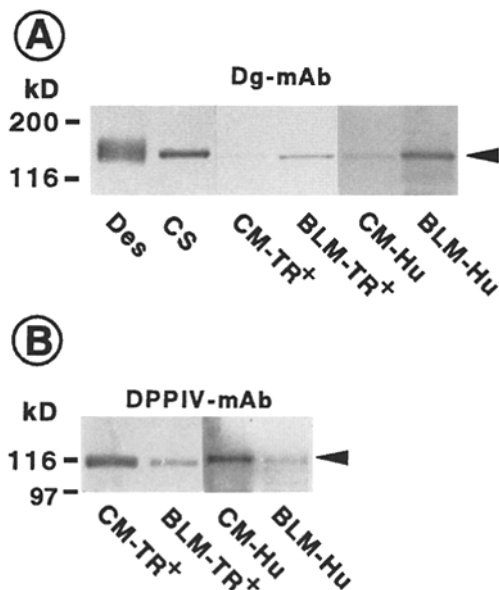


Figure 5. Immunoblot analysis of the domain-specific antigens desmoglein (*Dg*) and dipeptidyl-peptidase IV (*DPPIV*) in hepatocyte membrane preparations. Canalicular (*CM*) and basolateral (*BLM*) membrane preparations from rat and human liver (50 μ g of protein each) were subjected to SDS-PAGE and transferred to nitrocellulose membrane. The protein blot was developed using a desmoglein mAb. A desmosome preparation (*Des*) from bovine muzzle and a cytoskeleton preparation (*CS*) of A431 cells were used for comparison (see Materials and Methods). Arrowhead points to 165 kD (*A*). Protein blots were performed with *CM* and *BLM* from rat (5 μ g of protein) and human liver (50 μ g of protein). The blot from the rat membranes was developed with the mAb De 13.4 directed against rat *DPPIV*, the blot from human membranes was developed with a mAb against human *DPPIV* (see Materials and Methods). Arrowhead points to 120 kD (*B*).

180 kD was consistently detected by the 6KQ antibody in *BLM* from TR^- liver, but not in membranes from normal rat liver (Fig. 6).

Deglycosylation of MRP in Liver Membranes. The extent of glycosylation of the 190-kD protein in human liver and

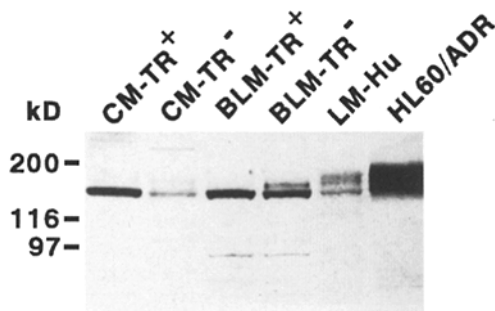


Figure 6. Immunodetection and altered distribution of *Mrp* in canalicular (*CM*) and basolateral (*BLM*) hepatocyte membrane preparations from transport mutant (TR^-) rats. The protein blots were incubated with the 6KQ antibody (see Materials and Methods). Membranes from human liver (*LM - Hu*) and from MRP-overexpressing cells (*HL60/ADR*) were analyzed for comparison. Membrane protein amounted to 5 μ g for *HL60/ADR* membranes and to 50 μ g for the other samples.

of the 170-kD protein in Wistar rat and TR^- liver was examined in membrane fractions digested with glycopeptide *N*-glycosidase and subjected to immunoblot analysis with the 6KQ antibody. In TR^+ and TR^- rat liver *CM* and *BLM* the 170-kD band shifted to about 165 kD after treatment with glycopeptide *N*-glycosidase, suggesting that the protein is only core-glycosylated. Deglycosylation of human liver *CM* and *BLM* resulted in a shift of about 20 kD from about 190 kD to about 170 kD (data not shown). This is in agreement with the molecular mass of 172 kD deduced from the primary amino acid sequence of *MRP* (Cole et al., 1992).

ATP-dependent [3H]LTC₄ Transport by Membrane Vesicles from Normal and Transport-deficient Liver

Primary-active ATP-dependent transport of [3H]LTC₄ into inside-out oriented vesicles of TR^+ and TR^- *CM* and *BLM* was studied over a 2-min period (Fig. 7). Membrane vesicles were incubated with 50 nM [3H]LTC₄ in the presence of 4 mM ATP or 4 mM 5'-AMP. 5'-AMP served as a control to differentiate between nucleotide-dependent binding of the substrate to the membrane vesicles and ATP-dependent uptake into the inside-out oriented vesicles. Transport rates were calculated from the difference in transport in the presence of 4 mM ATP and 4 mM 5'-AMP measured between 30 and 60 s. Significant uptake of [3H]LTC₄, with a transport rate of 6.8 pmol \times mg protein⁻¹ \times min⁻¹ (100%), was observed only with *CM* vesicles from normal (TR^+) Wistar liver. In the other membrane vesicle preparations transport rates were below 20% of the rate in TR^+ *CM* (Fig. 7).

Immunofluorescence and Confocal Laser Scanning Microscopy

The localization of *MRP* in intact liver tissue was visualized by immunofluorescence microscopy on cryosections of human liver, as well as normal (TR^+) and transport-

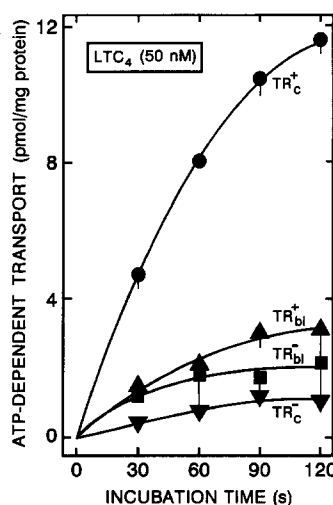


Figure 7. ATP-dependent transport of the endogenous glutathione conjugate LTC₄ by canalicular (*c*) and basolateral (*bl*) membrane vesicles prepared from normal (TR^+) and transport-deficient (TR^-) rat liver. Membrane vesicles (30 μ g of protein) were incubated with 50 nM [3H]LTC₄ in the presence of 4 mM ATP. Aliquots were taken at the indicated time points and the vesicle-associated radioactivity was measured as described in Materials and Methods. In control experiments ATP was replaced by 5'-AMP. The ATP-dependent uptake was

calculated by subtracting values obtained in the presence of 5'-AMP from those measured in the presence of ATP. Each point represents the mean from three different measurements. Vertical bars indicate SD. The membrane preparations were the same as the ones used in Fig. 6.

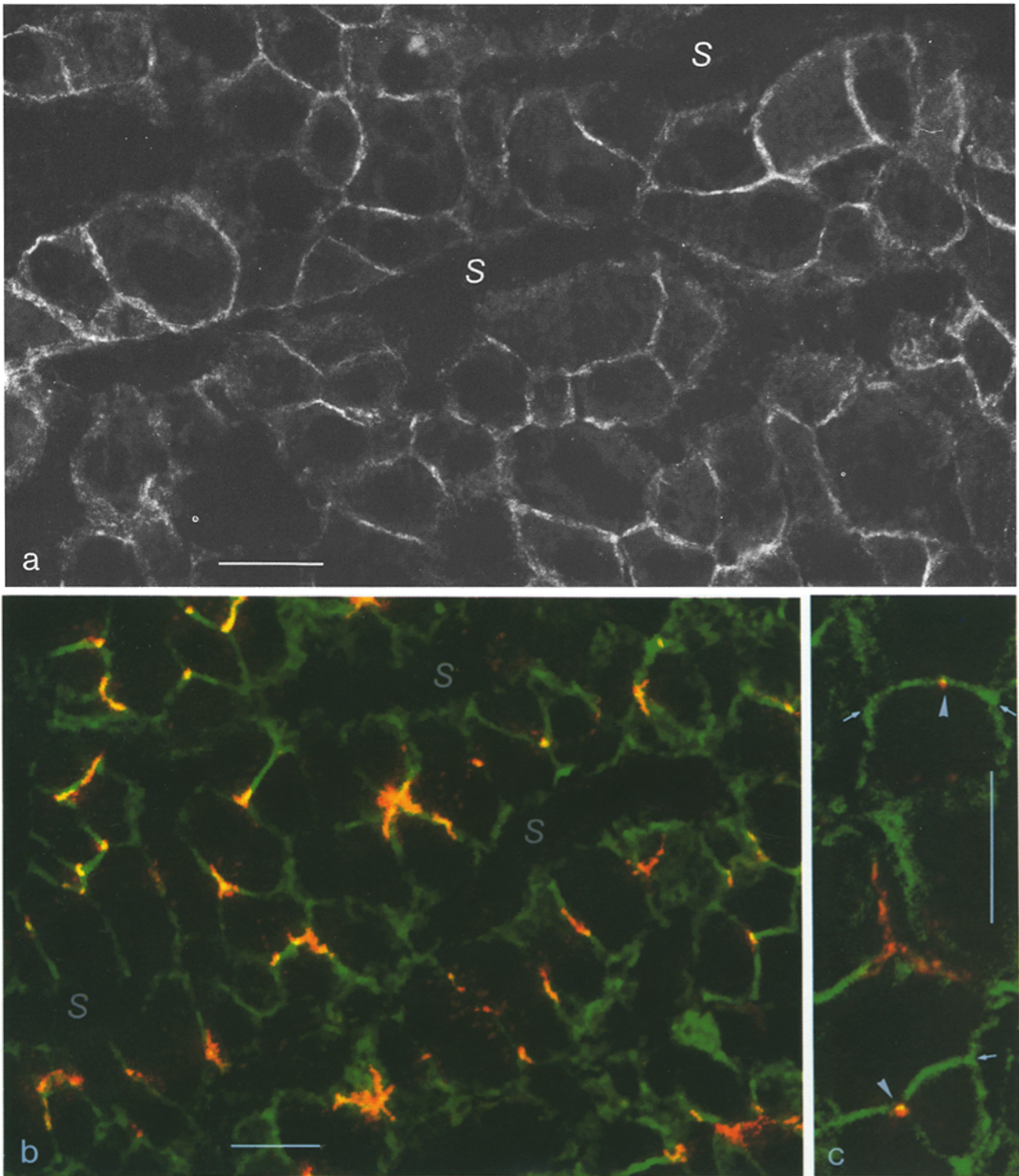


Figure 8. Immunofluorescence (a) and confocal laser scanning micrographs (b and c) of frozen sections of human liver after reaction with purified anti-MRP 6KQ antibody (a) and after double-labeling using the 6KQ antibody and anti-human DPPIV antibody (b and c). Sinusoidal spaces are indicated (S). Immunostaining of MRP appears as fluorescent lines at lateral cell membranes. None or only faint reaction products are seen at basal (sinusoidal) surfaces (a). Double-labeling demonstrates co-localization of bile canaliculi (DPPIV, red staining, e.g., *arrowhead* in c) and MRP-positive cell membranes (*green* staining, e.g., *arrows* in c) resulting in orange-yellow staining in the double-stained canalicular domains. Bars, 20 μ m.

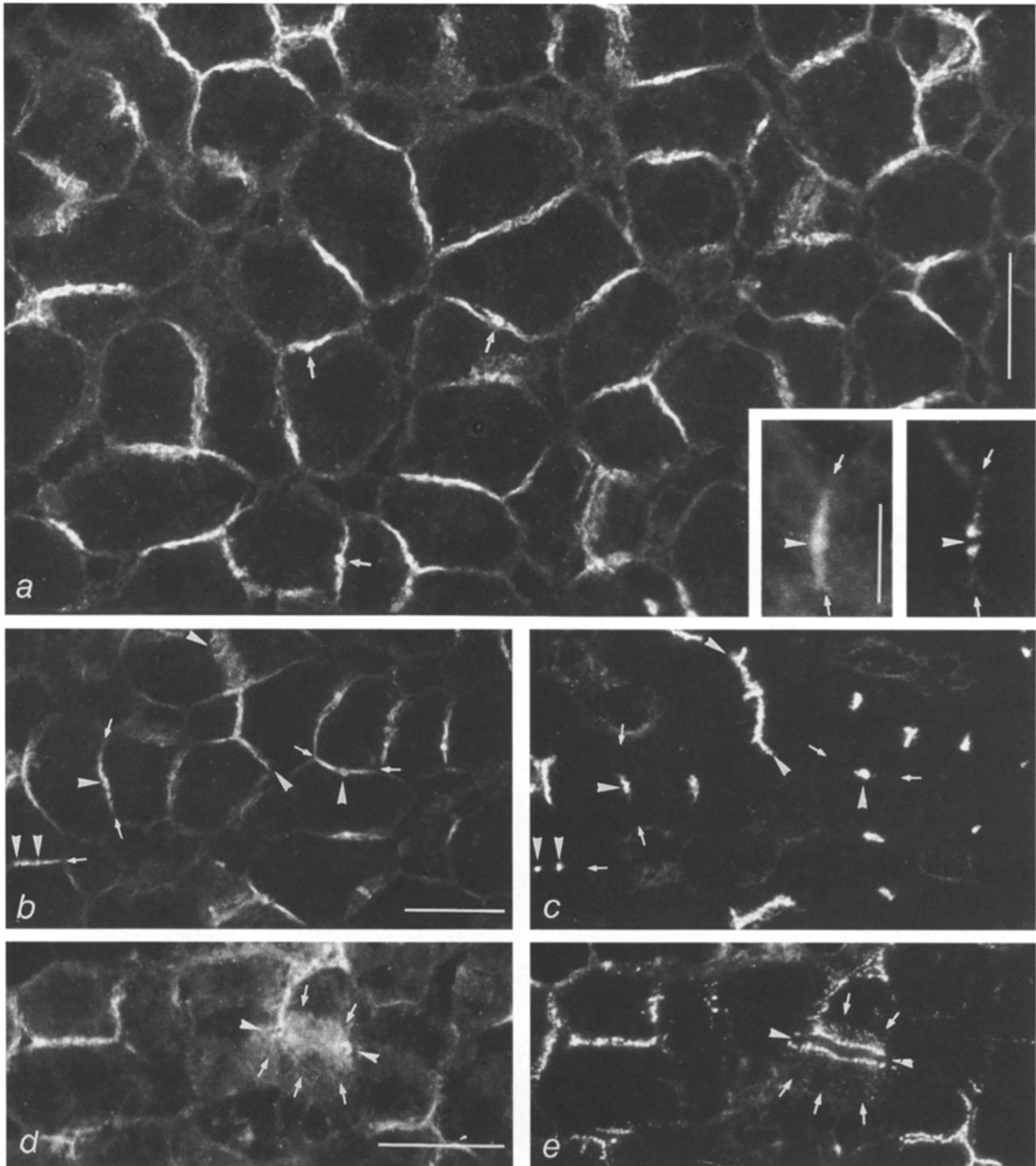
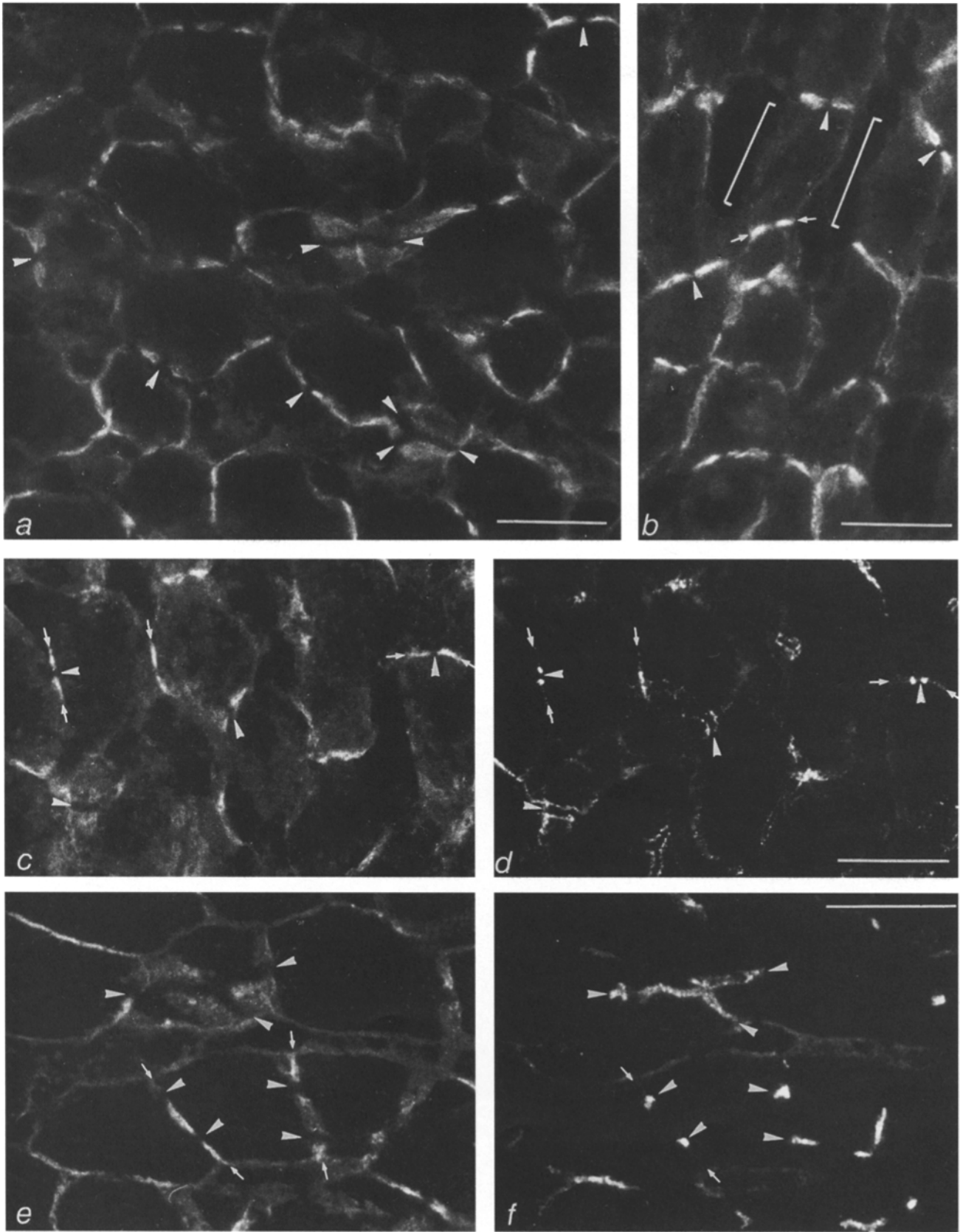


Figure 9. Immunofluorescence microscopy of normal rat liver after reaction with affinity-purified anti-MRP/Mrp 6KQ antibody (*a* and *b*), 6KQ antiserum (*left inset* in *a*, and *d*), desmoplakin antibody (*right inset* in *a*, and *e*), and anti-DPPIV antibody (*c*). Intense reaction with the purified 6KQ antibody demonstrates the distribution of Mrp at the lateral cell boundaries (*a*). Double-labeling of Mrp (*b*) and DPPIV (*c*) demonstrates that the Mrp-staining includes the structure of the bile canaliculi (*arrowheads*) and the total areas of the lateral plasma membranes (*arrows*). Comparable images are obtained after double-labeling of Mrp (*left inset* in *a*, and *d*) and of desmosomes bordering canalicular membranes (*right inset* in *a*, and *e*). Bars, 10 μm (inset) and 20 μm .

Figure 10. Immunofluorescence microscopy of rat liver from transport-deficient TR⁻ animals after reaction with the affinity-purified anti-MRP/Mrp 6KQ antibody (*a*, *b*, and *e*), 6KQ antiserum (*c*), desmoplakin antibody (*d*), and DPPIV antibody (*f*). Reaction with Mrp is visible at lateral plasma membranes (e.g., *arrows* in *b*, *c*, and *e*). Cross-sections reveal small fluorescence-negative gaps within the area of lateral plasma membranes (*left and upper right arrowheads* in *a*; *arrowheads* in *b* and *e*). On flat sections these fluorescence-negative



areas form elongated structures (e.g., *arrowheads* center and lower right in *a*, *upper arrowheads* in *e*). Double-label fluorescence demonstrates that Mrp-negative areas (*arrowheads* in *c-f*) are flanked by desmosomal structures (*arrowheads* in *d*). Mrp negative-gaps can be identified as bile canalicular structures as shown by double-labeling experiments with anti-Mrp and DPPIV antibodies (*arrowheads* in *e* and *f*). Brackets in *b* denote basal (sinusoidal) surfaces. Bars, 20 μm .

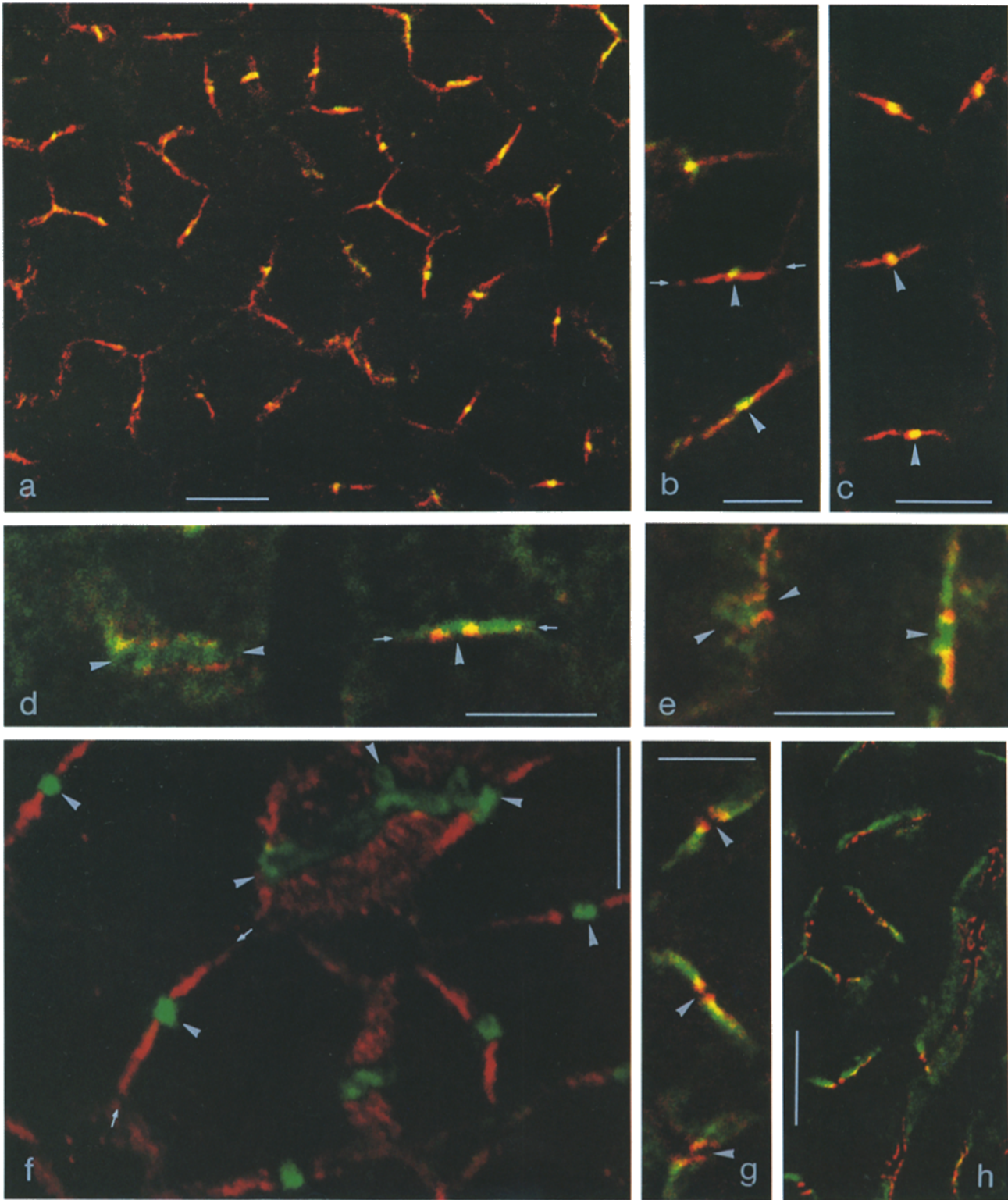


Figure 11. Confocal laser scanning micrographs of frozen sections of normal (*a-e*) and TR⁻ rat liver (*f-h*) after reaction with 6KQ anti-Mrp (red staining in *a-c*, *f*; green staining in *d*, *e* and *g*, *h*), anti-DPPIV (green staining *a-c*, *f*) and anti-desmoplakin antibodies (red staining in *d*, *e* and *g*, *h*). Note that in normal rat liver the red Mrp staining of the lateral cell membranes is turned into orange-yellow in the co-localization areas with green DPPIV reaction product (*a-c*). Double-labeling of Mrp (green staining; *d* and *e*) with desmosomes bordering bile canalicular structures (red staining; *d* and *e*) demonstrate the expression of Mrp in lateral as well as in bile canalicular membranes. In the TR⁻ mutant, Mrp (red staining in *f*, green staining in *g* and *h*) negative gaps are decorated with DPPIV reaction products (green staining in *f*) or are flanked by reaction products with desmoplakin antibody (red staining in *g* and *h*) leaving the Mrp-negative gap unstained. Arrows point to cross-sectioned lateral plasma membranes, arrowheads point to canalicular membranes. Bars, 10 μm.

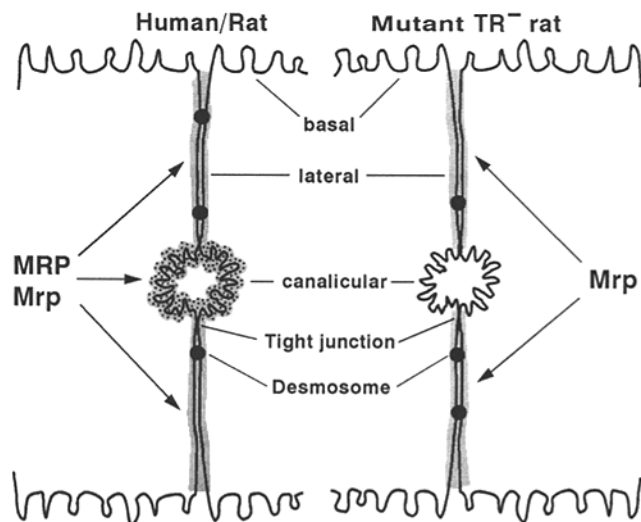


Figure 12. Scheme of the localization of human MRP and rat Mrp in hepatocyte membrane domains. In both species, this membrane transport protein is present in the lateral membrane domain but not detectable in the basal (sinusoidal) domain. In the transport-deficient TR^- rat hepatocyte Mrp is not detectable in the canalicular (apical) membrane, it is present, however, in this domain in normal liver where it functions in the hepatobiliary excretion of conjugates. The dotted canalicular area in the normal hepatocyte suggests that the canalicular protein may represent an isoform which is absent in the mutant hepatocyte.

deficient (TR^-) rat liver. In the three different tissues examined all hepatocytes reacted positively with the 6KQ MRP antibody. The staining product was localized in the cell periphery forming either fluorescent lines or, less intense, slightly extended plaque structures, indicative of either cross or tangentially sectioned plasma membranes (see e.g., Figs. 8, 9, and 10 a).

In order to distinguish between basal (sinusoidal) and lateral immunostaining by the MRP antibody we used antibodies recognizing desmosomal proteins to define the lateral plasma membrane domain of hepatocytes (Farquhar and Palade, 1963; Franke et al., 1981, 1982; Schwarz et al., 1990; Kartenbeck and Franke, 1993). Double-labeling experiments with antibodies directed against desmoplakin (Cowin et al., 1985) and MRP (Krishnamachary et al., 1994) revealed that MRP was present laterally where desmosomal structures are located as well. This colocalization was consistent for human liver (not shown), for normal rat liver (Fig. 9 a, insets; d and e), as well as for the transport-deficient TR^- mutant liver (Fig. 10, c and d). Basal hepatocyte plasma membranes showed no obvious reaction with MRP antibodies (see e.g., brackets on Fig. 10 b).

The canalicular membrane domain of human and rat (TR^+ and TR^-) liver was further identified by the appropriate anti-human and anti-rat DPPIV antibodies (Figs. 8, b and c; 9 c; 10 f; 11, a-c, and f). Double-labeling experiments using antibodies against MRP/Mrp and DPPIV demonstrated that the Mrp-staining along lateral plasma membranes includes the bile canalicular membranes in the normal (TR^+), but not in the transport-deficient TR^- mutant liver (Figs. 9, b and c; 10, c and d).

Desmosomes were concentrated close to the canalicular

membrane as shown by double-label fluorescence microscopy (see e.g., Fig. 9, d and e). This close association of desmosomes and canalicular areas (see especially Fig. 10, c and d for cross-sectioned bile canaliculi) allowed the identification of the limited area of the canalicular plasma membrane on sections stained simultaneously with antibodies against MRP and desmoplakin. The canalicular domain remained unstained in TR^- mutant liver.

These results were further confirmed for human liver, as well as normal (TR^+), and TR^- rat liver by confocal laser scanning microscopy. Superimposing the fluorescent images of MRP/Mrp and DPPIV reaction products allowed the direct identification of bile canalicular membranes on MRP/Mrp-positive structures in normal liver (Figs. 8, b and c; 11, a-c). In the canalicular domain of TR^- mutant liver only DPPIV but not Mrp was detected (Fig. 11 f). Immunoreaction with antibodies to desmosomal proteins and Mrp allowed, in addition, to define the Mrp-positive areas as lateral plasma membranes and the indirect localization of bile canaliculi.

Discussion

In this study we have elucidated the molecular identity of the ATP-dependent conjugate export pump in liver and shown that it is a product of the *MRP* gene. This is in line with our previous work in extrahepatic cells demonstrating that the primary-active ATP-dependent 190-kD export pump for glutathione *S*-conjugates and structurally related amphiphilic anions is encoded by the human *MRP* gene (Leier et al., 1994b; Jedlitschky et al., 1994b). Based on cDNA analysis by PCR (Fig. 1) and on Western blotting of human CM and BLM using four different antibodies (Fig. 3) we conclude that the 190-kD membrane glycoprotein, known as MRP (Cole et al., 1992; Krishnamachary and Center, 1993; Zaman et al., 1993; Hipfner et al., 1994; Krishnamachary et al., 1994), is also expressed in human liver. The presence of an MRP homolog in rat liver (Mrp) is indicated by PCR analysis of cDNA fragments (Fig. 1), by sequence comparison with human *MRP* cDNA (Fig. 2), and by immunoblotting of plasma membrane proteins using an antibody reactive with the peptide at the carboxyl terminus of MRP (Figs. 4 and 6). Moreover, immunofluorescence microscopy localized both human MRP and rat Mrp to the canalicular as well as to the lateral hepatocyte membrane (Figs. 8, 9, and 11). We have discovered a selective loss of Mrp in the canalicular membrane of hepatocytes from rats with a hereditary defect in the ATP-dependent transport of amphiphilic anionic conjugates from the liver into bile (Figs. 6, 7, and 10-11). This altered localization of Mrp in the transport-deficient hepatocyte indicates that the mutation in the TR^- rat selectively affects the appearance of the Mrp transporter in the canalicular membrane domain.

Detection and Localization of the MRP Gene-encoded Conjugate Export Pump in Human and Rat Liver

Our data on the expression of the *MRP* gene in human liver (Figs. 1, 3, and 8) differ from earlier results which had indicated insignificant amounts of *MRP* mRNA in human liver (Cole et al., 1992; Zaman et al., 1993). This apparent

discrepancy may be due to the high sensitivity of reverse transcription-PCR analysis and to the immediate freezing of the liver tissue prior to RNA extraction in our study. Moreover, previous investigations did not include immunodetection of MRP in liver. Rat liver Mrp has an apparent molecular mass around 170 kD and is thus below the apparent mass of MRP in human liver which is around 190 kD (Figs. 3, 4, and 6). Deglycosylation further reduced the apparent molecular mass of rat liver Mrp to about 165 kD. This corresponds to the molecular mass observed when MRP-overexpressing HL60/ADR cells were grown in the presence of tunicamycin (Krishnamachary and Center, 1993) or when MRP from transfected human carcinoma cells was deglycosylated (Zaman et al., 1994).

The localization of human MRP and rat Mrp in liver by immunofluorescence microscopy and double immunofluorescence labeling with confocal laser scanning microscopy indicated its presence in canalicular and lateral hepatocyte plasma membranes (Figs. 8, 9 *a*, 11 *a*). Comparative localization of MRP and Mrp with DPPIV and desmoplakin by double immunofluorescence labeling confirmed the conclusion that the protein is present in the canalicular as well as in the lateral plasma membrane domain (Figs. 8, *b-c*, 9, *b-e*, and 11, *a-e*). Desmoplakins, components of the cytoplasmic structure of desmosomes, are arranged at cell-to-cell boundaries (see e.g., Kartenbeck and Franke, 1993; Kartenbeck et al., 1993). DPPIV is a plasma membrane protein highly concentrated in the hepatocyte canalicular membrane (Becker et al., 1986; Hubbard et al., 1994). The presence of both human MRP and rat Mrp in the lateral plasma membrane (Figs. 8–12) is in line with their intense detection on immunoblots of basolateral plasma membrane preparations (Figs. 3, 4, and 6).

Function of the Conjugate Export Pump in Liver

Conjugation of lipophilic xenobiotics and endogenous compounds with glutathione has long been known as a prerequisite for their excretion across the canalicular membrane into bile (Combes, 1965; Wahlländer and Sies, 1979; Oude Elferink et al., 1989). This process has been recognized as an ATP-dependent primary-active transport (Kobayashi et al., 1988; Kitamura et al., 1990; Ishikawa et al., 1990; Akerboom et al., 1991) which is deficient in the liver of TR⁻ or GY, as well as Eisai hyperbilirubinemic mutant rats (Fig. 7; Kitamura et al., 1990; Ishikawa et al., 1990; Fernández-Checa et al., 1992). Analyses in bile and transport assays in canalicular plasma membrane vesicles from mutant and normal rat liver have been used to define the substrate specificity of this ATP-dependent transport system for conjugates of lipophilic compounds with glutathione, glucuronate, and sulfate, as well as for glutathione disulfide (for review see Oude Elferink and Jansen, 1994). The endogenous glutathione *S*-conjugate LTC₄ is an unmatched high-affinity substrate and competitor of this ATP-dependent transport in the canalicular membrane from rat (Ishikawa et al., 1990; Jedlitschky et al., 1994*a*) and human hepatocytes (Kadmon et al., 1993). The substrate specificities of the conjugate export pump in rat hepatocytes and the human MRP gene-encoded pump are very similar (compare data in Ishikawa et al., 1990, or Akerboom et al., 1991, with those in Leier et al., 1994*b*).

However, subtle differences in substrate specificity must be defined after cloning and expression of the rat *mrp* cDNA.

The important function of the MRP pump in the hepatobiliary excretion of conjugates is in accord with its localization to the canalicular membrane domain (Figs. 2, 3, 7, 8, and 11, *a-e*). Under some conditions, however, such as obstruction of the bile duct, the additional lateral localization of the conjugate export pump may be essential and provide a means to secrete conjugates across the lateral or basolateral hepatocyte membrane into the blood. This is illustrated by the renal elimination of leukotriene metabolites formed in hepatocytes of rats with bile duct ligation and in TR⁻ mutant rats (Huber et al., 1987; Guhlmann et al., 1995), or by secretion of these metabolites into the extracellular medium of hepatocytes isolated from TR⁻ mutant liver (Keppler et al., 1992). Moreover, the isolated perfused liver from TR⁻ mutant rats secretes about the same considerable quantity of *S*-(2,4-dinitrophenyl)glutathione, which is formed within hepatocytes, into the perfusion medium as does the normal control liver, whereas excretion of this conjugate into bile of the perfused TR⁻ mutant liver is reduced to less than 2% of normal (Oude Elferink et al., 1989). *S*-(2,4-Dinitrophenyl)glutathione is also secreted at a considerable rate into the extracellular medium of isolated TR⁻ mutant hepatocytes and it has been determined that 20% of this conjugate are effluxed across the basolateral membrane domain (Oude Elferink et al., 1989, 1994). These observations suggest that the Mrp pump in the lateral hepatocyte membrane of TR⁻ mutant rats is functionally active. The localization of MRP (and Mrp) to the lateral rather than to the basal (sinusoidal) hepatocyte plasma membrane may allow for uptake of compounds across the basal membrane followed by intracellular conjugation and subsequent ATP-dependent export across the lateral membrane, in addition to canalicular secretion. The low specific activity of ATP-dependent LTC₄ transport in basolateral membrane vesicle preparations from normal and TR⁻ mutant liver (Fig. 7) is in apparent contrast to the functional studies in isolated TR⁻ mutant hepatocytes mentioned above and to the amount of immunoreactive protein detected in this membrane fraction (Figs. 3 and 6). This may be explained by the difficulty of formation of inside-out-oriented vesicles from lateral plasma membranes which are stabilized by the neighboring lateral membrane via various intercellular junctional complexes and cannot form a single membrane inside-out vesicle. This orientation would be, however, the structural prerequisite for the measurement of ATP-dependent transport of labeled LTC₄ into the vesicle. Reconstitution of proteins, solubilized from liver plasma membrane preparations, into proteoliposomes and subsequent measurement of ATP-dependent LTC₄ transport has been achieved (Büchler et al., 1994) and may serve to determine the activity of the *mrp* gene-encoded conjugate export pump in basolateral membrane preparations from normal and TR⁻ mutant hepatocytes.

Altered Localization of the Mutant *mrp* Gene Product in Transport-deficient Rat Hepatocytes

Our study provides the first example of an altered localiza-

tion of a hepatocyte membrane transport protein (Figs. 6, and 10–12) resulting in impaired hepatobiliary excretion. The available evidence indicates that newly synthesized integral canalicular membrane glycoproteins are first transported to the basolateral hepatocyte membrane and then by transcytosis to the canalicular (apical) membrane domain (Hubbard et al., 1994; Maurice et al., 1994). Our results in TR⁻ mutant hepatocytes are consistent with a mutation causing the loss of one Mrp isoform containing the signal required for the trafficking of Mrp to the canalicular membrane domain. The mutation reflected by the lack of amplification of the 347-bp 3'-proximal cDNA fragment (Fig. 1) may also interfere with alternative splicing of *mrp* pre-mRNA leading to the loss of a canalicular Mrp isoform. It is of interest in this regard that alternative splicing of *MRP* mRNA with an internal deletion of 65 amino acids at the carboxy-proximal end of *MRP* has been described (Flens et al., 1994).

The functional defect in the TR⁻ rat mutant is considered to be very similar to the canalicular transport defect in patients with Dubin-Johnson syndrome (Oude Elferink and Jansen, 1994; Roy Chowdhury et al., 1994). It has been of interest, therefore, to examine the presence and localization of *MRP* in the liver of patients with Dubin-Johnson syndrome. Our recent immunofluorescence microscopy analyses in the liver from a patient with this syndrome indicate an altered localization of *MRP* similar as the one described above for TR⁻ mutant liver (Kartenbeck, J., U. Leuschner, R. Mayer, and D. Keppler; unpublished observation). The mislocalization of another member of the ATP-binding cassette superfamily of membrane transport proteins is the most frequent cause of cystic fibrosis (for review see Welsh and Smith, 1993). The $\Delta F508$ mutation in the first nucleotide-binding domain of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel results in defective processing and a failure of the protein to localize correctly at the apical plasma membrane domain (Cheng et al., 1990; Kartner et al., 1992). The analogy between mislocalized mutant CFTR, for instance in sweat glands (Kartner et al., 1992), and the altered localization of Mrp in TR⁻ hepatocytes (Figs. 10–12) may be examined in more detail when the mutation in the TR⁻ rat has been identified.

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