Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 317 (2004) 46–53
BBRC
www.elsevier.com/locate/ybbrc

Rat multidrug resistance protein 4 (Mrp4, Abcc4): molecular cloning, organ distribution, postnatal renal expression, and chemical inducibility

Chuan Chen and Curtis D. Klaassen*

Department of Pharmacology, Toxicology, and Therapeutics, University of Kansas Medical Center, Kansas City, KS 66160, USA

Received 2 March 2004

Abstract

In the present study, we report cloning of the rat Mrp4 cDNA. The cDNA is 4526 bp, containing a 3975 bp open reading frame. The deduced polypeptide has 1325 amino acids and is 83% and 91% identical to human MRP4 and mouse Mrp4, respectively. Phylogenetic analysis revealed that the cloned rat cDNA is closely related to human MRP4 and mouse Mrp4. Additionally, an alternatively spliced variant, 111 bp shorter than the full-length form, was cloned. Rat Mrp4 mRNA was detectable in 11 tissues examined, with levels being highest in kidney, and lowest in liver. Mrp4 mRNA levels in kidney were higher in males than females, and at birth were about half of adult levels. Mrp4 expression in liver and kidney of rats treated with six classes of microsomal enzyme inducers was examined. Mrp4 mRNA in liver was induced by two electrophile response element activators, namely ethoxyquin and oltipraz.

Keywords: Mrp4; Abcc4; Cloning; Rat; Ontogeny; Gender-difference; Microsomal enzyme inducers; Electrophile response element activators

Over 10 years ago, research on mechanisms for the acquired resistance to anticancer drugs in cell lines led to the discovery of a membrane-associated transporter known as multidrug resistance protein 1 (MRP1, ABCC1), which belongs to the ATP-binding cassette (ABC) family of transporters [1]. Since then, eight additional human MRPs have been cloned [2–7]. Thus, the human MRP family is currently composed of nine members, MRP1–9.

Structurally, MRP1, 2, 3, 6, and 7 are composed of an amino-terminal transmembrane domain (TMD) and two tandem repeats consisting of a TMD and a nucleotide-binding domain (NBD), whereas MRP4, 5, 8, and 9 lack the amino-terminal TMD found in other MRPs [8]. Functional studies showed that MRP1, 2, and 3 confer resistance to a variety of anticancer drugs including anthracyclines, vinca alkaloids, and methotrexate [9–12], and transport organic anions such as glutathione and glucuronide conjugates [11,13–15]. However, little is known about the functions of MRP6 and 7. Unlike MRP1, 2, and 3, MRP4 and 5 do not confer resistance to anthracyclines or vinca alkaloids [8]. Overexpression of MRP4 and 5 is associated with increased cellular efflux of purine analogs (e.g., 6-mercaptopurine and thioguanine) and nucleoside-based antiviral drugs [e.g., 9-(2-phosphonylmethoxyethyl)adenine (PMEA)], thus resulting in resistance to the cytotoxic effects of these agents, and a decrease in the antiviral efficacy of PMEA [16–18]. MRP4 and 5 were also reported to transport cyclic nucleotides, such as cAMP and cGMP [19,20]. Furthermore, several compounds of physiological and pharmacological importance, such as methotrexate, estradiol-17β-glucuronide, bile acids, prostaglandins, and dehydroepiandrosterone-3-sulfate, were recently shown to be transported by MRP4 [21–24].

Although progress has been made in understanding the functions of MRP4, little is known about the regulation of MRP4 expression. For example, whether MRP4 expression can be altered under pathophysiological conditions or following treatment with prototypical

* Corresponding author. Fax: 1-913-588-7501.
E-mail address: cklaasse@kumc.edu (C.D. Klaassen).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.03.014

microsomal enzyme inducers (MEIs) remains largely undetermined. Such information is typically obtained by conducting studies in rats and mice. So far, however, only human MRP4 cDNA has been cloned, thus prohibiting further characterization of MRP4 regulation in laboratory animal models.

The purpose of the present study was to clone rat Mrp4 cDNA and to characterize the tissue distribution, gender, and ontogenic expression patterns of rat Mrp4 mRNA, as well as to determine whether hepatic and renal expression of rat Mrp4 mRNA is inducible following treatment with different classes of prototypical MEIs. We chose to clone rat Mrp4, because rat is a commonly used rodent species in the study of xenobiotic metabolism and disposition. During the final stage of our cloning effort, two sequences defined as rat Mrp4 cDNA (GenBank Accession No. NM_224522 and XM_341375) were deposited in GenBank. These sequences were predicted by automated computational analysis of the draft version of rat genome sequence. However, these two sequences are poorly aligned to human MRP4 and the predicted mouse Mrp4 cDNA, indicating that the predicted cDNA sequences for rat Mrp4 were not correctly annotated. In the present study, we report the cloning of two forms of rat Mrp4 cDNA, the full-length form and an alternatively spliced variant, and the characterization of Mrp4 mRNA expression under various conditions.

Materials and methods

Materials. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and oltipraz (OLTI) were a generous gift from Dr. Karl Rozman (University of Kansas Medical Center, Kansas City, KS) and Dr. Ronald Lubet (National Cancer Institute, Bethesda, MD), respectively. All other chemicals were obtained from Sigma–Aldrich (St. Louis, MO).

Cloning of rat Mrp4 cDNA. Four PCRs were performed sequentially to obtain the complete open reading frame (ORF) and about 500 bp of the 3′-untranslated region (3′-UTR) of rat Mrp4 cDNA. No sequence from the 5′-untranslated region (5′-UTR) was obtained. The 3′-cDNA fragment of Mrp4 was obtained by 3′-RACE (3′-rapid amplification of cDNA ends), using rat kidney Marathon–Ready cDNA (Clontech, Palo Alto, CA). The reverse primer (adapter primer) (5′-CCATCCTAATACGACTCACTATAGGGC-3′) was provided with the Marathon–Ready cDNA system, and the gene-specific forward primer (5′-GACAGAGACAGTTAGTGTGCCTTG-3′) was designed based on a partial cDNA sequence of rat Mrp4 (GenBank Accession No. AF376781). The 3′-RACE product (~1.0-kb) was cloned into pCR4-TOPO TA vector (Invitrogen, Carlsbad, CA) and sequenced. The second PCR was performed with a reverse primer (5′-TTCAGAATTGCCCTCGCAAG-3′) designed based on the sequence obtained from the 3′-RACE. The forward primer of the second PCR (5′-CAGTACCTCAAAGCTGCAAG-3′) was designed based on a rat EST clone (CB609524). This EST sequence is 561-bp long and it shares about 84% and 92% nucleotide sequence identity to part of the human MRP4 (NM_005845) and mouse (XM_139262) Mrp4 cDNAs, respectively. The product of the second PCR (~1.8-kb) was cloned into pSTBlue-1 vector and sequenced. The third PCR was performed using a reverse primer (5′-TGCTTTCTGGCCTCCGCTCAGCG-3′) which was designed based on the sequence of rat EST clone CB609524, and a forward primer (5′-ATGCTGCCGGTGCACACCGA-3′). The forward primer contains the translation start site. The fourth PCR was carried out with a reverse primer (5′-GGAAGCAAGTCGTC CATGTGTCCG-3′) designed from the sequence of the second PCR product and a forward primer (5′-GGATCGCCTACGTTTCCCAG CAGCC-3′) designed from the sequence of the third PCR product. The resulting products of the third and fourth PCRs were ~1.6 and ~1.1-kb, respectively, and were cloned into pCR4Blunt-TOPO vector (Invitrogen) for sequencing. For PCRs #2, 3, and 4, reverse transcription for first-strand cDNA synthesis was performed with high-quality rat kidney poly(A)$^+$ RNA (Clontech) using ThermoScript RT-PCR System (Invitrogen). Reactions were primed by both random hexamers and Oligo(dT)$_{20}$ to ensure production of full-length cDNAs. Synthesis of oligonucleotide primers used for PCR and sequencing were carried out by the Biotech Support Facility, University of Kansas Medical Center (Kansas City, KS). To ensure the accuracy of the sequence, a total of three independent 3′-RACE reactions were performed, and each of the other three PCRs was carried out three times, using three first-strand cDNAs that were generated by independent reverse-transcription reactions. The reported sequence of rat Mrp4 cDNA was obtained by assembling the consensus of PCR products produced by each of the four sets of PCRs. Fig. 1 illustrates the strategy used to clone rat Mrp4 cDNA.

Tissue expression study. Male and female Sasco Sprague–Dawley (SD) rats weighing 200–250 g were obtained from Charles River Laboratories (Wilmington, MA) and acclimated for 1 week in the AAALAC-accredited animal care facility at the University of Kansas

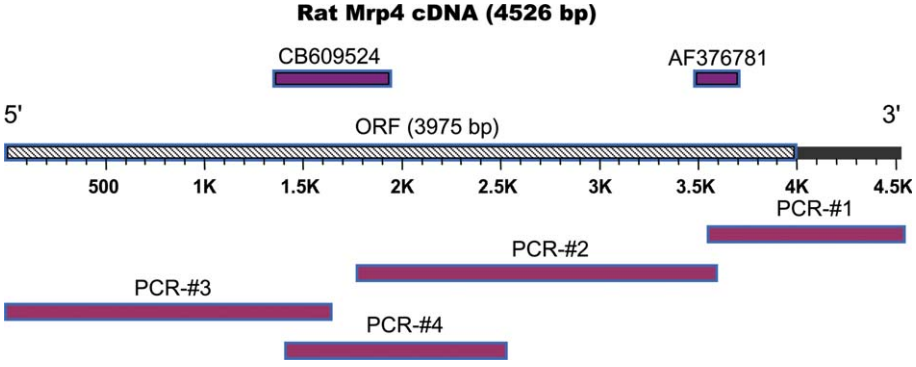

Fig. 1. Strategy for the cloning of rat Mrp4. The open reading frame (ORF) and 3′-untranslated region (3′-UTR) of rat Mrp4 are indicated by hedged and filled boxes, respectively. The two fragments above the ORF and 3′-UTR represent a partial rat Mrp4 cDNA (AF376781) and a rat EST clone (CB609524), which is highly similar to human MRP4 and mouse Mrp4. Also shown are the four PCR products from which the rat Mrp4 cDNA sequence reported here was assembled.

Table 1
Oligonucleotide probes generated for the quantification of rat Mrp4 mRNA levels using QuantiGene signal amplification assay

Function[a]	Sequence[b]	Target
CE	cggtttttcttcagaattgccTTTTTctcttggaaagaaagt	3567–3587
CE	cggattttctgttgtattaactcgTTTTTctcttggaaagaaagt	3636–3659
CE	tctgtgagcaatggtgagaacagTTTTTctcttggaaagaaagt	3680–3702
CE	ggtgagggcagcggcttTTTTTctcttggaaagaaagt	3839–3855
LE	ctcgcaaggcacactaactgtcTTTTTaggcataggacccgtgtct	3545–3566
LE	cagttgcttcatcaatgatcagtatcTTTTTaggcataggacccgtgtct	3588–3613
LE	tcagttctcggatccacatttgTTTTTaggcataggacccgtgtct	3614–3635
LE	tgcactgggcaaacttctccTTTTTaggcataggacccgtgtct	3660–3679
LE	cgctgtcaatgatggtgttcagTTTTTaggcataggacccgtgtct	3703–3724
LE	ctggattctgcagcaaaacatacTTTTTaggcataggacccgtgtct	3774–3796
LE	cgcccttacccagctgctTTTTTaggcataggacccgtgtct	3821–3838
LE	agtacgcctgttttgctgtttcTTTTTaggcataggacccgtgtct	3856–3877
BL	ccgaatccaaaaccattatcttgt	3725–3748
BL	ggctcatcatattctctcagtcttc	3749–3773
BL	gaaccatcttgtaaaagaggctct	3797–3820

[a] Function of the oligonucleotide probe in the QuantiGene assay: CE, capture extender; LE, label extender; and BL, blocker probe.
[b] Probes were designed with a T_m of about 63 °C, enabling hybridization conditions to be held constant (i.e., 53 °C) during the assay.

Medical Center. Animals (2–3 rats per cage) had free access to rodent chow (Harlan Sprague–Dawley, Indianapolis, IN) and water. Tissues (liver, kidney, lung, stomach, duodenum, jejunum, ileum, large intestine, cerebral cortex, cerebellum, and prostate) were removed, flash-frozen in liquid nitrogen, and stored at −80 °C until further use.

Ontogeny study. Pregnant SD rats were purchased at gestation day 13 (Charles River Laboratories) and housed in the aforementioned animal care facility. Livers and kidneys were removed from male and female SD rats at 0, 5, 10, 15, 20, 25, 30, 35, 40, and 45 days of age ($n = 5$/gender/age), frozen in liquid nitrogen, and stored at −80 °C until further use.

Inducibility by xenobiotics. Male SD rats (200–250 g) were treated with the following prototypical microsomal enzyme inducers that can be divided into six classes based on the mechanisms for cytochrome P450 (CYP) induction: aryl hydrocarbon (Ah) receptor ligands: TCDD (3.9 μg/kg/day × 1 day, i.p., in corn oil) and β-naphthoflavone (BNF, 100 mg/kg/day, i.p., in corn oil); constitutive androstane receptor (CAR) activators: phenobarbital (PB, 80 mg/kg/day, i.p., in saline) and diallyl sulfide (DAS, 500 mg/kg/day, i.p., in corn oil); pregnane X receptor (PXR) ligands: pregnenolone-16α-carbonitrile (PCN, 50 mg/kg/day, i.p., in corn oil), spironolactone (SPIRO, 75 mg/kg/day, i.p., in corn oil), and dexamethasone (DEX, 40 mg/kg/day, i.p., in corn oil); peroxisome proliferator-activated receptor α (PPARα) ligands: clofibrate (CFB, 200 mg/kg/day, i.p., in saline), diethylhexylphthalate (DEHP, 1200 mg/kg/day, in corn oil), and perfluorodecanoic acid (PFDA, 40 mg/kg/day × 1 day, i.p., in corn oil); electrophile response element (EpRE) activators: ethoxyquin (ETHOX, 50 mg/kg/day, p.o., in corn oil) and oltipraz (OLTI, 150 mg/kg/day, p.o., in corn oil); and CYP2E1 inducers: isoniazid (INH, 200 mg/kg/day, i.p., in saline), acetylsalicylic acid (ASA, 500 mg/kg/day, p.o., in corn oil), and streptozotocin (STREP, 100 mg/kg/day × 1 day, i.p., in 100 mM sodium citrate). These treatment regimens were previously shown to result in marked increases in the expression of the hallmark inducible genes for each class of inducers: CYP1A1 induction by Ah receptor ligands, CYP2B1/2 induction by CAR activators, CYP3A1/23 induction by PXR ligands, CYP4A2/3 induction by PPARα ligands, quinone reductase induction by EpRE activators, and CYP2E1 induction by its inducers [25]. Groups of controls received corn oil (i.p. or p.o.) or saline (i.p.). All animals were treated for 4 days unless otherwise noted and injection volume was 5 ml/kg. Tissues were collected as described above.

Total RNA isolation. Total RNA was isolated from frozen tissue samples using RNA-Bee reagent (Tel-Test, Friendswood, TX) according to the manufacturer's protocol. After spectrophotometrical quantification of RNA concentrations, samples were diluted with diethyl pyrocarbonate (DEPC)-treated water to a final concentration of 1 μg/μl. The integrity of the RNA samples was determined by visual examination of the integrity of 18S and 28S rRNA bands after resolving total RNA on 1.2% denaturing agarose gel. Purity of the isolated RNA samples was determined from the A_{260}/A_{280} ratio.

Measurement of rat Mrp4 mRNA levels with Quantigene branched DNA signal amplification assay. Oligonucleotide probes specific for rat Mrp4 mRNA used in the Quantigene branched DNA (bDNA) signal amplification assay (Genospectra, Fremont, CA) were designed using ProbeDesigner Software, Version 1.0 (Bayer Diagnostics Division, Tarrytown, NY). The detailed sequence information of these probes is listed in Table 1. Total RNA (10 μg per sample) was used to determine the levels of Mrp4 mRNA in a 96-well format as described in detail previously [26]. Data are presented as relative light units (RLU) per 10 μg total RNA.

Statistical analysis. Results of Mrp4 mRNA levels were expressed as means ± standard error (SE). Renal and hepatic levels of Mrp4 mRNA following treatment with various xenobiotics were analyzed by one-way analysis of variance (ANOVA) followed by the Newman–Keuls test. Mrp4 mRNA expression in male and female rats was compared with Student's t test. Differences were considered significant at $p < 0.05$.

Results and discussion

Characterization of rat Mrp4 cDNA

The total length of rat Mrp4 cDNA obtained in the present study from four separate PCRs is 4526 bp (Fig. 1). This sequence contains a 3975-bp long open reading frame (ORF) and a 551-bp 3′-UTR. Although the 3′-UTR ended with a 32-bp poly(A) stretch, the highly conserved polyadenylation sequence, AATAAA, was not found in the 3′-UTR. The deduced amino acid sequences of the rat Mrp4 cloned in the present study (AY533524), human MRP4 (NM_005845), and mouse Mrp4 (XM_139262) all have 1325 amino acid residues

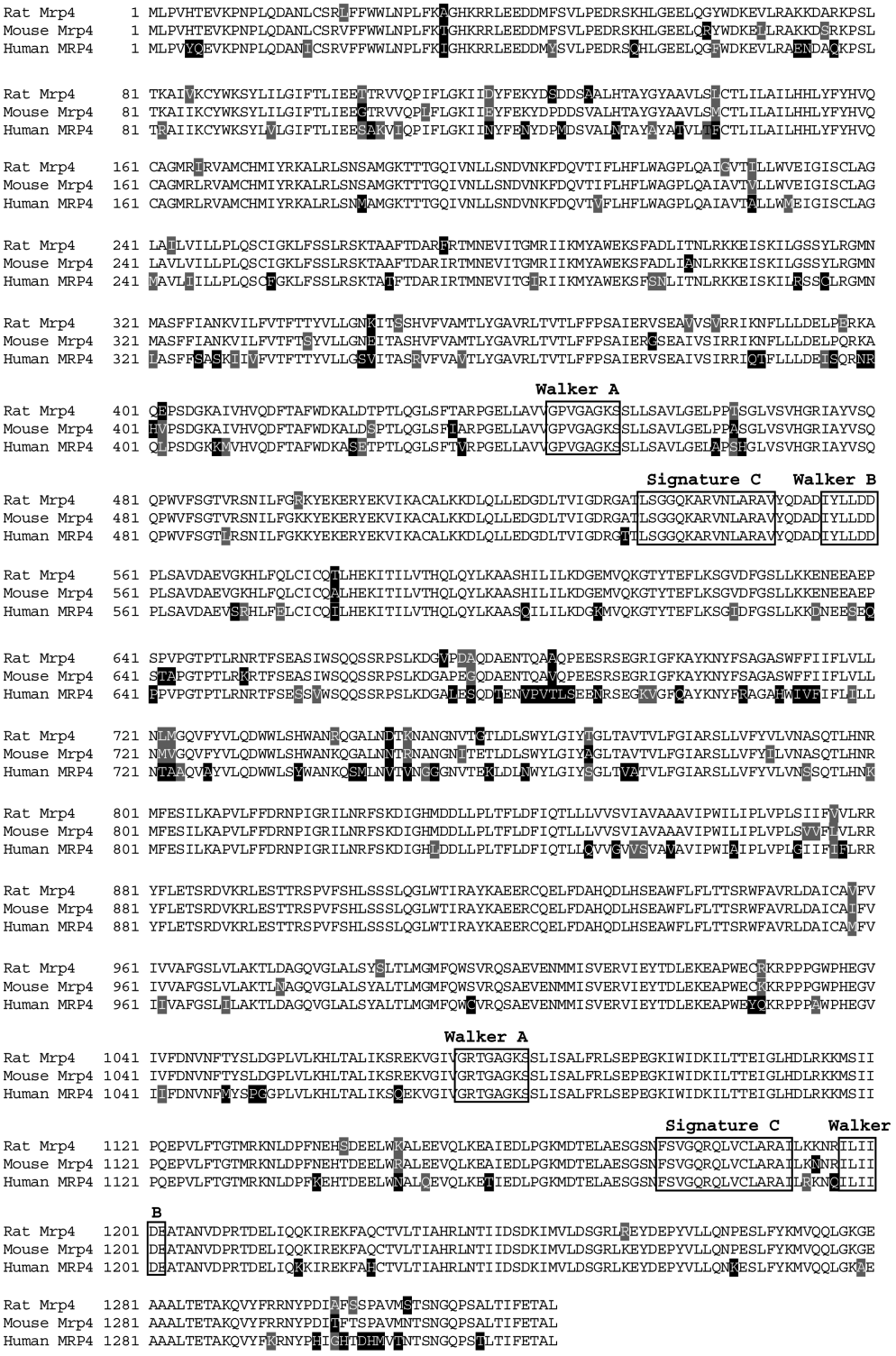

Fig. 2. Alignment of the deduced amino acid sequence of rat Mrp4, mouse Mrp4, and human MRP4. Identical residues are shown with white background and black letters, similar residues with gray background and white letters, and dissimilar residues with black backgrounds and white letters. Identity between rat and mouse sequences is 91%, and between the rat and human sequences is 83%. The highly conserved motifs within the nucleotide-binding domain of ATP-binding cassette transporters, such as Walker A and B, and Signature C are indicated by boxes.

(Fig. 2). It should be noted that molecular cloning of mouse Mrp4 has not been reported in the literature. The cDNA sequence under GenBank Accession No. XM_139262 is a predicted sequence of mouse Mrp4, which was deduced by annotation of the mouse genome from the mouse genome project. The amino acid

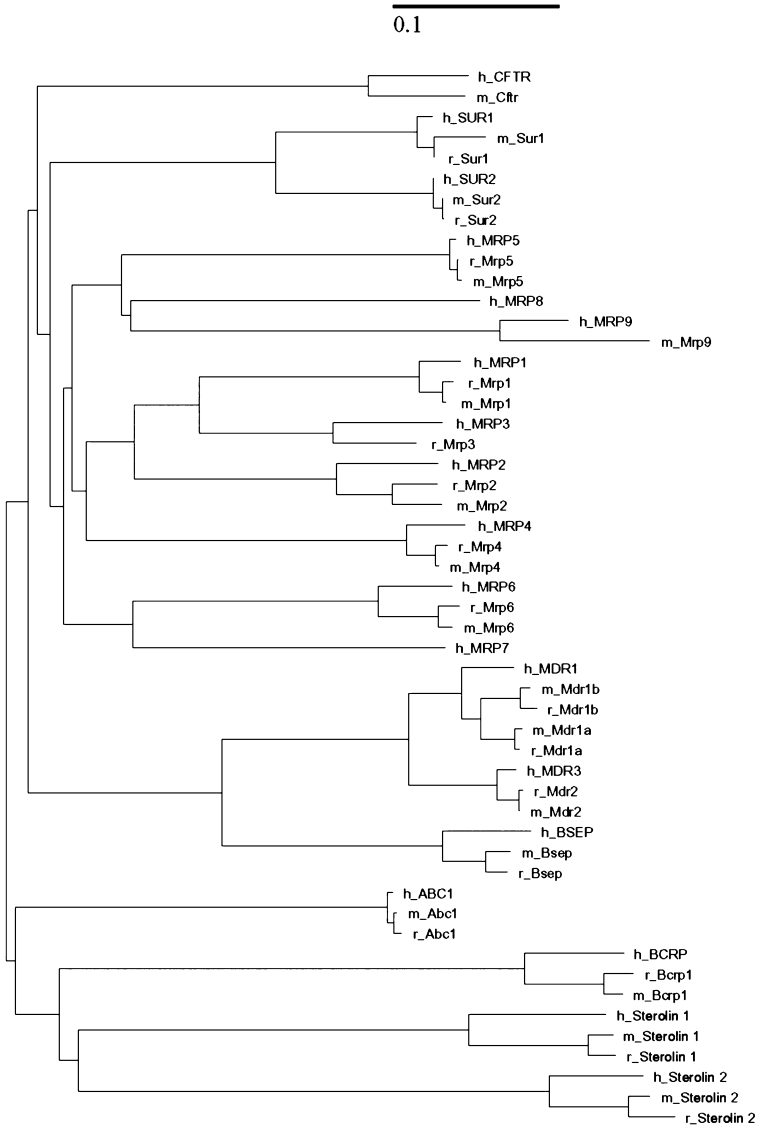

Fig. 3. Phylogenetic analysis of the known MRPs and other related transporters belonging to the family of ATP-binding cassette transporters, from human, mouse, and rat. Phylogenetic tree was constructed by the neighbor-joining method using CLUSTAL W [32]. Branch lengths are drawn to scale.

sequence identity between rat Mrp4 and human MRP4 is 83%. In addition, rat Mrp4 shares 91% amino acid sequence identity to mouse Mrp4. As shown in Fig. 2, the deduced amino acid sequences of rat, human, and mouse MRP4 contain two sets of ATP-binding cassettes, identified by the presence of highly conserved motifs known as Walker A and B, and Signature C. Phylogenetic analysis of the known MRPs and other related transporters belonging to the family of ATP-binding cassette transporters from human, mouse, and rat reveals that rat Mrp4 is closely related to human MRP4 and mouse Mrp4, and diverged with the rest of the MRP family members from the same root (Fig. 3). Taken together, it is reasonable to conclude that the rat Mrp4 reported in the present study is the ortholog of human MRP4 and mouse Mrp4.

Splice variant of rat Mrp4

During the cloning of rat Mrp4 cDNA, one splice variant of rat Mrp4 was obtained (AY533525). This variant differs from the rat Mrp4 cDNA sequence discussed earlier in that it is 111 bp shorter. Fig. 4 shows the exon boundaries at the 5′-end of the two forms of rat Mrp4 cDNA, revealed by comparison of the sequence of Mrp4 cDNA with that of Mrp4 gene. It is evident that the short form is an alternatively spliced variant of the full-length rat Mrp4 cDNA, and one exon is spliced out in the short form. To further examine which form of rat Mrp4 is predominantly expressed, we performed reverse transcription-PCR using primers that flank the spliced-out exon. From the expected amplicon size, we found that the full-length form is the predominantly expressed

```
Rat Mrp4 (long)     1 ATGCTGCCGGTGCACACCGAGGTGAAACCCAACCCGCTGCAGGACGCCAA
Rat Mrp4 (short)    1 ATGCTGCCGGTGCACACCGAGGTGAAACCCAACCCGCTGCAGGACGCCAA

                                          ↓
Rat Mrp4 (long)    51 CCTCTGCTCGCGCTTGTTCTTCTGGTGGCTCAACCCGTTGTTTAAAGCTG
Rat Mrp4 (short)   51 CCTCTGCTCGCGCTTGTTCTTCTG-------------------------
                                          ↑
Rat Mrp4 (long)   101 GACATAAGCGGAGATTGGAAGAAGATGACATGTTTTCAGTGCTTCCAGAA
Rat Mrp4 (short)   77 -------------------------------------------------

                                                  ↓
Rat Mrp4 (long)   151 GATCGCTCAAAGCACCTTGGAGAGGAGTTGCAAGGGTACTGGGATAAAGA
Rat Mrp4 (short)   77 ----------------------------------GTACTGGGATAAAGA
                                                  ↑
Rat Mrp4 (long)   201 AGTTCTGCGAGCCAAGAAGGACGCTCGGAAGCCTTCCTTAACGAAGGCAA
Rat Mrp4 (short)   90 AGTTCTGCGAGCCAAGAAGGACGCTCGGAAGCCTTCCTTAACGAAGGCAA

Rat Mrp4 (long)   251 TCGTGAAGTGTTACTGGAAATCTTACCTGATTTTGGGAATTTTTACGTTA
Rat Mrp4 (short)  140 TCGTGAAGTGTTACTGGAAATCTTACCTGATTTTGGGAATTTTTACGTTA

                        ↓
Rat Mrp4 (long)   301 ATTGAGGAGAACCACCCGAGTAGTTCAGCCCATATTTTTAGGGAAAA
Rat Mrp4 (short)  190 ATTGAGGAGAACCACCCGAGTAGTTCAGCCCATATTTTTAGGGAAAA
```

Fig. 4. Exon boundaries at the 5′-end of the two forms of rat Mrp4 cDNA, the full-length form (long) and an alternatively spliced form (short). Only the coding sequence (sense strand) is shown. The arrows indicate exon breakpoints. The exon that is absent in the splice variant is indicated with gaps (–).

form of rat Mrp4 in kidney, while the splice variant is undetectable (results not shown). Thus, the full-length form appears to be the physiologically relevant one.

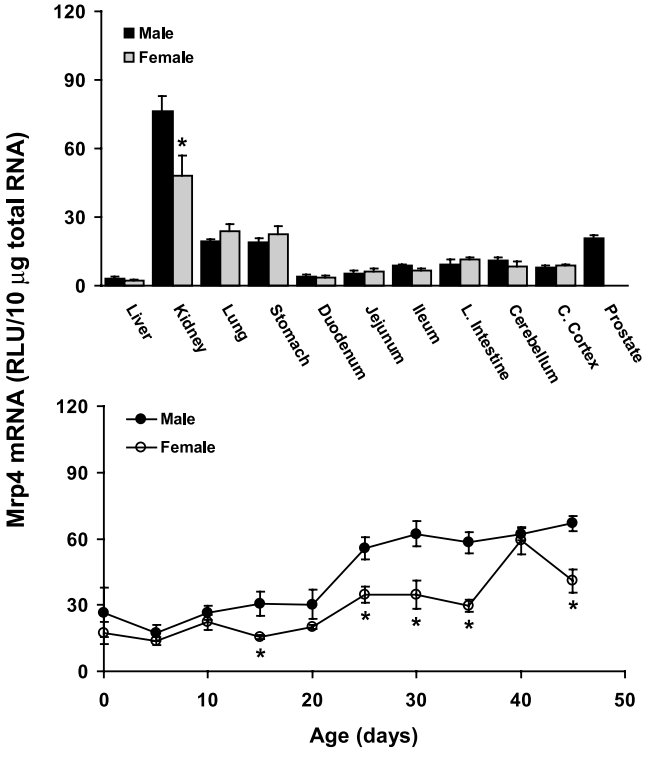

Fig. 5. Tissue distribution of rat Mrp4 mRNA in adults (top panel) and postnatal expression of Mrp4 mRNA in kidney (bottom panel). Relative light units (RLU) are expressed as the means ± SE of 5–6 rats. Asterisk indicates values significantly different from those of males.

Mrp4 mRNA tissue distribution and postnatal renal expression

Of the 11 tissues examined, the highest levels of Mrp4 mRNA were detected in both male and female kidney, with the expression in males higher than in females (Fig. 5, top panel). Mrp4 mRNA was expressed at similar levels in lung and prostate. In the gastrointestinal tract, the highest levels of Mrp4 mRNA were found in stomach. Levels of Mrp4 mRNA in the three segments of small intestine (i.e., duodenum, jejunum, and ileum) and large intestine were about 20–50% of those in stomach. Mrp4 mRNA was also expressed in cerebellum and cerebral cortex. Mrp4 mRNA expression in liver was lower than that in the other 10 tissues examined. In general, the tissue distribution of rat Mrp4 mRNA in the present study is in agreement with that of human MRP4 [3,5]. In two recent publications from our group, mRNA levels of rat Mrp4 in choroid plexus and placenta were determined with the same method used in the present study. We found that levels of Mrp4 in choroid plexus are similar to that in kidney, while placental expression of Mrp4 is lower than in kidney but higher than that in liver [27,28].

Because MRP4 was highly expressed in both rat (present study) and human [29] kidney, the pattern for postnatal renal expression of Mrp4 mRNA was examined. After birth, Mrp4 was expressed in kidneys of both genders (Fig. 5, bottom panel). Its level of expression remained relatively constant until postnatal day 20 in both genders. At postnatal day 25, the first time point

examined after weaning of rats, Mrp4 expression in both genders was about twice that seen in neonatal rats. Thereafter, Mrp4 expression remained relatively constant. A gender difference in renal Mrp4 expression was seen as early as 15 days of age (Fig. 5, bottom panel). At day 45, Mrp4 expression in male kidneys was about 50% higher than that in females.

Among the MRP family members, human MRP4 has a unique substrate profile in that it mediates cellular efflux of purine analogues used in cancer chemotherapy and nucleoside-based antiviral drugs. The difference in MRP4 expression in tissues may have an impact on therapeutic outcomes by affecting the accumulation of these drugs in specific tissues. The developmental and gender-specific expression patterns of MRP4 may lead to age- and gender-related differences in pharmacokinetics of these drugs.

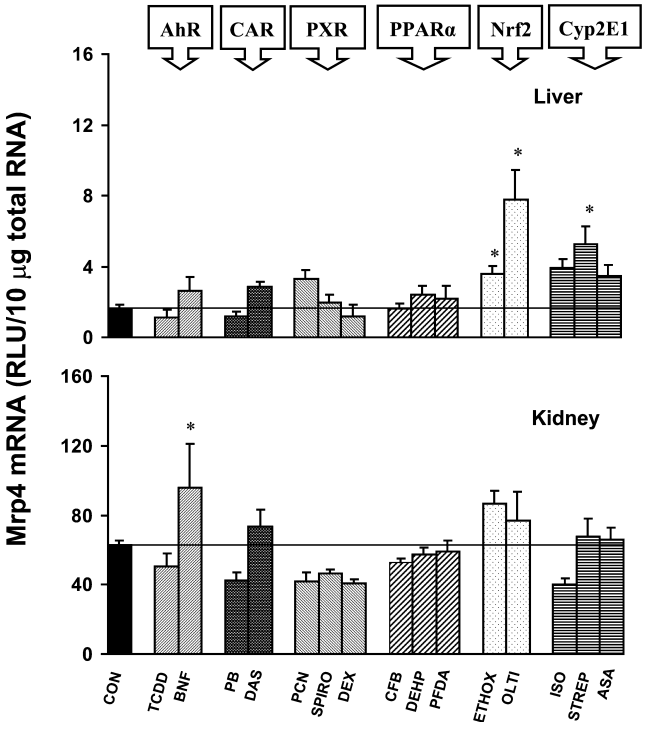

Fig. 6. Rat Mrp4 mRNA expression after treatment with microsomal enzyme inducers (MEIs). Mrp4 mRNA levels were quantified in liver (top panel) and kidney (bottom panel) of rats treated with six classes of MEIs as described in detail in the Materials and methods section. Controls received corn oil (i.p. or p.o.), or saline (i.p.), and were grouped together because no differences in Mrp4 mRNA expression were seen between these control groups. Ah receptor ligands: tetrachlorodibenzo-*p*-dioxin (TCDD) and β-naphthoflavone (BNF); CAR activators: phenobarbital (PB) and diallyl sulfide (DAS); PXR ligands: pregnenolone 16α-carbonitrile (PCN), spironolactone (SPIRO), and dexamethasone (DEX); PPARα ligands: clofibrate (CFB), diethylhexylphthalate (DEHP), and perfluorodecanoic acid (PFDA); EpRE activators: ethoxyquin (ETHOX) and oltipraz (OLTI); and CYP2E1 inducers: isoniazid (INH), acetylsalicylic acid (ASA), and streptozotocin (STREP). Relative light units (RLU) are expressed as the means ± SE of 5–6 rats. Asterisk indicates values significantly different from controls.

Inducibility of Mrp4 in kidney and liver by xenobiotics

The inducible expression of phase I and phase II biotransformation enzymes following exposure to prototypical microsomal enzyme inducers (MEIs) has been extensively examined over the past several decades. Recent data indicate that some classes of MEIs are capable of inducing Mrp2 and 3 hepatic expression [4,30]. In the present study, hepatic levels of Mrp4 mRNA were significantly increased by the EpRE activators ethoxyquin (ETHOX) and oltipraz (OLTI) (Fig. 6, top panel). While Mrp4 mRNA levels were elevated in liver of rats treated with three CYP2E1 inducers [isoniazid (INH), acetylsalicylic acid (ASA), and streptozotocin (STREP)], a statistically significant increase was only seen in STREP-treated animals. Treatment of rats with Ah receptor ligands [tetrachlorodibenzo-*p*-dioxin (TCDD) and β-naphthoflavone (BNF)], CAR activators [phenobarbital (PB) and diallyl sulfide (DAS)], PXR ligands [pregnenolone 16α-carbonitrile (PCN), spironolactone (SPIRO), and dexamethasone (DEX)], PPARα ligands [clofibrate (CFB), diethylhexylphthalate (DEHP), and perfluorodecanoic acid (PFDA)] did not have significant effects on the expression of Mrp4 mRNA in liver. In kidneys, Mrp4 expression was significantly increased only by BNF treatment, whereas the expression was consistently lower following treatment with PXR ligands ($p > 0.05$) (Fig. 6, bottom panel). Overall, these data indicate that rat Mrp4 mRNA expression in liver or kidney is not subjected to regulation by most of the MEIs. Because two inducers belonging to EpRE class of inducers (i.e., ETHOX and OLTI) moderately increased rat Mrp4 expression in liver, the EpRE signaling pathway may play a role in such induction.

Many chemicals are known to induce drug biotransformation enzymes and transporters, leading to an accelerated metabolism and excretion of many drugs. For example, induction of *P*-glycoprotein (Pgp) in enterocytes and CYP3A in hepatocytes by the herbal supplement St. John's Wort may be the cause of decrease in blood concentration and efficacy of cyclosporine, indinavir, and digoxin [31]. Although our results indicate that rat Mrp4 in liver and kidney is not readily inducible by several classes of prototypical MEIs, it is still possible that MRP4 could be induced by the treatment with some as yet untested chemicals. Given the importance of this transporter in the disposition of some of the anticancer and antiviral drugs, further research on the chemical induciblitliy of MRP4 is necessary.

Acknowledgments

The authors thank Dr. Nathan J. Cherrington (University of Arizona, Tucson, AZ) for his technical help during the initial stage of this work, and Dr. Supratim Choudhuri (US FDA, College Park, MD) for his advice on 3′-RACE and for his helpful comments during

preparation of the manuscript. The authors also thank members of Klaassen laboratory for their technical assistance in the animal studies. Work was supported by NIH Grant ES-090716.

References

[1] S.P. Cole, G. Bhardwaj, J.H. Gerlach, J.E. Mackie, C.E. Grant, K.C. Almquist, A.J. Stewart, E.U. Kurz, A.M. Duncan, R.G. Deeley, Science 258 (1992) 1650–1654.
[2] C.C. Paulusma, P.J. Bosma, G.J. Zaman, C.T. Bakker, M. Otter, G.L. Scheffer, R.J. Scheper, P. Borst, R.P. Oude Elferink, Science 271 (1996) 1126–1128.
[3] M. Kool, M. de Haas, G.L. Scheffer, R.J. Scheper, M.J. van Eijk, J.A. Juijn, F. Baas, P. Borst, Cancer Res. 57 (1997) 3537–3547.
[4] Y. Kiuchi, H. Suzuki, T. Hirohashi, C.A. Tyson, Y. Sugiyama, FEBS Lett. 433 (1998) 149–152.
[5] K. Lee, M.G. Belinsky, D.W. Bell, J.R. Testa, G.D. Kruh, Cancer Res. 58 (1998) 2741–2747.
[6] H.H. Kao, J.D. Huang, M.S. Chang, Gene 20 (286) (2002) 299–306.
[7] J. Tammur, C. Prades, I. Arnould, A. Rzhetsky, A. Hutchinson, M. Adachi, J.D. Schuetz, K.J. Swoboda, L.J. Ptacek, M. Rosier, M. Dean, R. Allikmets, Gene 273 (2001) 89–96.
[8] G.D. Kruh, M.G. Belinsky, Oncogene 20 (22) (2003) 7537–7552.
[9] S.P. Cole, K.E. Sparks, K. Fraser, D.W. Loe, C.E. Grant, G.M. Wilson, R.G. Deeley, Cancer Res. 54 (1994) 5902–5910.
[10] K. Koike, T. Kawabe, T. Tanaka, S. Toh, T. Uchiumi, M. Wada, S. Akiyama, M. Ono, M. Kuwano, Cancer Res. 57 (1997) 5475–5479.
[11] Y. Cui, J. Konig, J.K. Buchholz, H. Spring, I. Leier, D. Keppler, Mol. Pharmacol. 55 (1999) 929–937.
[12] N. Zelcer, T. Saeki, G. Reid, J.H. Beijnen, P. Borst, J. Biol. Chem. 276 (2001) 46400–46407.
[13] I. Leier, G. Jedlitschky, U. Buchholz, S.P. Cole, R.G. Deeley, D. Keppler, J. Biol. Chem. 269 (1994) 27807–27810.
[14] G. Jedlitschky, I. Leier, U. Buchholz, K. Barnouin, G. Kurz, D. Keppler, Cancer Res. 56 (1996) 988–994.
[15] T. Hirohashi, H. Suzuki, Y. Sugiyama, J. Biol Chem. 274 (1999) 15181–15185.
[16] J.D. Schuetz, M.C. Connelly, D. Sun, S.G. Paibir, P.M. Flynn, R.V. Srinivas, A. Kumar, A. Fridland, Nat. Med. 5 (1999) 1048–1051.
[17] K. Lee, A.J. Klein-Szanto, G.D. Kruh, J. Natl. Cancer Inst. 92 (2000) 1934–1940.
[18] P.R. Wielinga, G. Reid, E.E. Challa, I. van der Heijden, L. van Deemter, M. de Haas, C. Mol, A.J. Kuil, E. Groeneveld, J.D. Schuetz, C. Brouwer, R.A. De Abreu, J. Wijnholds, J.H. Beijnen, P. Borst, Mol. Pharmacol. 62 (2002) 1321–1331.
[19] G. Jedlitschky, B. Burchell, D. Keppler, J. Biol. Chem. 275 (2000) 30069–30074.
[20] Z.S. Chen, K. Lee, G.D. Kruh, J. Biol. Chem. 276 (2001) 33747–33754.
[21] G. Reid, P. Wielinga, N. Zelcer, I. van der Heijden, A. Kuil, M. de Haas, J. Wijnholds, P. Borst, Proc. Natl. Acad. Sci. USA 100 (2003) 9244–9249.
[22] Z.S. Chen, K. Lee, S. Walther, R.B. Raftogianis, M. Kuwano, H. Zeng, G.D. Kruh, Cancer Res. 62 (2002) 3144–3150.
[23] M. Rius, A.T. Nies, J. Hummel-Eisenbeiss, G. Jedlitschky, D. Keppler, Hepatology 38 (2003) 374–384.
[24] N. Zelcer, G. Reid, P. Wielinga, A. Kuil, I. van der Heijden, J.D. Schuetz, P. Borst, Biochem. J. 371 (2003) 361–367.
[25] N. Li, D.P. Hartley, N.J. Cherrington, C.D. Klaassen, J. Pharmacol. Exp. Ther. 301 (2002) 551–560.
[26] D.P. Hartley, C.D. Klaassen, Drug Metab. Dispos. 28 (2000) 608–616.
[27] T.M. Leazer, C.D. Klaassen, Drug Metab. Dispos. 31 (2003) 153–167.
[28] S. Choudhuri, N.J. Cherrington, N. Li, C.D. Klaassen, Drug Metab. Dispos. 31 (2003) 1337–1345.
[29] R.A. van Aubel, P.H. Smeets, J.G. Peters, R.J. Bindels, F.G. Russel, J. Am. Soc. Nephrol. 13 (2002) 595–603.
[30] J.L. Staudinger, A. Madan, K.M. Carol, A. Parkinson, Drug Metab. Dispos. 31 (2003) 523–527.
[31] D. Durr, B. Stieger, G.A. Kullak-Ublick, K.M. Rentsch, H.C. Steinert, P.J. Meier, K. Fattinger, Clin. Pharmacol. Ther. 68 (2000) 598–604.
[32] N. Saitou, M. Nei, Mol. Biol. Evol. 4 (1987) 406–425.