

# Translational Regulation of Rat Multidrug Resistance-Associated Protein 2 Expression Is Mediated by Upstream Open Reading Frames in the 5' Untranslated Region

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## ABSTRACT

Multidrug resistance-associated protein 2 (Mrp2/Abcc2), an organic anion transporter present in the apical membrane of hepatocytes, renal epithelial cells, and enterocytes, is postulated to undergo translational regulation. Transcription of rat hepatic Mrp2 mRNA is initiated at multiple sites (–213, –163, –132, and –98 nucleotides relative to the Mrp2 ATG) and contains potential upstream open reading frames (uORFs) in the 5' untranslated region (UTR) starting at –213, –149 and –109 nucleotides. Ribonuclease protection assays demonstrated that transcription of the *Mrp2* gene at the various initiation sites was tissue-specific, with the major initiation site in the liver and kidney being –98 and –132 nucleotides, respectively. In the jejunum, the primary and secondary initiation sites were –98 and –132 nucleotides, respectively, with the con-

verse true in the ileum. The relative abundance of these Mrp2 transcripts expressed in tissues varied with age from birth to the adult. HepG2 transient expression assays and in vitro translation assays in which the 5'UTRs were fused with a luciferase reporter showed that the 5'UTR without any uORF (–98 nucleotide) expressed maximal luciferase activity compared with those with one (–132 nucleotides), two (–163 nucleotides), or three (–213 nucleotides) uORFs. Disruption of the uORF by site-directed mutagenesis at nucleotide –109 enhanced luciferase activity 2- to 3-fold, whereas disruption of the uORF at nucleotide –149 had little effect. We conclude that among the uORFs in the Mrp2 5'UTR, the uORF starting at nucleotide –109 probably plays an important role in the regulation of Mrp2 protein expression.

The multidrug resistance-associated protein 2 (rat Mrp2 or human MRP2), a member of the ATP-binding cassette gene superfamily of transport proteins, is present in the apical membrane of hepatocytes, enterocytes, and renal proximal tubules. Mrp2 protein mediates efflux of organic anions, such as glutathione, glucuronide, and sulfate conjugates, against a concentration gradient from hepatocytes into bile (Jansen et al., 1987; Paulusma et al., 1996; König et al., 1999; Gerk and Vore, 2002) and also contributes to bile flow by mediating the canalicular excretion of glutathione (GSH) (Ballatori and Truong, 1992).

Regulation of Mrp2 expression has been characterized primarily at the transcriptional level in rats and mice. Exposure to ligands for the nuclear receptors, such as Nrf2, constitutive androstane receptor, and PXR, increases Mrp2 protein expression in rat primary hepatocyte cultures (Kast et al.,

2002), whereas studies in mice have shown that constitutive androstane receptor and Nrf2 agonists, but not PXR agonists, increase Mrp2 mRNA expression (Maher et al., 2005; Wagner et al., 2005), supporting the importance of transcriptional regulation. However, in rats treated with pregnenolone-16 $\alpha$ -carbonitrile (PCN), a PXR agonist, hepatic Mrp2 mRNA expression is unchanged, whereas Mrp2 protein expression is increased 2- to 3-fold (Johnson and Klaassen, 2002; Johnson et al., 2002; Jones et al., 2005). Ethinyl estradiol treatment markedly decreases Mrp2 protein in rat liver, whereas Mrp2 mRNA remains unchanged (Trauner et al., 1997). Likewise, hepatic Mrp2 protein in the pregnant rat is significantly decreased by 50%, whereas Mrp2 mRNA is unchanged (Cao et al., 2001, 2002). Along the rat small intestine, Mrp2 protein is decreased by 90% in the distal ileum relative to that in the jejunum, whereas Mrp2 mRNA does not change significantly (Mottino et al., 2000). The inconsistency between the changes in Mrp2 mRNA and protein expression indicates that under some conditions, rat Mrp2 protein expression undergoes post-transcriptional regulation.

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**ABBREVIATIONS:** Mrp2 or MRP2, rat or human multidrug resistance-associated protein 2; PXR, pregnane X receptor; PCN, pregnenolone-16 $\alpha$ -carbonitrile; UORF, upstream open reading frame; UTR, untranslated region; RPA, ribonuclease protection assay; PCR, polymerase chain reaction; SV40, simian virus 40.

Post-transcriptional regulation of protein expression can occur through changes in mRNA stability, in the rate of protein degradation, or in the rate of protein synthesis. The minimal changes in Mrp2 mRNA expression in female control, pregnant, and PCN-treated rats argue against significant differences in Mrp2 mRNA stability as being likely to contribute to the mechanism of post-transcriptional regulation of rat Mrp2 that could account for the 4- to 5-fold differences in Mrp2 protein expression among these groups. We recently showed that altered rates of hepatic Mrp2 protein degradation cannot explain the differences in its protein expression in control, pregnant, and PCN-treated rats, whereas decreased and increased rates of Mrp2 protein synthesis were observed in pregnant and PCN-treated rats, respectively (Jones et al., 2005). In the present studies, we therefore focused on the potential mechanism for translational regulation of Mrp2 protein synthesis.

Accumulating evidence indicates that upstream open reading frames (uORFs) are important regulators of mRNA translation (Gray and Wickens, 1998; van der Velden and Thomas, 1999; Morris and Geballe, 2000), which can be explained by the ribosomal scanning model. Translation of the downstream main ORF of a gene by ribosomes occurs through leaky scanning of any AUGs in the 5' untranslated region (5'UTR) when the sequence around the upstream AUGs is suboptimal (Kozak, 1986), or through reinitiation when the translation machinery is not dissociated from the mRNA chain after termination of translation of uORFs (Morris and Geballe, 2000). We identified four transcription initiation sites in rat hepatic Mrp2 cDNA that occur at -213, -163, -132, and -98, where the ATG of the Mrp2 coding gene is numbered +1, +2, and +3 (Jones et al., 2005) (Fig. 1A). In the present study, we fused these Mrp2 5'UTRs upstream of the luciferase reporter gene and investigated their effect on luciferase expression in HepG2 cells in transient expression assays and on the translation efficiency of the luciferase transcript in *in vitro* translation assays. We also used ribonuclease protection assay (RPA) to identify the transcription initiation sites in rat liver, kidney, and small intestine and during postnatal development. We found that these tissues use different Mrp2 transcription initiation sites, and that translation from these transcripts is greatly influenced by the presence of the uORF at -109 nucleotides.

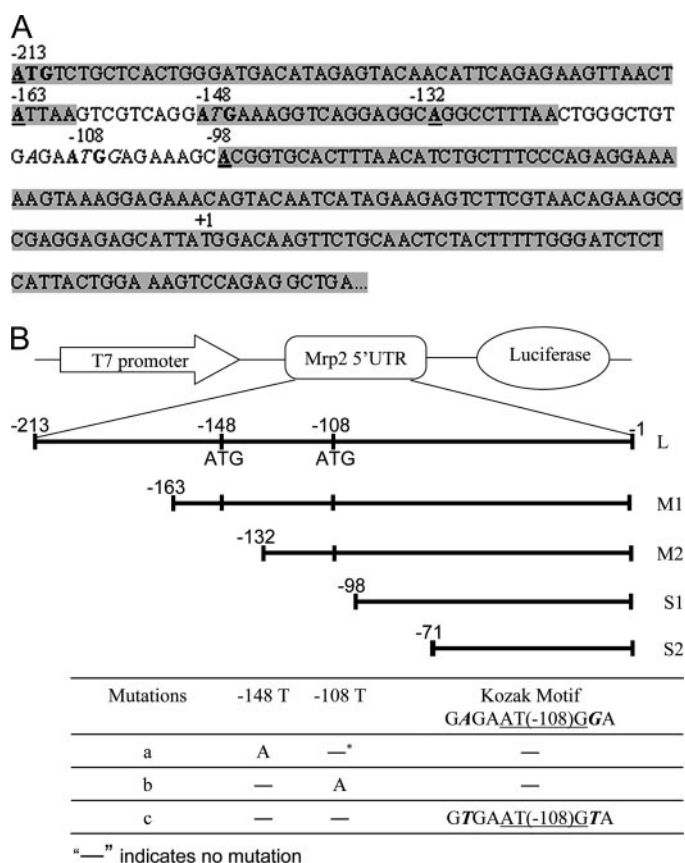
## Materials and Methods

**Materials.** [ $\alpha$ - $^{32}$ P]UTP (800 Ci/mmol) and [ $^{35}$ S]methionine (1000 Ci/mmol) were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Unless otherwise noted, all other chemicals were of analytical grade and of cell culture grade from Sigma Chemical Co. (St. Louis, MO), Invitrogen (Carlsbad, CA), Roche Diagnostics (Indianapolis, IN), and Fisher Scientific (Pittsburgh, PA). Restriction enzymes were obtained from Invitrogen and Promega (Madison, WI).

**Animals.** Adult female Sprague-Dawley rats whose weights were  $215 \pm 25$  g were obtained from Harlan Industries (Indianapolis, IN). The rats had free access to water and food and were maintained on an automatically timed 12-h light/12-h dark cycle. All experimental protocols involving animals were approved by the Institutional Animal Care and Use Committee of the University of Kentucky and conducted according to National Institutes of Health guidelines for the care and use of laboratory animals. To determine postnatal changes, female pups were removed at various times after birth, and tissues were immediately removed and frozen in liquid nitrogen until isolation of RNA.

**Ribonuclease Protection Assay.** Postnatal and control (adult) rat liver, kidney, placenta, lung, and small intestine were removed immediately after decapitation and frozen in liquid nitrogen. Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions.

The luciferase control vector (Promega) was fused at cloning sites HindIII and BamHI with the Mrp2 5'UTR cDNA sequence starting at -1 to -214 relative to the ATG (numbered as +1, +2, and +3) of the Mrp2 coding region. A double-stranded 280-base pair fragment containing the T7 promoter and the Mrp2 5'UTR was purified after the fusion luciferase vector was digested with PvuII and BamHI. [ $\alpha$ - $^{32}$ P]UTP labeled Mrp2 probe was prepared according to the instructions of MAXIscript T7 kit (Ambion, Austin, TX) using the 280-base pair fragment as template. RPA was performed according to manufacturer's instructions (RPA III Kit; Ambion). In brief, total RNA was incubated with the Mrp2 probe in the mixture containing 0.5 M ammonium acetate and 2.5 volumes of ethanol. After coprecipitation of the probe with total RNA after incubation at -80°C for 90 min, the RNA pellet was washed once with 75% ethanol and



**Fig. 1.** A, the full-length 5'UTR cDNA of rat Mrp2. Transcription initiation sites are underlined and in bold and are located at -213, -163, -132, and -98, relative to the A<sup>+</sup>TG, which is the translation start codon of the rat *Mrp2* gene. The shaded sequences represent the uORFs, and their start codons ATGs are in bold. The uORF<sup>-109</sup> starts at -109; uORF<sup>-149</sup> starts at -149. The first uORF starting at -213 is in-frame; the uORF<sup>-109</sup> and uORF<sup>-149</sup> are out-of-frame with the Mrp2 ORF. The uORF<sup>-109</sup> terminates at +60 inside the Mrp2 coding region and has a perfect Kozak motif. The italicized nucleotides were mutated to disrupt the uORFs or Kozak motif. B, schematic representation of Mrp2 5'UTR-luciferase constructs. The inserts are shown to be fused in the pGL3 vector or in the T7 control vector between the T7 promoter and the luciferase protein-coding region. Wild-type Mrp2 5'UTRs are shown as the bold lines to scale. The point mutations in the wild type 5'UTRs are listed in the table. a, mutation of the nucleotide T at -148 to A (disruption of the uORF<sup>-148</sup>); b, mutation of the nucleotide T to A (disruption of the uORF<sup>-109</sup>); c, mutation of both the nucleotides A at -112 and G at -106 to T (disruption of Kozak motif).

dissolved in hybridization solution. The hybridization reaction was incubated at gradient annealing temperature from 56°C to 36°C at the rate of 2°C per 2 h. Single-stranded RNA was digested by RNase A/T1 mix at 37°C for 1 h. The fragments protected from RNase digestion were identified by electrophoresis on 6% polyacrylamide, denaturing gels.

**Plasmid Construction.** The Mrp2 5'UTR cDNA is shown in Fig. 1A. cDNA sequences of the wild-type Mrp2 5'UTRs (Fig. 1B) L, M1, M2, and S1 were PCR-amplified using the forward primers TRF1, TRF2, TRF3, and TRF4, respectively (Table 1). The reverse primer for L and M1 PCR amplification was CONR. The reverse primer for amplifying M2 and S1 was T7R1. 5'UTR cDNAs were ligated upstream of the ATG of the firefly luciferase reporter gene into the pGL3 control vector (Promega) for transient cotransfection assays in HepG2 cells, and into the T7 control vector (Promega) for in vitro translation assays.

cDNA sequences of the 5'UTRs deL, deM, and S2 were PCR-amplified using forward primers TRF1, TRF2, and TRF5, respectively, and the reverse primer TRRR that deleted one nucleotide from T7R1 (Table 1), resulting in uORF<sup>-109</sup> being in-frame with the luciferase reporter gene ORF. These fragments were cloned into the T7 control vector (Promega).

uORFs were disrupted by introducing a point mutation into start codons, ATG→AAG, using the corresponding wild-type constructs as templates. The point mutations of the nucleotides T at -148 and -108 to A were termed "a" and "b," respectively; the mutation of the Kozak motif flanking the ATG at -109 was termed "c" (Fig. 1B). Mutagenesis was performed with the use of the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. All the structures of plasmids were confirmed by DNA sequencing.

**HepG2 Cell Transient Cotransfection Assays.** HepG2 cells were cultured in Dulbecco's modified Eagle's medium/Ham's F12 media (1:10) supplemented with 10% charcoal-stripped fetal bovine serum (Hyclone Laboratories, Logan, UT), 3.58 mM glutamine, 55 µg/ml gentamycin, and 1 µg/ml insulin (Invitrogen). One day before transfection, culture medium was replaced by phenol red-free Dulbecco's modified Eagle's medium supplemented with 10% charcoal stripped fetal bovine serum, glutamine, and gentamycin. The plasmids (1 µg) were transfected by the ProFectin mammalian transfection system-calcium phosphate (Promega) into HepG2 cells together with 30 ng of pSV40-Ren (Promega), used as an internal control for transfection efficiency. After 5 to 6 h of incubation, the transfection medium was replaced with maintenance medium. Cells were harvested 24 h later for measurement of the firefly and *R. reniformis* luciferase activities by the Dual-luciferase reporter assay system (Promega). The firefly luciferase activity was normalized to *R. reniformis* luciferase activity.

**In Vitro Translation Assays.** Each tested Mrp2 5'-UTR-luciferase construct was linearized by PvuII and SacI. Capped and [ $\alpha$ -<sup>32</sup>P]UTP-labeled firefly luciferase transcripts that were fused with Mrp2 5'UTRs were synthesized in vitro with the PvuII-SacI fragments as templates, using the mMESSAGE mMACHINE T7 kit

(Ambion), according to the manufacturer's instructions. Transcription efficiency was quantified by liquid scintillation counting of <sup>32</sup>P incorporation into RNA. The integrity and size of the luciferase transcripts were verified by formaldehyde-agarose gel electrophoresis. The luciferase protein was synthesized from the capped luciferase transcripts in vitro, according to the manufacturer's procedure of the rabbit reticulocyte lysate system (Promega). In brief, 0, 2, 4, 10, or 20 ng of the luciferase transcript was added to a reaction mixture. The translation reaction was immediately incubated at 30°C for 60 min and terminated by moving onto ice. The firefly luciferase activity was measured by the Luciferase Assay System (Promega) according to the manufacturer's instructions.

**In Vitro Expression in Coupled Transcription/Translation System.** The TNT quick coupled transcription/translation system (Promega) was used to translate the luciferase protein using tested constructs as templates. In accordance with the manufacturer's instructions, a reaction mixture (50 µl) containing 40 µl of TNT Master Mix, 2 µl of [<sup>35</sup>S]methionine was incubated at 30°C for 90 min. The translated products were separated on 4 to 20% gradient denaturing SDS-PAGE, and data was processed using the STORM 840 PhosphorImager (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

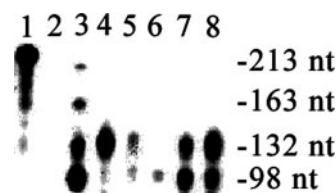
**Data Calculation.** RPA bands were quantified by densitometry using Quantity One 1-D Analysis Software (Bio-Rad Laboratories). Linear regression analysis was performed by Prism 4.0 (GraphPad Software, San Diego, CA).

## Results

### Identification of Rat Mrp2 Transcription Initiation Sites and Their Abundance in Rat Tissues by RPA.

Four transcription initiation sites have been identified at -213, -163, -132, and -98 in the rat hepatic Mrp2 5'UTR (Fig. 1A) (Jones et al., 2005). We next investigated the transcription initiation sites in various rat tissues to determine whether their use might be tissue-specific. Four transcription initiation sites were detected in rat liver; the site at -98 was the primary site, and the site at -132 was the secondary site (Fig. 2). The transcription initiation sites in other tissues were different from that in the liver. In the placenta and kidney, the primary site was located at -132; other sites were not detected. The primary site in the lung was at -98; other transcription initiation sites were not detected. In the jejunum, the primary and secondary sites were located at -98 and -132, but were located at -132 and -98, respectively, in the ileum; other sites were not detected.

We next investigated whether utilization of the transcription initiation sites might vary with age. In the liver, the



**Fig. 2.** Identification of transcription initiation sites of the *Mrp2* gene and the relative abundance in rat tissues by RPA. The [ $\alpha$ -<sup>32</sup>P]UTP-labeled Mrp2 probe of 280 nucleotides was synthesized and contained the Mrp2 5'UTR from -1 to -214 and the T7 promoter. Total RNA was incubated with the Mrp2 radiolabeled probe. After coprecipitation and hybridization, the single strand RNA was degraded by RNase A/T1. The fragments protected from RNase digestion were identified by electrophoresis on 6% polyacrylamide denaturing gel. Lane 1, Mrp2 probe (20 µg of yeast without RNase treatment); lane 2, 20 µg of yeast RNA; lane 3, 10 µg of liver RNA; lane 4, 80 µg of kidney RNA; lane 5, 80 µg of placenta RNA; lane 6, 80 µg of lung RNA; lane 7, 40 µg of jejunum RNA; lane 8, 40 µg of ileum RNA. A figure representative of four experiments is shown.

TABLE 1

Primers used for plasmid construction

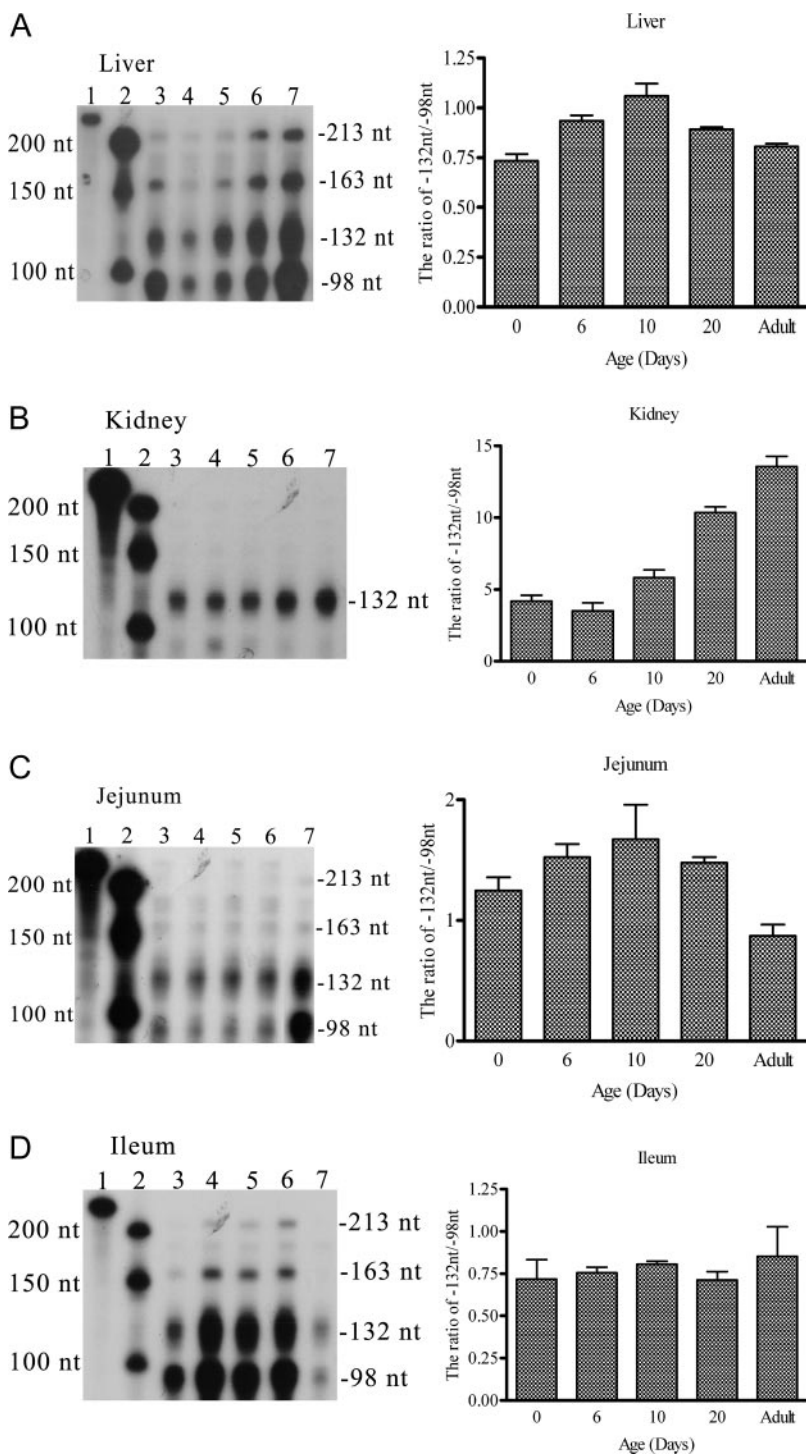
Listed primers used for PCR amplification of rat Mrp2 5'UTR cDNAs are shown, with the cloning sites underlined.

Name	Sequence (5'-3')	Cloning Site
TRF1	5'- <u>ggaagctt</u> atgtctgctcactgga-3'	HindIII
TRF2	5'- <u>ggaagctt</u> attaagtcgcaggatga-3'	HindIII
TRF3	5'- <u>tcaaaagctt</u> aggcctttaactgggctg-3'	HindIII
TRF4	5'- <u>ggaagctt</u> acgggtgcactttaacatctg-3'	HindIII
TRF5	5'- <u>ggaagctt</u> agaggaaaaagtaaggag-3'	HindIII
CONR	5'- <u>acccatggt</u> aatgctctcctcgcgc-3'	NcoI
T7R1	5'- <u>ggggatccc</u> aatgctctcctcgcgc-3'	BamHI
T7R2	5'- <u>tttggatcca</u> atgctctcctcgcgc-3'	BamHI
TRRR	5'- <u>ggggatccc</u> aatgctctcctcgcgc-3'	BamHI



ratio of expression of the transcript starting at  $-132$  to the transcript starting at  $-98$  was  $0.73 \pm 0.06$  at day 0, increased to  $1.06 \pm 0.10$  at day 10, and then decreased to  $0.80 \pm 0.03$  in the adult (Fig. 3A). In the kidney, the ratio was similar at days 0 and 6 (approximately 4) and increased to  $13.4 \pm 1.2$  in adulthood (Fig. 3B). In the jejunum, the ratio was  $1.2 \pm 0.19$  at day 0, increased to  $1.5 \pm 0.02$  at day 20, and then decreased to  $0.87 \pm 0.1$  in the adult (Fig. 3C). In the ileum, the ratio was very similar from day 0 to adulthood ( $0.70$ – $0.85$ ) (Fig. 3D). The data indicated that the changes in expression of Mrp2 transcripts with age were relatively minor compared with those among the tissues.

**Effect of the Rat Mrp2 5'UTR on Expression of the Luciferase Reporter Gene in Transiently Transfected HepG2 Cells.** To determine whether the various 5'UTRs differentially influenced Mrp2 protein expression, the effect of the Mrp2 5'UTR on expression of the firefly luciferase reporter gene was determined in transiently transfected HepG2 cells. Fusion plasmids were constructed by inserting the Mrp2 5'UTRs into the pGL3 control vector immediately upstream of the luciferase reporter gene. In addition, the wild-type 5'UTRs were altered by disruption of the uORFs to determine the influence of the uORFs on protein expression. Fusion plasmids were transiently cotransfected individually



**Fig. 3.** The relative abundance of transcription initiation sites of *Mrp2* gene in various postnatal rat tissues and adult by RPA. Rats were decapitated at days 0, 6, 10, 20, and adult. Liver (A), kidney (B), jejunum (C), and ileum (D) were taken from rats and frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until total RNA isolation. Liver RNA ( $10\text{ }\mu\text{g}$ ) and  $20\text{ }\mu\text{g}$  of kidney, jejunum, and ileum RNA were used. Lane 1, Mrp2 probe ( $10\text{ }\mu\text{g}$  of yeast RNA without Rnase treatment); lane 2, labeled RNA ladders ( $200\text{nt}$ ,  $150\text{nt}$ ,  $100\text{nt}$ ); lane 3, day 0; lane 4, day 6; lane 5, day 10; lane 6, day 20; lane 7, adult. Ribonuclease protection assay representative of three similar experiments obtained at various time points is shown. The histograms represent the ratio obtained from densitometric band quantification of the expressed transcript starting at  $-132$  to the transcript starting at  $-98$ . The data are represented as mean  $\pm$  S.D. of three separate experiments.

with pSV40-Ren into HepG2 cells, and firefly luciferase activity normalized to *Renilla reniformis* luciferase activity of cell extracts.

Compared with pGL3 control vector without any Mrp2 5'UTR (Fig. 4A), L decreased the luciferase activity by 60%, whereas  $L^b$  increased the luciferase activity 2-fold relative to L.  $L^{a+b}$  did not change luciferase activity relative to  $L^b$ . Likewise, the wild-type M1 expressed the lowest luciferase activity (25% of pGL3) (Fig. 4B).  $M1^b$  increased the luciferase activity by 3-fold, compared with M1, whereas  $M1^{a+b}$  resulted in luciferase activity similar to that of  $M1^b$ . Taken together, these data implied that the uORF<sup>-109</sup> was more important in regulation of expression compared with the uORF<sup>-149</sup>.

**Effect of the Rat Mrp2 5'UTR on the Translation Efficiency of the Luciferase Transcript by in Vitro Translation Assays.** Because the luciferase protein expression in HepG2 cells required both transcription and translation processes, we next investigated the influence of the various rat Mrp2 5'UTRs on the translation efficiency of mRNA. The capped luciferase transcripts were prepared using PvuII-SacI fragments as templates in which Mrp2 5'UTRs were located immediately upstream of the luciferase. The capped luciferase transcripts were added to the rabbit reticulocyte lysate to determine the effect of 5'UTRs on translation efficiency under conditions of linearity with respect to transcript concentration. Translation efficiency was

calculated from the linear relationship between the luciferase activities and mRNA concentrations.

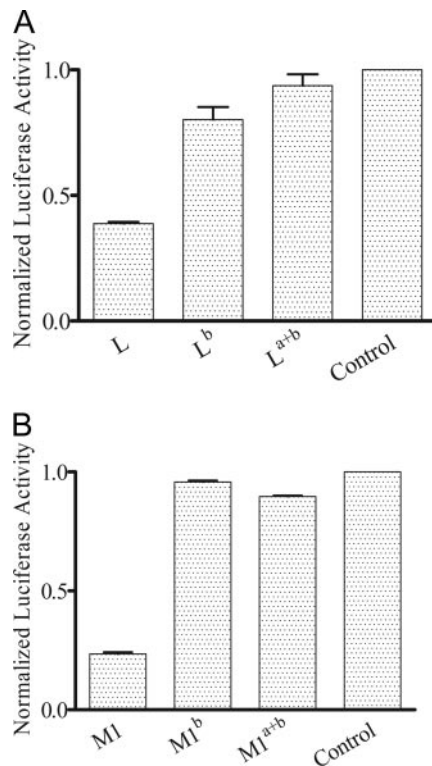
Translation efficiency of the capped luciferase transcripts with S1, M2, and L was 67-, 37-, and 15-fold higher than that of M1 (Fig. 5A). We next investigated contributions of the uORFs to the marked differences in translation efficiency of the various transcripts.  $L^b$  increased translation efficiency 3-fold, whereas  $L^a$  decreased translation efficiency 80% compared with the wild-type L (Fig. 5B). Translation efficiency of wild-type M1 was 31- and 6-fold lower than that of  $M1^b$ , and  $M1^a$  (Fig. 5C). Compared with the wild-type M2,  $M2^b$  increased translation efficiency by 4-fold, whereas disruption of the Kozak motif in  $M2^c$  increased translation efficiency only 1.7-fold (Fig. 5D).

**In Vitro Expression in Coupled Transcription/Translation System.** The marked effect of the uORF<sup>-109</sup> on translation efficiency suggested that this uORF serves as a translation start site. To determine whether translation could be initiated at uORF<sup>-109</sup>, we inserted the Mrp2 5'UTRs into the T7 control vector in such a way that the uORF<sup>-109</sup> was in-frame with the luciferase ORF. When the plasmids containing deL and deM1 were used as templates, a peptide of a higher molecular weight was produced in the coupled transcription/translation system (Fig. 6, lanes 1 and 5). The higher molecular weight peptide was not produced when the plasmid containing S2 was used as template (lane 3) or when the uORF<sup>-109</sup> in deL and deM1 was disrupted (Fig. 6, lanes 2 and 4). Detection of a higher molecular weight peptide when wild-type plasmids containing the uORF<sup>-109</sup> were used as templates indicated that the AUG at -109 can be used as an efficient translation initiation site.

## Discussion

This study shows for the first time that 1) the Mrp2 5'UTRs are involved in translational regulation of Mrp2 protein expression and 2) the uORF<sup>-109</sup> has an inhibitory effect on translation. Disruption of the uORF<sup>-109</sup> abolished the inhibitory effect of the wild type 5'UTRs (-213, -163, and -132) on translation of the luciferase reporter gene in HepG2 cell transient transfection assays (Fig. 4) and in vitro translation assays (Fig. 5, B-D). These data suggested that translation was efficiently initiated at the uORF<sup>-109</sup> and was confirmed by demonstration of translation of a higher molecular weight peptide when this uORF was fused in-frame with the luciferase ORF (Fig. 6). Thus, the absence of uORF<sup>-109</sup> explained why the luciferase transcript containing S1-5'UTR was translated much more efficiently than the luciferase transcripts containing L-, M1-, and M2-5'UTR (Fig. 5). Recognition and translation initiation of ribosomes at the AUG<sup>-109</sup> was also indirectly verified by disruption of the Kozak motif, because the mutation *GAITGAATGGTA* (Fig. 1) increased translation efficiency compared with the wild-type M2-5'UTR (Fig. 5D).

There are three uORFs in the 5'UTR of Mrp2 (GenBank accession number Y14995; Fig. 1A). The first uORF, uORF<sup>-213</sup>, is in-frame with the Mrp2 ORF, whereas uORF<sup>-109</sup> and uORF<sup>-149</sup> are out-of-frame with the Mrp2 ORF. The uORF<sup>-109</sup> has a perfect Kozak motif, and overlaps the Mrp2 coding region by terminating at +60. Therefore, translation of the downstream Mrp2 ORF must occur through partially leaky scanning of the uORF<sup>-109</sup>, despite its



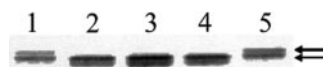
**Fig. 4.** Effect of the Mrp2 5'UTR on expression of the luciferase reporter gene in HepG2 cell transient cotransfection assays. Mrp2 5'UTR-luciferase constructs were cotransfected into HepG2 cells with pSV40-Ren. After 24 h, the firefly and *R. reniformis* luciferase activities were measured. The effect of various 5'UTRs (A, L, -213 nucleotides; B, M1, -163 nucleotides) on luciferase expression is represented as the ratio of the firefly luciferase activity/*R. reniformis* luciferase activity. The assays were performed in triplicate. The data are represented as mean  $\pm$  S.E.M. and normalized to the Luc/Ren ratio for the pGL3 control vector.

perfect Kozak motif, not through reinitiation of ribosomes. That is, a portion of the ribosomes must bypass the AUG at  $-109$  to translate the Mrp2 ORF. In addition to leaky scanning, there are other possible mechanisms for the uORF $^{-109}$  to exert an inhibitory effect on translation of Mrp2 protein. As pointed out by Morris (Morris and Geballe, 2000), nascent peptides encoded by uORFs can mediate regulation through interfering with translation elongation or termination. As a result, ribosomes stall and translation of the downstream ORF is inhibited. These mechanisms have been identified with the peptide products of various prokaryotic and eukaryotic uORFs (Cao and Geballe, 1996; Lovett and Rogers, 1996). In the present study, we truncated the native uORF $^{-109}$  at  $-1$  by fusing it to the luciferase gene, so that the inhibitory effect of the uORF $^{-109}$  could be exerted by competition for ribosomes with the downstream luciferase ORF, or by the sequence-dependent information in the first 36 amino acids of the nascent peptide. We are currently investigating whether the entire peptide of 56 amino acids encoded by the uORF $^{-109}$  might mediate any sequence-dependent regulation of translation of Mrp2 protein.

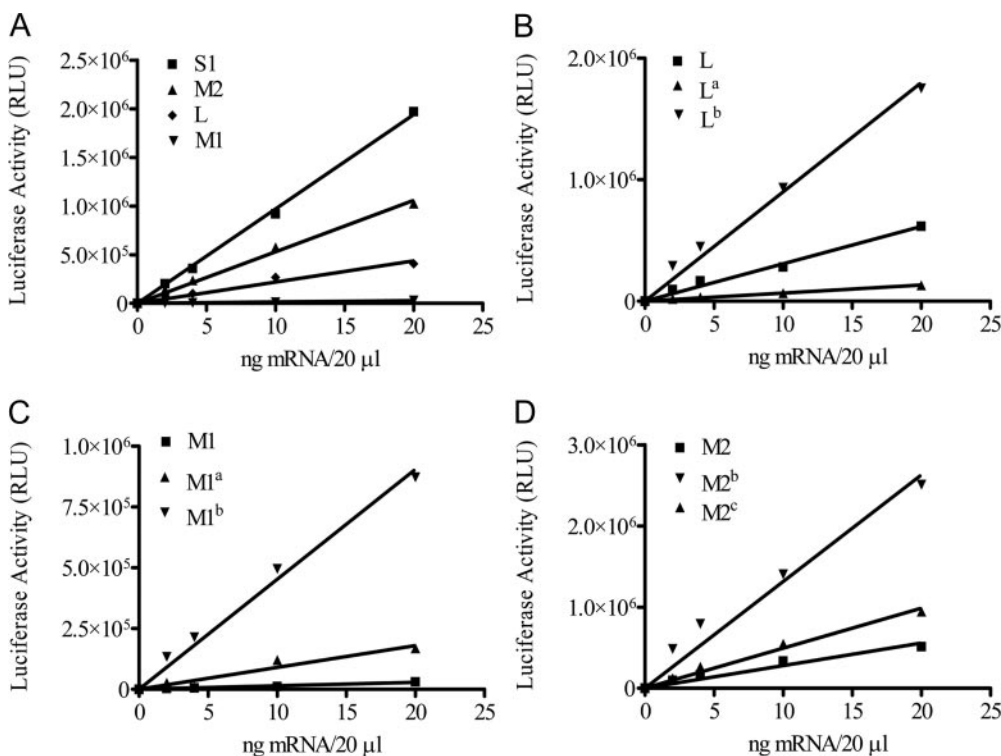
Disruption of the uORF $^{-149}$  alone (Fig. 5B, C, and D) or together with disruption of the uORF $^{-109}$  (Fig. 4) showed a minimal effect on translation in *in vitro* translation assays or in transiently transfected HepG2 cells, indicating that the AUG at  $-149$  is successfully bypassed by ribosomes, most likely because of the absence of the Kozak sequence. Because the AUG at  $-213$  is the first codon at the 5' end of the Mrp2 messenger, and ribosomes poorly recognize AUG start codons close to the 5' end of a cDNA (Kozak, 1991), it is unlikely that the 40S subunits can recognize it and initiate translation. Therefore, we did not investigate this AUG start codon. Taken together, the data indicate that among the three AUG start codons identified in the Mrp2 5'UTR, only the AUG at  $-109$  serves as an efficient translation initiation site and

plays an important role in the translational regulation of Mrp2 protein expression.

The distance between the cap site and the initiation codon is very important for recognition of the uORF by 40S subunits. A study of mammalian *S*-adenosylmethionine decarboxylase shows that recognition of the single uORF in the *S*-adenosylmethionine decarboxylase 5'UTR in nonlymphoid cells is increased by extending the space between the cap site and the upstream AUG codon from 14 to 47 nucleotides, leading to suppression of translation (Ruan et al., 1994). The distance between the cap sites and the AUG at  $-109$  in L, M1, and M2 5'UTR are 104, 54, and 23 nucleotides, respectively. The order of suppression of translation of the four wild-type Mrp2 5'UTRs from high to low was M1, L, M2, and S1 (Fig. 5A). This implies that recognition and translation initiation of the AUG at  $-109$  are most efficient when there are 54 nucleotides between the cap and the initiation site, as in the transcript M1, but less efficient when the distance is reduced to 23 nucleotides (i.e., M2) or increased to 104 nucleotides (L). It is likely that extending the distance from 23 to 54 nucleotides gives the 40S subunits the time needed to recruit additional translational factors, resulting in more efficient recognition. However, we cannot explain at this time



**Fig. 6.** *In vitro* expression in coupled transcription/translation system. The rat Mrp2 5'UTR cDNA sequences were inserted into T7 control vector in a way that the uORF $^{-109}$  was in-frame with the luciferase reporter reading frame. deL and deM1 contained the uORF $^{-109}$ , whereas S2 did not. The point mutation of AT $^{-108}$ G $\rightarrow$ AAG disrupted the uORF $^{-109}$  in deL and deM1, resulting in deL $^b$  and deM1 $^b$  constructs. The TNT quick-coupled transcription/translation system was used to express the luciferase protein *in vitro* using plasmids as templates. The translated products were separated on 4–20% gradient reductive SDS-PAGE. Data were processed using STORM 840 PhosphorImager. Lane 1, deL; lane 2, deL $^b$ ; lane 3, S2; lane 4, M1 $^b$ ; lane 5: deM1.



**Fig. 5.** Effect of the Mrp2 5'UTR on translation efficiency of the luciferase reporter transcript by *in vitro* translation assays. Mrp2 5'UTR-luciferase constructs were linearized by restriction enzymes PvuII and SacI. The PvuII-SacI fragments were used as templates to synthesize the capped, Mrp2 5'UTRs-fused luciferase transcripts. Figure shows translation of wild-type 5'UTRs (A), L and its mutants (B), M1 and its mutants (C), and M2 and its mutants (D). The luciferase transcripts (0, 2, 4, 10, or 20 ng) were added to a rabbit reticulocyte lysate mixture. The translation reaction was incubated at 30°C for 60 min and terminated on ice. The firefly luciferase activity was measured. The linear lines represent the relationship of luciferase activity with respect to transcript concentration.



why extending the distance from 54 (M1) to 104 (L) nucleotides impairs recognition, although potential secondary structures formed by the additional sequence could act to decrease translation efficiency.

We also showed for the first time that the transcription initiation sites were differentially used in the rat liver, kidney, small intestine, lung and placenta, and that their use varied with age in the liver, kidney, and jejunum and ileum (Figs. 2 and 3). In the jejunum, the primary transcription initiation site was -98 and the secondary site was -132, whereas in the ileum, the primary site was -132 and the secondary site was -98. Considering the fact that Mrp2 protein expression is decreased 90% in the distal ileum relative to that in the jejunum, with mRNA unchanged (Mottino et al., 2000), these data suggest that the uORF<sup>-109</sup> exerts an inhibitory effect on translation of Mrp2 protein expression in the ileum. In the kidney, the transcription initiation site at -98 was barely detectable, with the site at -132 predominating. In the liver, the site at -98 was the primary site; however, the longer transcripts could all be detected. Low expression of Mrp2 protein in kidney may therefore be due in part to suppression of translation of Mrp2 by the uORF<sup>-109</sup>. Further studies are needed to understand the basis and physiologic implications for use of multiple transcription initiation sites by *Mrp2*, and why their use varies among tissues, and to a lesser extent, with age. Our early studies demonstrated that use of transcription start sites in rat liver was not altered by treatment with PCN or in pregnancy, implying that this is a fundamental property of the gene that is not readily modified.

The function of the uORF in rat *Mrp2* is not known. However, this feature is conserved in human MRP2, which also has an uORF with a perfect Kozak motif at -104 nucleotides; interestingly, the major transcription start site in human MRP2 occurs at -247 nucleotides (Tanaka et al., 1999), implying that the uORF in MRP2 could also be important in regulating MRP2 protein expression. Analysis of murine *Mrp2* gene (GenBank accession number AY905402) also indicates the presence of a uORF with a perfect Kozak motif at -110 nucleotides; however, there is apparently no available information regarding Mrp2 transcription start site(s) in mice. The conservation of these uORFs in rat, mouse, and human does suggest a function that merits further investigation.

In summary, our present studies showed clearly that expression of various rat Mrp2 transcripts was tissue-specific. More importantly, the Mrp2 5'UTRs differentially influenced translation because the uORF<sup>-109</sup> had a marked inhibitory effect on translation. These data provide a new rationale for the high tissue expression of Mrp2 in liver and proximal intestine, because the primary transcription start of -98 nucleotides in these tissues lacks the inhibitory uORF<sup>-109</sup>.

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