Congenital Jaundice in Rats with a Mutation in a Multidrug Resistance–Associated Protein Gene

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The human Dubin-Johnson syndrome and its animal model, the TR$^-$ rat, are characterized by a chronic conjugated hyperbilirubinemia. TR$^-$ rats are defective in the canalicular multispecific organic anion transporter (cMOAT), which mediates hepatobiliary excretion of numerous organic anions. The complementary DNA for rat *cmoat*, a homolog of the human multidrug resistance gene (h*MRP1*), was isolated and shown to be expressed in the canalicular membrane of hepatocytes. In the TR$^-$ rat, a single-nucleotide deletion in this gene resulted in a reduced messenger RNA level and absence of the protein. It is likely that this mutation accounts for the TR$^-$ phenotype.

The liver plays a major role in the detoxification of many endogenous and xenobiotic, lipophilic compounds. Detoxification is accomplished by transferase-mediated conjugation with glutathione, glucuronide, or sulfate moieties, resulting in negatively charged, amphiphilic compounds that are efficiently secreted into bile or urine. Hepatobiliary excretion of these conjugates is mediated by an adenosine triphosphate (ATP)–dependent transport system, cMOAT, located in the apical (canalicular) membrane of the hepatocyte (*1*). The identification of a transport-deficient mutant rat strain, the TR$^-$ rat (*2*), has contributed to the functional characterization of cMOAT (*1*). These rats have an autosomal recessive defect in the hepatobiliary excretion of bilirubin glucuronides (*3*) and other multivalent organic anions, including glutathione-S-conjugates (for example, leukotriene C_4) and 3-OH–glucuronidated and –sulfated bile salts (*4*). Thus far, neither the protein nor the complementary DNA (cDNA) encoding cMOAT have been identified. Transport studies in plasma membrane vesicles from cells overexpressing the human multidrug resistance–associated protein 1 (hMRP1) (*5*) have demonstrated a role for hMRP1 in the ATP-dependent transport of the glutathione conjugates LTC_4 and dinitrophenyl glutathione (GS-DNP) (*6*). Because these substrates are also transported by the putative cMOAT protein, *MRP1* is a possible candidate gene for cMOAT. The extremely low expression of MRP1 in liver (*5*, *7*) and the recently demonstrated lateral localization of MRP1 (*8*), however, renders it unlikely that this gene product is responsible for biliary organic anion secretion. Furthermore, the transport defect in the TR$^-$ rat appears to be specific for liver (*9*), whereas MRP1 is expressed in all human tissues (*7*).

We hypothesized that cMOAT might be a liver-specific homolog of MRP1. To obtain a rat *mrp1* probe, we subjected rat lung cDNA to polymerase chain reaction (PCR) with nested degenerate oligonucleotide primers that were based on the highly conserved first ATP-binding cassette of the hMRP1 sequence (*10*). The resulting 213–base pair (bp) product shared 83% amino acid sequence identity with the corresponding region of the hMRP1 sequence. When analyzed on Northern (RNA) blot, this PCR fragment hybridized with a single, 9.5-kb transcript in all Wistar and TR$^-$ rat tissues examined, with high expression in lung and testis but no detectable expression in liver. Because this expression pattern resembled that of h*MRP1* in human tissues (*7*), we assumed that we had isolated a part of the rat homolog of h*MRP1*, rat *mrp1* (*rmrp1*). In order to find the putative cMOAT gene, we screened two rat liver cDNA libraries, using the *rmrp1* fragment obtained as a probe (*11*). This resulted in the isolation of a full-length cDNA with a single open reading frame of 1541 amino acids (Fig. 1). On the basis of similarity searches (*12*), the protein was identified as a member of the ABC transporter family (*13*), with highest overall identity to hMRP1 (47.6%) (*5*), yeast cadmium factor 1 (41.8%) (*14*), and the human cystic fibrosis transmembrane conductance regulator (30.2%) (*15*). The amino acid sequence

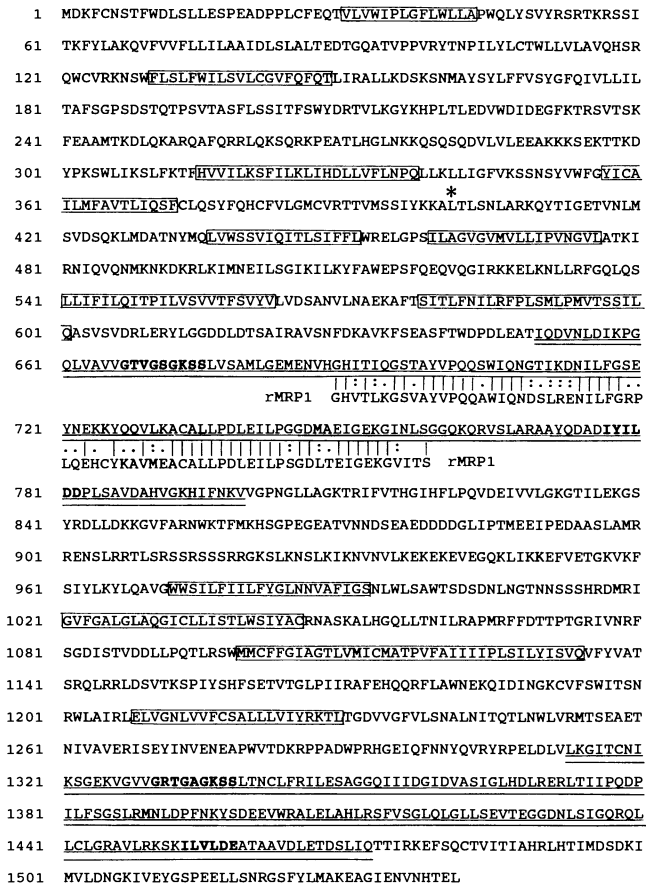

Fig. 1. Deduced amino acid sequence of rat cMOAT (*25*) and alignment with the deduced 70–amino acid sequence of the translated 213-bp putative rat *mrp1* cDNA. The alignment was generated with the PILEUP program from the GCG package (*26*) with a gap weight of 3.0 and a gap length weight of 0.1. Alignment starts at amino acid 689 and ends at amino acid 758 of the cMOAT protein. The nucleotide-binding domains are double-underlined; Walker A and B consensus sequences are in boldface. The predicted transmembrane regions are enclosed in boxes (*27*). The asterisk indicates the location (amino acid 401) at which a stop codon is introduced by the single-nucleotide deletion in TR$^-$ rat *cmoat*.

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identity with hMRP1 ranged from 38 to 61% outside the ATP-binding domains to 67 and 75% in the first and second ATP-binding domain, respectively. Northern blot analysis of rat tissues with a 1-kb restriction fragment of this cDNA revealed three different transcripts, ranging from approximately 6.5 to 9.5 kb, with high expression only in liver and low expression in kidney, duodenum, and ileum (Fig. 2A). The abundance of these transcripts was strongly decreased (but not absent) in liver (Fig. 2B) and other tissues of the TR$^-$ rat, which suggests that these transcripts were related to the defect in the TR$^-$ rat. The three transcripts observed were probably derived from a single gene, because the level of all three transcripts was decreased in the TR$^-$ rat. The decreased abundance of this transcript in TR$^-$ liver suggests that the isolated cDNA encoded cMOAT.

To examine the expression level and cellular localization of the cMOAT protein in hepatocytes, we produced a monoclonal antibody (mAb M_2 III-5) to a bacterial fusion protein containing the 202–amino acid COOH-terminal end of the sequence (*16*). On protein blots, this antibody detected a protein of ~200 kD in the canalicular, but not the basolateral, plasma membrane fraction of the Wistar rat liver (Fig. 3). This molecular size was similar to that of hMRP1 and in good agreement with the predicted molecular size of the cMOAT protein. The 200-kD protein was completely absent from the canalicular membrane fraction of the TR$^-$ rat (Fig. 3), which correlated with the decreased mRNA level in TR$^-$ rat liver (Fig. 2B). Again, this finding was in good agreement with the defect observed in TR$^-$ rats, which lack a functional transport system for organic anions in the canalicular membrane.

Thus, it is likely that we have isolated the cDNA encoding the cMOAT protein, which is deficient in the TR$^-$ rat. Because the *cmoat* mRNA was not completely absent in TR$^-$ liver, it was possible to amplify the complete cDNA by PCR (*11*) with various specific primer sets. To identify the nature of the genetic defect in TR$^-$ rats, we sequenced the obtained cDNA. Analysis revealed a 1-bp deletion at amino acid 393, which results in a frameshift and subsequent introduction of a stop codon at position 401 (Fig. 1). This deletion results in the destruction of an Nla III restriction site, which provided a means to quickly confirm the presence of the mutation in cDNAs from various tissues (*17*). The low mRNA expression in TR$^-$ rats (Fig. 2B) might be due to the fact that the frameshift causes premature termination of translation and subsequent increased degradation of the mRNA.

Our results imply a correlation between the presence of the *cmoat* gene, the absence of the gene product from the canalicular membrane, and the defined congenital transport defect in TR$^-$ rats. A recent study suggested lateral as well as canalicular localization of the rMRP1 gene product in Wistar liver, but only a lateral localization in TR$^-$ liver (*18*), and suggested a role for MRP1 in the (defective) hepatobiliary excretion of organic anions in TR$^-$ rats. We have demonstrated an exclusive canalicular localization of cMOAT (Fig. 3). On the other hand, hMRP1 is routed only to the lateral domain of the plasma membrane of pig kidney epithelial cells (*8*). This finding suggests a differential localization of MRP1 (basolateral) and cMOAT (canalicular) and implies that cMOAT and not MRP1 is involved in biliary organic anion transport. It has also been suggested (*18*) that an isoform of MRP1 exists in rat liver that is derived from the same gene by alternative splicing based on the detection of two different sequences for the second ATP-binding domain and only one for the first ATP-binding domain. Our data, however, show that there are two different sequences for the first ATP-binding domain in *mrp1* and *cmoat*, which suggests that MRP1 and cMOAT are encoded by two different genes.

We conclude that the MRP homolog, identified here, encodes cMOAT, and that a 1-bp deletion, resulting in a truncated, nonviable protein, is responsible for the impaired transport of organic compounds from liver to bile in the TR$^-$ rat. TR$^-$ rats have the same phenotype as patients with the Dubin-Johnson syndrome, characterized by mild chronic conjugated hyperbilirubinemia (*19*). Isolation of the human homolog of cMOAT will be required to elucidate the nature of the defect in humans. Overexpression of hMRP1 confers resistance of human tumor cells to a number of cytostatic drugs (*20*, *21*), which is dependent on intracellular glutathione levels (*22*). Apparently, both MRP1 and cMOAT are involved in the excretion of organic anions from cells. Thus, overexpression of cMOAT, like that of MRP1, might also confer resistance to cancer cells against cytostatic drugs or their metabolites.

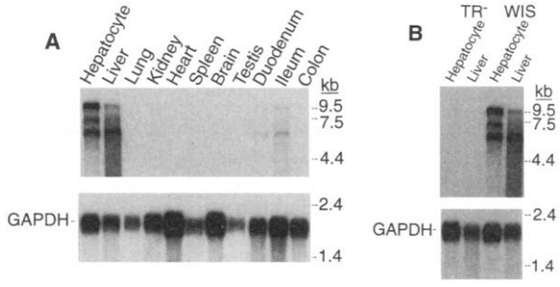

Fig. 2. (**A**) Northern (RNA) blot analysis of 2 μg of poly(A)$^+$ RNA from Wistar rat tissues hybridized to a 1-kb Hind III–Ava II cDNA fragment of *cmoat*. RNA was analyzed as described (*28*). Prolonged exposure of the film revealed no detectable expression in tissues other than kidney, duodenum, and ileum. (**B**) Northern blot analysis of 2 μg of poly(A)$^+$ RNA from Wistar and TR$^-$ rat liver and hepatocytes hybridized with the same probe as in (A). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signal is shown at the bottom. Molecular size standards are indicated at the right in kilobases.

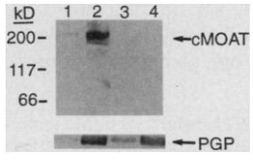

Fig. 3. Immunoblot analysis of cMOAT and P-glycoprotein in canalicular and basolateral membrane fractions of Wistar and TR$^-$ rat hepatocytes. Lane 1, Wistar basolateral membranes; lane 2, Wistar canalicular membranes; lane 3, TR$^-$ basolateral membranes; and lane 4, TR$^-$ canalicular membranes. (Upper panel) The blot was incubated with mAb M_2 III-5 specific for cMOAT (*29*). This antibody did not cross-react with the hMRP1 protein as tested in total lysates from the MRP-overexpressing cell line GLC4/ADR (*20*). (Lower panel) Immunodetection of P-glycoproteins with mAb C219 in the same membrane preparations. The 150-kD P-glycoproteins are expressed exclusively in canalicular membranes (*30*). Differential staining of the two fractions demonstrates the separation of the two membrane domains, with slight contamination of the basolateral fraction by canalicular membranes. Molecular size markers are indicated on the left in kilodaltons.

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10. A 213-bp PCR product was obtained from rat lung cDNA after first-round amplification with degenerate primers corresponding to amino acids 678 to 648 (forward) and 770 to 776 (reverse) and subsequent

second-round amplification with nested primers corresponding to amino acids 694 to 700 (forward) and 760 to 766 (reverse) of the hMRP1 sequence (*5*).
11. Partial cDNA clones were isolated from a rat hepatocyte cDNA library (*23*) that was screened with the 213-bp probe according to standard procedures (*24*). From a 4.5-kb positive clone, a 5′-located, 0.6-kb Hph I restriction fragment was used to screen a λGT10 5′ stretch rat liver cDNA library (Clontech, Palo Alto, CA). A 0.8-kb overlapping clone was obtained from which a 0.6-kb Ava II probe was isolated to rescreen the same library, resulting in the isolation of another overlapping clone. The 5′ end of the cDNA was obtained by use of the anchored PCR procedure [M. A. Frohman, M. K. Dush, G. R. Martin, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8998 (1988)]. cDNA synthesis was carried out with 5 μg of total RNA isolated from Wistar rat liver and random hexamer primers with Superscript Reverse Transcriptase II (Life Technologies, Gaithersburg, MD). After purification, the cDNAs were tailed with a synthetic oligonucleotide anchor sequence with the use of a 5′-RACE kit (Life Technologies). Two rounds of nested PCR (96°C, 30 s; 60°C, 30 s; 72°C, 45 s) with an anchor-specific primer and two cMOAT-specific primers [5′-TGTCCAGTATCTTCTGTGAGCG-3′ (first round) and 5′-AACACGACGAACACCTGCTTGGC-3′(nested)] resulted in the isolation of the missing 5′ sequence. Probes were labeled with [α-^{32}P]deoxycytidine triphosphate (dCTP) with the use of random primers. Hybridization of the filters was done at 65°C in 0.5 M $NaPO_4$ (pH 7.0), 2 mM EDTA, and 7% SDS (hybridization solution) for 20 hours. Filters were washed four times in 2× saline sodium citrate (SSC), 1% SDS for 30 min at 65°C and autoradiographed. Nucleotide sequences were determined by the dideoxynucleotide chain method [F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 463 (1977)]. The *cmoat* cDNA sequence has been deposited with GenBank (accession number L49379).
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16. A fusion gene, consisting of the gene for the *Escherichia coli* maltose-binding protein and the 3′ part of the *cmoat* cDNA corresponding to amino acids 1340 to 1541, was constructed in pMal-c [C. V. Maina *et al.*, *Gene* **74**, 365 (1977)]. The fusion protein was produced in *E. coli* strain JM101 and purified by amylose resin affinity chromatography. Mice were injected three times during 6 weeks with 200 μg of the purified protein. The first injection was in the presence of Freund's complete adjuvant, and the subsequent boosts in Freund's incomplete adjuvant. Two weeks after the final boost with a glutathione-S-transferase–cMOAT fusion protein, splenocytes were isolated and fused with myeloma cells. Hybridomas were screened by enzyme-linked immunosorbent assay with the glutathione-S-transferase–cMOAT fusion protein and subsequently tested for positivity in protein immunoblots.
17. *cmoat* cDNA was amplified from liver, kidney, ileum, and duodenum from both Wistar and TR$^-$ rats with primers corresponding to amino acids 366 to 375 (forward) and 451 to 458 (reverse) of the cMOAT sequence. The resulting PCR product was digested with Nla III. PCR products from TR$^-$ rat digestion produced two bands of 206 and 66 bp, whereas in the Wistar rats three bands of 83, 122, and 67 bp were observed.
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25. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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28. Total RNA was extracted according to the acid-phenol single-step method [P. Chomczynski and N. Sacchi, *Anal. Biochem.* **8**, 148 (1987)]. Polyadenylated [poly(A)$^+$] RNA was isolated with the polyAtract mRNA system III (Promega). RNA was fractionated on a 0.8% denaturating agarose gel, transferred to Hybond N$^+$ nitrocellulose membrane filters, and hybridized with a [α-^{32}P]dCTP-labeled 213-bp rat lung *mrp1* probe and a 1-kb Hind III–Ava II fragment of *cmoat* in hybridization solution (*11*) for 20 hours at 65°C. Filters were washed 4× for 30 min in 0.2× SSC–0.1% SDS at 65°C and autoradiographed. A ^{32}P-labeled 1.2-kb Pst I fragment of the rat glyceraldehyde-3-phosphate dehydrogenase cDNA [Ph. Forth *et al.*, *Nucleic Acid Res.* **13**, 1431 (1985)] was used to estimate variations in RNA loading.
29. Canalicular and basolateral membranes were isolated as described [P. J. Meier, E. S. Sztul, A. Reuben, J. L. Boyer, *J. Cell Biol.* **98**, 991 (1984)]. Membranes, containing 50 μg of protein, were fractionated by 7.5% SDS–polyacrylamide gel electrophoresis, transferred by electrophoresis to nitrocellulose filters, blocked for at least 2 hours in PBS/M/T [phosphate-buffered saline containing 1% bovine serum albumin, 1% milk powder, and 0.05% Tween-20), and incubated with the mAb (M_2 III-5 hybridoma culture medium diluted eightfold with PBS/M/T) for 2 hours. Immunoreactivity was visualized with peroxidase-conjugated rabbit antibody to mouse immunoglobulin G (anti–mouse IgG) and subsequent staining with 3,3′-diaminobenzidine and 4-chloro-1-naphthol substrate. P-glycoproteins were detected with mAb C219 and peroxidase-conjugated rabbit anti–mouse IgG. Immune complexes were visualized by enhanced chemiluminescence.
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IRAK: A Kinase Associated with the Interleukin-1 Receptor

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The pleiotropic biological activities of interleukin-1 (IL-1) are mediated by its type I receptor (IL-1RI). When the ligand binds, IL-1RI initiates a signaling cascade that results in the activation of the transcription regulator nuclear factor kappa B (NF-κB). A protein kinase designated IRAK (IL-1 receptor–associated kinase) was purified, and its complementary DNA was molecularly cloned. When human embryonic kidney cells (cell line 293) overexpressing IL-1RI or HeLa cells were exposed to IL-1, IRAK rapidly associated with the IL-1RI complex and was phosphorylated. The primary amino acid sequence of IRAK shares similarity with that of Pelle, a protein kinase that is essential for the activation of a NF-κB homolog in *Drosophila*.

Interleukin-1 is a proinflammatory cytokine that functions in the generation of systemic and local responses to infection, injury, and immunological challenges. Produced mainly by activated macrophages and monocytes, IL-1 participates in lymphocyte activation, fever, leukocyte trafficking, the acute phase response, and cartilage remodeling (*1*). The biological effects of IL-1 are mediated by IL-1RI located on the plasma membrane of responsive cells (*2*). Binding of IL-1 to its receptor triggers activation of NF-κB (*3*). NF-κB constitutes a family of related transcription factors that regulate the expression of genes bearing cognate DNA binding sites (*4*). In most cells, NF-κB is retained in the cytoplasm by inhibitory proteins designated IκB's (*5*). In response to a variety of extracellular stimuli (including IL-1, tumor necrosis factor, mitogens, oxidative stress, lipopolysaccharide, and double-stranded RNA), IκB's are degraded, releasing NF-κB to enter the nucleus where it activates an array of genes (*6*).

Genetic studies examining the formation of dorsoventral polarity of the *Drosophila* embryo have shed light on the intracellular signaling pathway leading to NF-κB activation. The protein Dorsal, a homolog of NF-κB, is activated during embryogenesis to regulate gene expression essential for establishing dorsoventral polarity (*7*). Like NF-κB, Dorsal activity is suppressed by an IκB-like molecule designated Cactus (*8*). Activation of Dorsal is initiated by the interaction of an extracellular ligand designated Spaetzle with a membrane-bound receptor designated Toll (*9*). A potential connection between the IL-1 and Spaetzle signaling pathways was found on the basis of the sequence similarity shared by the intracellular domains of IL-1RI and Toll (*10*). Two other genetically identified molecules, Tube and Pelle, function downstream of

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