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The Role of Protein Synthesis and Degradation in the Post-Transcriptional Regulation of Rat Multidrug Resistance-Associated Protein 2 (Mrp2, Abcc2)

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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 post-transcriptional regulation. We hypothesized that Mrp2 protein undergoes altered rates of protein synthesis or degradation consistent with different Mrp2 protein expression. We analyzed Mrp2 synthesis, expression, and degradation in control female, 19- and 20-day pregnant, and pregnenolone-16α-carbonitrile (PCN)-treated rats using in vivo metabolic-labeling studies with [^{35}S]cysteine/methionine or [^{14}C]$NaHCO_3$, polysomal distribution analyses and ribonuclease protection assays (RPA). Mrp2 protein was significantly increased in rats treated with PCN for 2 days but significantly decreased in 19-day pregnant rats relative to controls; no significant differences were observed in Mrp2 mRNA expression among these groups. The measured half-lives of ^{14}C-labeled Mrp2 in control, pregnant, and PCN-treated rats were 27, 36, and 22 h, respectively, and were not significantly different. The rate of incorporation of ^{35}S into Mrp2 was highest in PCN-treated rats. Polysomal distribution analysis of Mrp2 mRNA was consistent with increased Mrp2 protein synthesis after PCN treatment. The major transcription-initiation site for rat liver determined by RPA was −98 nucleotides (nt), with other start sites observed at −213, −163, −132, and −71 nt; use of transcription sites did not differ among the groups. Differences in the degradation of Mrp2 protein cannot explain the post-transcriptional regulation of Mrp2 in control, pregnant, and PCN-treated rats. Rather, the observed difference in protein synthesis suggests an intrinsic role for the translational regulation of rat Mrp2 protein.

The formation of bile in the liver is critical for the elimination of numerous environmental contaminants and endogenous substrates (Trauner and Boyer, 2003). The generation of bile flow is an osmotic secretory process dependent on the coordinated synergy of a number of transporter proteins located in the sinusoidal and canalicular membranes of the hepatocyte (Meier and Stieger, 2002; Oude Elferink and Groen, 2002). The canalicular multidrug resistance protein 2 (rat Mrp2; Abcc2), a 190-kDa membrane glycoprotein located in the canalicular membrane, mediates the ATP-dependent excretion of a wide spectrum of organic anions into bile, including glutathione, glucuronide, and sulfate conjugates (Jansen et al., 1987; Paulusma et al., 1996; Konig et al., 1999; Gerk and Vore, 2002). Mrp2 also contributes to bile acid-independent bile flow by mediating the canalicular excretion of glutathione (Ballatori and Truong, 1992).

Mrp2 expression and function has been studied extensively during a variety of conditions, including pregnancy, models of intrahepatic and obstructive cholestasis (e.g., ethinylestradiol treatment), common bile duct ligation, after endotoxin exposure, and treatment with classic liver enzyme inducers (Bossard et al., 1993; Roelofsen et al., 1994; Bolder et al., 1997; Trauner et al., 1997; Paulusma et al., 2000; Johnson and Klaassen, 2002). Several lines of evidence from these studies suggest that expression of Mrp2 is under post-transcriptional regulation in addition to classic transcriptional regulation. Pregnancy represents a major physiological event in which the biliary secretion of many organic anions and glucuronide conjugates is significantly decreased (Gallagher et al., 1966; Vore et al., 1978, 1979; Auansakul and Vore, 1982). We have demonstrated a significant decrease in Mrp2 protein expression during pregnancy, consistent with the decreased biliary excretion of organic anions at this time; however, mRNA levels are unchanged (Cao et al., 2001,

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ABBREVIATIONS: Mrp, multidrug resistance-associated protein; PCN, pregnenolone-16α-carbonitrile; PXR, pregnane X receptor; RPA, ribonuclease protection assay; kb, kilobase; nt, nucleotide; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PAGE, polyacrylamide gel electrophoresis; RACE, rapid amplification of cDNA ends.

2002). The specific down-regulation of hepatic Mrp2 at both the RNA and protein levels in common bile duct ligation-treated and endotoxemic rats provides an explanation for the observed impairment of biliary transport in these two cholestatic models (Bolder et al., 1997; Trauner et al., 1997). However, ethinylestradiol treatment of rats, although significantly decreasing hepatic Mrp2 protein expression, has no effect at the mRNA level, similar to the changes observed in pregnancy (Trauner et al., 1997). Furthermore, treatment with pregnane X receptor (PXR) ligands, such as pregnenolone-16α-carbonitrile (PCN), significantly increases hepatic Mrp2 protein expression but not Mrp2 mRNA in both male and female rats (Johnson et al., 2002; Johnson and Klaassen, 2002). In rat small intestine, Mrp2 protein expression decreases from a maximum in the duodenum and jejunum to approximately 5% of this level in the terminal ileum; in contrast, Mrp2 mRNA expression in the terminal ileum is not significantly decreased relative to that in the duodenum-jejunum (Mottino et al., 2000). Cholestasis promotes the down-regulation of MRP2 in the intestine of both rats and humans similar to that observed with rat Mrp2 down-regulation in liver (Trauner et al., 1997; Dietrich et al., 2004). In particular, a slow transcriptional down-regulation of rat intestinal Mrp2 expression is observed during cholestasis that is insufficient to explain the rapid decrease in Mrp2 protein that occurs within 3 days. Together, these data have led to the hypothesis that the selective down-regulation of Mrp2/MRP2 is mediated by species-specific transcriptional and post-transcriptional mechanisms (Dietrich et al., 2004).

Mrp2/MRP2 expression is responsive to a number of drugs used in the treatment of disease and in diseases affecting the liver, with a corresponding impact on the disposition of drugs and endogenous substrates. Mrp2/MRP2 function is regulated at a number of levels, including its transcriptional regulation, translational regulation, and endocytic retrieval from the canalicular membrane of the hepatocyte (Gerk and Vore, 2002). In the present studies, we investigated the post-transcriptional regulation of rat Mrp2 expression by examining the rates of synthesis and degradation of Mrp2 protein in pregnant and PCN-treated rats compared with female controls. We also examined the polysomal distribution of Mrp2 mRNA in control, pregnant, and PCN-treated rats as another estimate of Mrp2 mRNA translation. Finally, the transcription initiation sites of rat Mrp2 were determined, and their use was compared in control, pregnant, and PCN-treated rats by ribonuclease protection assay. The present data demonstrate that translational regulation of Mrp2 is likely to be an important determinant of its function.

Materials and Methods

Materials

[^{35}S]Methionine/cysteine (Expre^{35}S^{35}S protein label, 1175 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA) and [^{14}C]Na-HCO_3 (50–62 mCi/mmol) from Amersham Biosciences Inc. (Piscataway, NJ). All other chemicals were of analytical grade and were obtained from Invitrogen (Carlsbad, CA), Roche Diagnostics (Indianapolis, IN), or Sigma Chemical (St Louis, MO). Mouse monoclonal antibody against the carboxyl terminus of human MRP2 (M_2III-6) was purchased from Alexis Biochemicals (San Diego, CA).

Animals

Male and female Sprague-Dawley rats (Harlan, Indianapolis, IN) were used throughout the study. Pregnant rats were timed according to the first day that sperm was detected (day 0) and were used at 17 to 20 days of pregnancy, as indicated in the figure legends. For the PCN induction experiments, rats received an injection of PCN (75 mg/kg/day i.p.) or vehicle (corn oil) for 2 days or as indicated in the figure legends. The rats had free access to food and water and were maintained on a 12-h/12-h timed light/dark cycle. All procedures involving animals were conducted in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the University of Kentucky (Lexington, KY).

Metabolic Labeling

Synthesis of Mrp2. Groups of five female rats (control, pregnant, and PCN-treated; 250–300 g) were anesthetized with urethane (50 mg/kg i.p.) and administered 4 mCi of [^{35}S]methionine/cysteine intravenously in 0.87 ml of phosphate-buffered saline (PBS). To investigate the incorporation of [^{35}S]methionine/cysteine into Mrp2 protein, livers were removed after 20 min, 40 min, 1 h, 2 h, and 3 h, rinsed with ice-cold PBS, and portions (~1 g) were frozen in liquid nitrogen. Frozen liver pieces were homogenized in 5 volumes of homogenization buffer (0.3 M sucrose) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin A, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml antipain) and filtered through a double layer of cheesecloth. The homogenates were stored at −80°C until used to measure the entire pool of newly synthesized Mrp2 protein.

Degradation Half-Life of Mrp2. Female rats (control, pregnant, and PCN-treated) were briefly anesthetized with ether and were administered [^{14}C]$NaHCO_3$ (6 mCi/rat i.p. in 3.3 ml of saline). The animals were killed at 2, 6, 12, 24, 48, 72, and 96 h thereafter, and mixed liver plasma membranes were prepared using a discontinuous sucrose density gradient centrifugation method as described previously (Cao et al., 2001).

Immunoprecipitation from Homogenate. All procedures were carried out at 4°C unless otherwise specified. Whole liver homogenate (10 mg of protein) was solubilized for 1 h in 1 ml of buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, pH 7.5, and protease inhibitors. The mixture was centrifuged at 13,000*g* for 10 min, and the supernatant was precleared by adding 60 μl of protein A-agarose suspension, rotated for 180 min, and centrifuged for 5 min at 5000*g*. The precleared supernatant was used for immunoprecipitation with monoclonal anti-human MRP2 antibody M_2III-6 (20 μg). The lysates were supplemented with bovine serum albumin to 0.5% and incubated with the antibody for 1 h, with rotation. Protein A-agarose (60 μl) was added, and incubation with shaking was continued overnight. The complex was sedimented for 5 min at 2050*g*, washed twice by rotation for 20 min in 1 ml of the lysis buffer, and centrifuged for 5 min at 2050*g*. The agarose was further washed two times (20 min) in 1 ml of wash buffer 2 (50 mM Tris-HCl, 500 mM NaCl, 0.1% Nonidet P-40, and 0.05% sodium deoxycholate, pH 7.5) and once in 1 ml of wash buffer 3 (50 mM Tris-HCl, 0.1% Nonidet P-40, and 0.05% sodium deoxycholate, pH 7.5) with centrifugation for 5 min at 2050*g* after each wash. Gel loading buffer (40 μl; 50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 10% β-mercaptoethanol, and 0.1% bromphenol blue) was added to the agarose pellet and heated to 100°C for 4 min. The protein A-agarose was removed by centrifugation for 20 s at 12,000*g*, and the supernatant was analyzed by SDS-PAGE on an 8% gel (Laemmli, 1970). The gel was fixed with methanol/water/glacial acetic acid (20:70:10) and dried on Whatman paper (80°C, 2 h, vacuum) (Whatman, Clifton, NJ). The dried gel was scanned and quantified using an Instant Imager (PerkinElmer).

Immunoprecipitation from Mixed Liver Plasma Membranes. All procedures were carried out at 4°C unless indicated

otherwise. Aliquots (1–2 mg of protein) of mixed liver plasma membranes were solubilized in 1.2 ml of radioimmunoprecipitation assay buffer (0.1 M PBS, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 10% glycerol) containing the protease inhibitors for 1 h. The mixture was centrifuged at 15,000*g* for 10 min, and the supernatant was precleared by adding 20 μg of normal mouse IgG and 200 μl of protein A/G-agarose, incubating for 30 min, and centrifuging at 15,000*g* for 1 min. The precleared supernatant was used in immunoprecipitation experiments with 40 to 50 μg of mouse anti-human MRP2 antibody M_2III-6 by incubation for 2 h with rotation. Protein A/G-agarose (800 μl) was added, and incubation with shaking continued overnight. After sedimentation of the immune complexes at 5000*g* for 5 min, the immunoprecipitates were washed four times with 1 ml of radioimmunoprecipitation assay buffer containing the protease inhibitors. Protein was eluted from the agarose by boiling for 5 min in 30 to 40 μl of gel loading buffer (125 mM Tris-HCl, pH 6.8, 2.5% SDS, 20% glycerol, 5% β-mercaptoethanol, and 0.005% bromphenol blue) and centrifuging at 10,000*g* for 1 min. The supernatant, containing the immunoprecipitated proteins, was resolved by SDS-PAGE on an 8% gel. The gel was fixed and dried as described previously for the liver homogenate immunoprecipitate. The dried gel was subjected to autoradiography by exposing to Kodak Bio-Max MR film at −80°C for 2 to 4 weeks (Eastman Kodak, Rochester, NY). The signals were quantified using ImageQuant software, and the data were analyzed by Prism software (GraphPad Software Inc., San Diego, CA) to determine the degradation half-life and 95% confidence intervals.

Immunoblot Analysis

For immunoblot analyses, rat liver homogenate or mixed plasma membrane samples were suspended in gel loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 10% β-mercaptoethanol, and 0.1% bromphenol blue). Samples (50–100 μg, without boiling) were separated by a 6 or 8% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked with Tris-buffered 5% nonfat milk overnight at 4°C and then incubated with the anti-MRP2 antibody (1:5000 dilution) for 1 to 2 h at room temperature. The blots were then washed three times (for 5 min each) in Tris-buffered saline/Tween solution (25 mM Tris, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) and incubated with a peroxidase-conjugated anti-mouse secondary antibody for 1 to 2 h. The blots were further washed three times (5 min each) in Tris-buffered saline/Tween solution and then visualized using the enhanced chemiluminescence detection system (ECL Plus; Amersham Biosciences) for 5 min and exposed to Kodak film.

Polysomal Distribution Analysis

Polysomes were prepared according to modifications of Ruan et al. (1997). In brief, 0.5 g of tissue was homogenized in 5 ml of lysis buffer containing 20 mM HEPES, pH 7.6, 100 mM KCl, 1.5 mM $MgCl_2$, 20 mg/ml torula yeast RNA, 0.5 mM cycloheximide, 10 mM dithiothreitol, 5 mg/ml sodium heparin, and 420 U/ml RNase inhibitor in diethyl pyrocarbonate-treated H_2O. The homogenate was centrifuged at 10,000*g* for 10 min at 4°C, and sodium cholate and Nonidet P-40 was added to the supernatant to a final concentration of 0.5%. The lysate was layered onto a linear 20 to 47% sucrose gradient prepared as described by Ruan et al. (1997) and centrifuged at 30,000*g* in an SW41 rotor for 150 min at 4°C. The gradient was collected from the bottom of the tube in 0.5-ml portions into a tube containing an equal volume of TRIzol reagent (Invitrogen) and incubated at room temperature for 5 min. Chloroform (0.1 ml) was added, shaken vigorously for 15 s, and then centrifuged at 10,000*g* for 15 min at 4°C. The aqueous phase was transferred to a new tube, and 0.5 ml of isopropanol was added and mixed. RNA was further purified according to the manufacturer's instructions, and Mrp2 mRNA was quantified by Northern analysis.

Northern Analysis

Total RNA was isolated from rat livers according to Chomczynski and Sacchi (1987), and poly(A)$^+$ RNA was purified using the PolyATtract mRNA Isolation System I kit (Promega, Madison, WI). Total RNA (20 μg/lane) or Poly(A)$^+$ (5 μg/lane) was denatured and separated using a 1% agarose-formaldehyde 3-(*N*-morpholino)propanesulfonic acid gel and transferred to a Duralon UV membrane by overnight capillary blotting. Hybridization and posthybridization washes were performed as described previously (Cao et al., 2001). To correct for the variance in total RNA loading and transfer, a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) oligoprobe was used. Signals were visualized using a PhosphorImager and quantified using the ImageQuant software (both from Amersham Biosciences). The blots were also exposed to Bio-Max MR-2 film. The amount of Mrp2 mRNA is expressed as relative density adjusted by the density of GAPDH RNA and normalized by the values of control female rats.

Ribonuclease Protection Assay

The 5′-untranslated region of rat Mrp2 (+1/−214) was inserted into luciferase T7 control vector (Invitrogen). After linearization, a 280-base pair fragment (rat Mrp2 5′-untranslated region + T7 promoter region) was used as template for RPA probe preparation. The probe was radiolabeled using the MAXIscript in vitro transcription kit (Ambion, Austin, TX) and PAGE-purified. The rat GAPDH RPA probe (847–1098) was isolated, radiolabeled, and purified in the same manner. RNase protection assays were performed using the RPA III kit (Ambion), with 20 μg of total RNA from male and female pregnant and PCN-treated rat livers, according to the manufacturer's instructions.

5′ Rapid Amplification of cDNA Ends

Analysis of the 5′-untranslated region of rat Mrp2 mRNA was carried out using the GeneRacer kit (Invitrogen). Total RNA was isolated from female control Sprague-Dawley rats using an Rnase kit (Qiagen, Valencia, CA). The RNA ligase-mediated reactions and oligonucleotide-capping rapid amplification of the cDNA ends (RACE) were performed according to the manufacturer's instructions. In brief, the ligated mRNA was reverse-transcribed using the gene-specific primer 5′-gccaaggagccaggagccaaagaa-3′ and the following conditions: 94°C for 2 min; 5 cycles at 94°C for 30 s, 72°C for 90 s; 5 cycles at 94°C for 30 s, 70°C for 30 s, 72°C for 90 s; then 30 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 90 s; and final elongation at 72°C for 5 min. To obtain 5′ ends, the first-strand cDNA was amplified using the GeneRacer 5′-primer and the reverse gene-specific primer 5′-cgggatccaatgctctcctcgcgcttc-3′. The following parameters were used: 94°C for 2 min; 5 cycles at 94°C for 30 s, 58°C for 30 s, 72°C for 90 s; 30 cycles at 94°C for 30 s, 62°C for 30 s, 72°C for 90 s; with a final elongation at 72°C for 5 min. The 5′-RACE polymerase chain reaction products were sequenced using the T7 Sequenase Quick-Denature plasmid sequencing kit from U.S. Biochemical Corporation (Cleveland, OH).

Statistical Analysis

Data represent mean values ± S.E. and were analyzed by analysis of variance followed by Bonferroni or Dunnett's multiple comparisons test where appropriate. Statistical significance was considered at P values of <0.05.

Results

Mrp2 mRNA Expression in Female Control, PCN-Treated, and Pregnant Rats. Northern analyses were initially performed to characterize Mrp2 mRNA expression in pregnant and control female rats and in rats pretreated with corn oil or PCN for 2 days. We examined each of the major mRNA transcripts of Mrp2 (5.5, 6.5, and 8.3 kb), analyzing

them separately in addition to considering total Mrp2 mRNA content. The relative proportion (mean ± S.E., $n = 3$; expressed as a percentage of total mRNA) of each of the three transcripts in the control group was 8.3 kb, 41.9 ± 9%; 6.5 kb, 8.3 ± 1.5%; and 5.5 kb, 49.8 ± 10.5%. As shown in Fig. 1, total Mrp2 mRNA levels did not significantly differ in pregnant or vehicle-treated rats relative to female controls. Furthermore, no differences were observed in the relative proportions of the three transcripts among the groups. Mrp2 mRNA expression was slightly elevated in PCN-treated rats for the 5.5-kb mRNA transcript and total Mrp2 mRNA; however, it did not reach statistical significance (Fig. 1). The data indicate that there are no differences in the relative proportion of the different 3′-untranslated regions transcripts that can explain the significant differences observed in expression of Mrp2 protein among these groups. Our results are in agreement with the observations of Johnson and Klaassen (2002) and Johnson et al. (2002), who showed no significant induction of Mrp2 mRNA expression in rat liver at various times during a 4-day PCN treatment schedule.

Mrp2 Protein Expression in Female Control, PCN-Treated, and Pregnant Rats. Mrp2 protein was analyzed in control, vehicle-treated, 19-day pregnant, and PCN-treated female rats (Fig. 2). As shown in Fig. 2, Mrp2 protein expression was significantly decreased (~50%) in liver homogenate preparations from pregnant rats relative to controls. We have reported previously a decrease in Mrp2 protein expression during pregnancy (Cao et al., 2001), and the data are presented here again for comparative purposes. In contrast, Mrp2 protein levels were elevated at both 12 and 24 h after a single dose of PCN, with expression becoming significantly increased above that of controls 24 h after a 2-day PCN dosing regimen (Fig. 2C). Mrp2 protein expression was also analyzed after a 4-day PCN dosing schedule and was further elevated relative to the 2-day treatment regimen. These data indicated that active Mrp2 protein synthesis was occurring after 2 days of PCN treatment.

Degradation Half-Life of Mrp2 Protein in Female Control, Pregnant, and PCN-Treated Rats. We investigated the degradation half-life of Mrp2 protein using minimally reusable [^{14}C]bicarbonate, because it is primarily incorporated into the guanidino group of arginine and the ω-carboxyl groups of aspartate and glutamate. The overall recycling of ^{14}C is therefore significantly lower (~7%) than would be observed with [^{35}S]methionine or [^{35}S]cysteine (Swick and Ip, 1974). Rats were sacrificed at selected time points after [^{14}C]$NaHCO_3$ administration, and mixed liver plasma membranes were isolated. Mrp2 protein was subsequently immunoprecipitated from mixed membranes of each rat. Mrp2 protein levels did not differ in mixed membranes

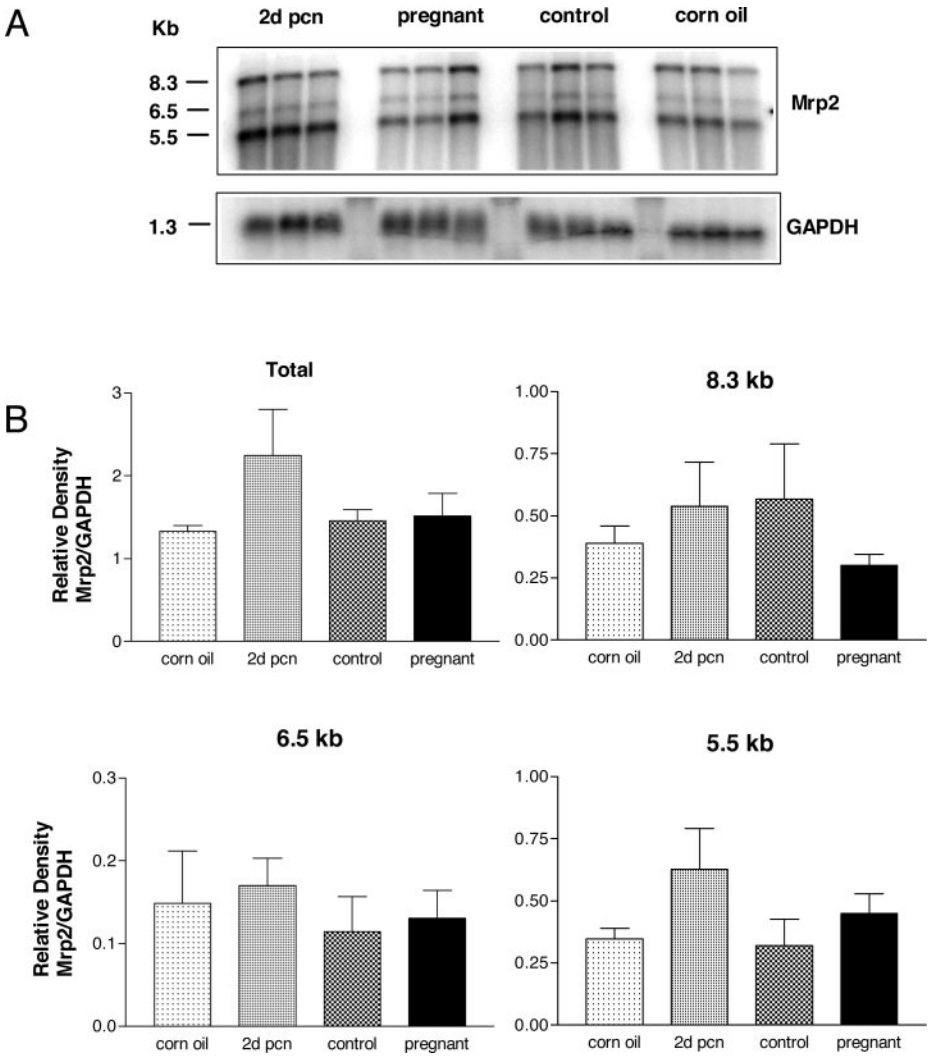

Fig. 1. Northern blot analysis of hepatic Mrp2 mRNA expression from control, 19-day pregnant, 2-day PCN-treated (2d pcn) and corn oil (vehicle)-treated rats. A, Northern blot analysis and the corresponding GAPDH control. Each lane represents a single liver RNA sample. B, densitometry analysis for each of the different transcripts and the total content. The amount of Mrp2 mRNA is expressed as relative density adjusted by the density of GAPDH RNA. The values are expressed as mean ± S.E. $P < 0.05$.

from each of the time points (Fig. 3A) or in the immunoprecipitate (Fig. 3B) that was subjected to SDS-PAGE and exposed to film. A sample autoradiogram from control rats is shown in Fig. 3C and demonstrates that only Mrp2 was precipitated from incubation with the M_2III-6 antibody. Densitometry analysis of the single band was used to calculate the degradation half-life (Fig. 3D). The same methods were used for determination of the degradation half-lives in pregnant and PCN-treated rats. The calculated degradation half-lives (and 95% confidence intervals of the slope of the regression line) of Mrp2 protein, as determined by autoradiography, were 27 (15.6–108), 36 (25.1–64.5), and 22 (19.0–25.7) h in control female, pregnant, and PCN-treated rats, respectively (Fig. 3D). The 95% confidence intervals on the

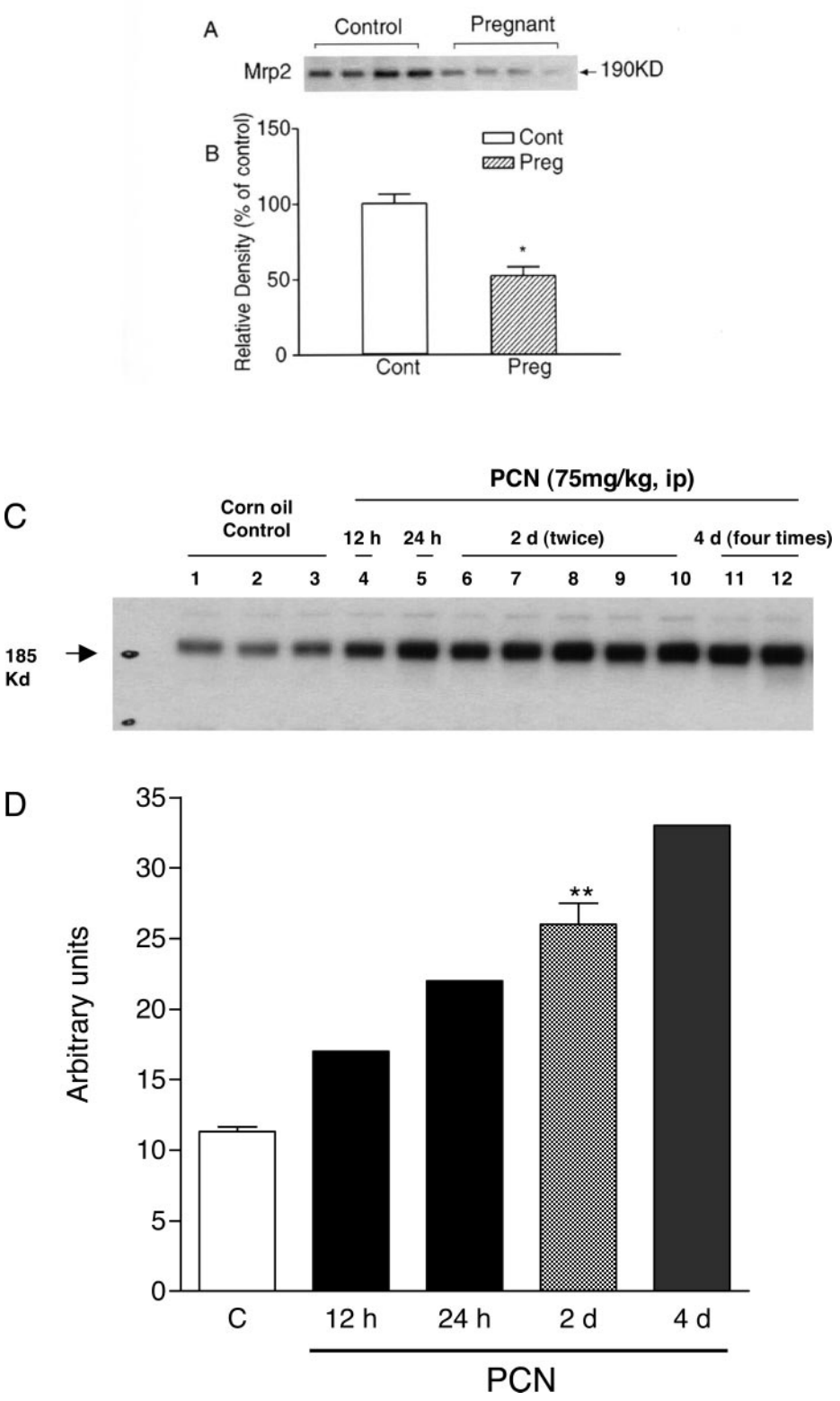

Fig. 2. Western blot analysis of hepatic Mrp2 expression from control, PCN-treated, and 19-day pregnant rats. A, typical immunoblot performed with total homogenate prepared from control and 19-day pregnant individual liver samples. B, densitometry was performed in four animals per group, and the resulting values were normalized to the mean of the control group. [A and B are reprinted from Cao J, Stieger B, Meier PJ, and Vore M (2002) Expression of rat hepatic multidrug resistance-associated proteins and organic anions in pregnancy. *Am J Physiol* **283:**G757–G766. Copyright © 2002 by the American Physiological Society. Used with permission.] C, representative Western blot of Mrp2 protein in total liver homogenate 12 and 24 h after a single PCN dose or 24 h after treatment with PCN for 2 or 4 days. D, results of densitometry analysis of signals in C. The resulting values were expressed in arbitrary units and represent mean ± S.E. ⋆, $P < 0.05$, significantly different from controls.

degradation half-lives overlapped, indicating no significant differences in the degradation half-lives of Mrp2 protein in control, pregnant, and PCN-treated rats.

Efficiency of Immunoprecipitation with MRP2 (M_2III-6) Antibody. We performed a set of quantification experiments to investigate the efficiency and detection limits of the anti-human MRP2 antibody (M_2III-6) in the immunoprecipitation procedure with liver homogenate. Lysates of total homogenate (3 mg) that had been immunoprecipitated with M_2III-6 using the same conditions as performed in the metabolic-labeling experiments were reprobed with the antibody to detect any remaining antigen. For comparison, control experiments were conducted in which lysates of 3 mg of homogenate were immunoprecipitated with an aliquot of phosphate-buffered saline (Fig. 4A). Quantification of the blots using Bio-Rad (Hercules, CA) densitometry software revealed that after immunoprecipitation with M_2III-6, at least 85% of Mrp2 protein was immunoprecipitated from the lysate.

Measurement of Mrp2 Protein Synthesis in Control, Pregnant, and PCN-Treated Rats. To test whether the synthesis of Mrp2 protein is altered under these different conditions, thus providing an explanation for its altered expression, rats were in vivo metabolically labeled with [^{35}S]methionine/cysteine. The incorporation of label into newly synthesized Mrp2 protein was determined by immunoprecipitation followed by autoradiography (Fig. 4B). Furthermore, SDS-PAGE analysis of each immunoprecipitated sample revealed a single band corresponding in size to Mrp2 (data not shown). The use of total liver homogenate precluded problems related to localization of newly synthesized Mrp2 in various subcellular organelles. The initial rate of Mrp2 synthesis was greater in PCN-treated rats relative to controls, as determined from the initial slope of ^{35}S incorporation (Fig. 4B). Because of the very low level of incorporation of ^{35}S into Mrp2 protein in pregnant rats, it was not possible to obtain an initial rate of Mrp2 synthesis in this group. These data are consistent with the greater amount of Mrp2 protein in PCN-treated rats and lesser amounts in pregnant rats.

Polysomal Distribution Analysis of Hepatic Mrp2 mRNA in Control Female, Pregnant, and PCN-Treated Rats. We characterized the polysomal distribution of Mrp2 mRNA as an additional measure of the translation of Mrp2. Translation is primarily controlled at the level of initiation, when the ribosome is recruited to an mRNA and positioned at the initiation codon. Translational initiation is catalyzed by numerous eukaryotic initiation factors and commences by dissociation of the 80S ribosome into the 60S and 40S components and association of the 40S ribosome with specific initiation factors, GTP, and the methionine-charged initiator tRNA to form the 43S preinitiation complex. The 43S complex is recruited to the 5′-end of the mRNA with the aid of additional initiation factors and then migrates along the 5′-leader sequence in an ATP-dependent scanning process until it encounters an initiator AUG codon. After AUG recognition, initiation factor release occurs, the 60S subunit enters, and elongation begins. In active translation, a new ribosome can attach onto the 5′-end of the mRNA molecule almost as soon as the preceding ribosome is out of the way. Such mRNA molecules are thus found in the cell as polyribosomes, or polysomes, formed by several ribosomes spaced as close as 80 nucleotides along a single messenger molecule.

Polysomal distribution analysis entails separation of the polyribosomes from single ribosomes and their subunits by differential sedimentation through a sucrose gradient. RNA purified from individual fractions of the gradient is then used for Northern analysis. We characterized the polysomal distribution of Mrp2 mRNA in livers from pregnant, PCN-treated, and control female rats. Figure 5 shows the polysomal distribution of Mrp2 mRNA, expressed as a percentage of the total Mrp2 mRNA on the gradient. PCN treatment markedly shifted Mrp2 mRNA to the bottom of the gradient, consistent with its association with a large number of ribosomes, and thus increased protein synthesis relative to control rats. For pregnant rats, we did not observe major

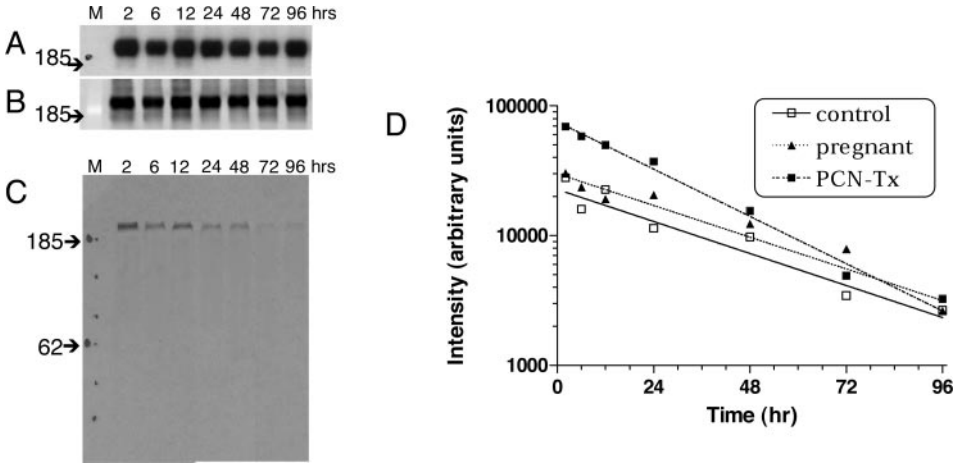

Fig. 3. Measurement of the degradation half-life of Mrp2 protein in control, pregnant, and PCN-treated rats. Rats were administered [^{14}C]$NaHCO_3$, and livers were removed for analysis at 2, 6, 12, 24, 48, 72, and 96 h. Pregnant rats were administered [^{14}C]$NaHCO_3$ on day 17 of pregnancy. A, immunoblot was performed with mixed membranes from control liver samples at various times after [^{14}C]$NaHCO_3$ administration and probed with mouse anti-human MRP2 antibody M_2III-6. B, immunoblot of control samples after the immunoprecipitation of Mrp2 protein from mixed liver plasma membranes. C, representative autoradiogram of Mrp2 immunoprecipitated from control mixed liver plasma membranes at the indicated times after administration of [^{14}C]$NaHCO_3$. ^{14}C in immunoprecipitates from control, pregnant, and PCN-treated liver membrane samples, separated by SDS-PAGE and detected by autoradiography, was quantified using ImageQuant software. The positions of known protein standards are indicated by the arrows. D, relative intensity of immunoprecipitated ^{14}C-labeled Mrp2 in control, pregnant, and PCN-treated rats plotted versus time to determine the degradation half-life of the protein.

changes in distribution of Mrp2 mRNA along the gradient, compared with controls, despite numerous modifications in the sucrose gradient (data not shown). However, more Mrp2 mRNA was observed at the very top of the gradient, where mRNA that is associated with a single ribosome sediments, consistent with decreased Mrp2 protein synthesis in liver from pregnant rats.

Analysis of corn oil-treated rats also revealed no significant differences in Mrp2 mRNA distribution compared with controls (data not shown). The polysomal distribution of GAPDH did not differ between control, corn oil, pregnant, or PCN-treated rats (data not shown).

Identification of Mrp2 Transcription Initiation Sites in Rat Liver. To identify the sequence corresponding to the 5′-end of the Mrp2 mRNA, we used a 5′-RACE cloning strategy. Analysis of 33 clones obtained by the RACE procedure showed five transcriptional start sites, at −213, −163, −132, −98, and −71 nt, respectively (Table 1). The most frequent capping site was identified as −98 nt. We next compared the major Mrp2 transcription initiation start sites between male, female, pregnant, and PCN-treated rats using ribonuclease protection assays (Fig. 6). The −98-nt site was confirmed as the major Mrp2 transcription initiation start site in each of the groups examined. The data also indicate that Mrp2 has multiple transcription start sites. However, we did not detect any differences between groups in the use of the various transcription initiation sites.

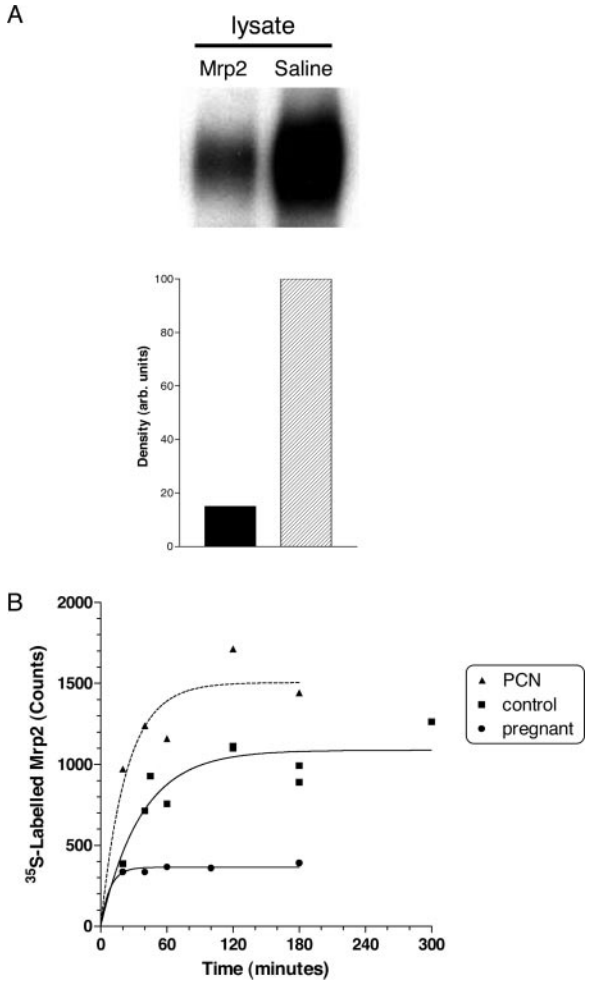

Fig. 4. Efficiency of M_2III-6 antibody and measurement of Mrp2 protein synthesis in control, 20-day pregnant, and PCN-treated female rats. A, whole liver homogenate samples were immunoprecipitated with M_2III-6 (control immunoprecipitation with phosphate-buffered saline) as described under *Materials and Methods*. The remaining lysates were reprobed with antibody, dissolved in gel loading buffer, and subjected to SDS-PAGE. The blots were then probed with M_2III-6. The amount of antigen was quantified using Bio-Rad densitometry software. B, rats were labeled with [^{35}S]methionine for 20 min, 40 min, 1 h, 2 h, and 3 h. ^{35}S-labeled Mrp2 in immunoprecipitates from liver homogenates separated by SDS-PAGE was detected and quantified with an InstantImager. The relative intensity of ^{35}S-labeled Mrp2 was plotted versus time to determine the rate of synthesis of the protein. Data presented are typical results observed in at least two sets of five rats each.

Discussion

Mrp2 plays a vital role as a canalicular multispecific organic anion transporter in the elimination of both endogenous and xenobiotic conjugates (Jansen et al., 1987; Konig et al., 1999). Several PXR ligands, including PCN, dexamethasone, and spironolactone, increase rat Mrp2 protein expression in vivo, with no significant changes observed in Mrp2 mRNA levels, even when examined at various times after treatment (Johnson and Klaassen, 2002; Johnson et al., 2002). In support of these observations, we demonstrated that PCN significantly increased rat Mrp2 protein, but not mRNA, after a 2-day dosing regimen (Figs. 1 and 2). Mrp2 mRNA in rats consists of three different transcripts (5.5, 6.5, and 8.3 kb), reflecting differences in the 3′-untranslated region (Ito et al., 1997). Analysis of the major Mrp2 mRNA transcripts between controls and PCN-treated animals confirmed the absence of any significant induction or changes in the relative proportions of each transcript (Fig. 1A). In contrast, PCN and dexamethasone both significantly increase Mrp2 mRNA levels when incubated with primary rat hepatocytes in culture (Kast et al., 2002). The potential mechanisms underlying these regulatory differences in Mrp2 expression in vivo and in vitro have been discussed previously (Johnson and Klaassen, 2002) but remain unclear. Although the ability of three PXR ligands to increase Mrp2 protein expression is suggestive of a role for PXR, it is not clear whether this transcription factor is, in fact, important in mediating the increases in protein expression.

Decreased expression of rat Mrp2 protein expression in the absence of changes in mRNA has been well documented (i.e., after pregnancy or treatment with ethinylestradiol), where Mrp2 mRNA levels are unchanged relative to controls, but Mrp2 protein levels are decreased significantly (Trauner et al., 1997; Cao et al., 2001). Likewise, in the terminal small intestine, Mrp2 protein expression is decreased by more than 90% relative to that in the duodenum and jejunum, whereas Mrp2 mRNA is not significantly decreased (Mottino et al., 2000).

In the present study, we examined several factors potentially important in the post-transcriptional regulation of rat Mrp2 expression. Such post-transcriptional regulation could occur via changes in mRNA stability, protein stability, or rates of protein synthesis. The lack of effect of pregnancy and PCN treatment on expression of Mrp2 mRNA transcripts, despite decreases and increases in Mrp2 protein, respectively, indicates that differences in the stability of Mrp2 mRNA are not likely to contribute to the differences observed in expression of Mrp2 protein.

We examined the stability of Mrp2 protein in female con-

trol, pregnant, and PCN-treated rats and found that the calculated degradation half-lives of Mrp2 protein in these three groups did not differ significantly (Fig. 3D). The minor differences observed cannot explain the marked changes in protein expression levels. The shorter half-life observed in PCN-treated rats infers increased protein degradation that would lead to lower protein expression unless protein synthesis were increased to a greater extent. Likewise, the longer Mrp2 half-life in pregnant rats indicates decreased protein degradation that would culminate in increased Mrp2 protein expression if protein synthesis rates were unchanged. The present data represent the first determination of the rates of protein degradation in liver for any ATP-binding cassette transporter family member. The half-lives of selected rat hepatic integral plasma membrane glycoproteins CE9, HA4, and HA321 have been described to have apparent half-lives of between 4 and 5 days (Scott and Hubbard, 1992). In addition, dipeptidyl peptidase IV, an apical protein that cleaves dipeptides from proteins containing a penultimate proline, had an estimated half-life of 9 days (Scott and Hubbard, 1992). However, as noted by the authors, the calculated half-lives for the selected proteins were determined through the use of the reusable amino acids [^{35}S]methionine and [^{35}S]cysteine and are therefore lengthened because of label reincorporation (Schimke, 1975; Scott and Hubbard, 1992). The actual half-lives were estimated to be 1.5 to 2.5 times shorter than the uncorrected values (Elovson, 1980; Scott and Hubbard, 1992). The half-lives of 22 to 36 h for Mrp2 are shorter than those predicted for other apical proteins, but because we used minimally reusable [^{14}C]bicarbonate, they represent an accurate assessment of the Mrp2 degradation half-life. Indeed, Swick and Ip (1974) concluded that by using [^{14}C]bicarbonate as a tracer for hepatic protein turnover, extensive manipulations of the data to correct for reuse of labeled amino acids are unnecessary.

Because no differences were observed in the rates of Mrp2 protein degradation, we next examined the potential differences in rates of protein synthesis. The initial rate of Mrp2 protein synthesis was increased in PCN-treated animals relative to controls (Fig. 4B) and is consistent with the greater association of Mrp2 mRNA with a large number of ribosomes, as determined by the polysomal distribution analysis (Fig. 5). These data support the hypothesis that increased Mrp2 protein levels in PCN-treated rats is caused by increased protein synthesis, particularly in view of the somewhat faster rate of degradation after PCN treatment. Together, these data suggest a faster turnover of Mrp2 after PCN treatment. Because of the minimal levels of ^{35}S incorporation into Mrp2 protein in pregnant rats, we were unable to obtain initial rates of protein synthesis for this group (Fig. 4B). Nevertheless, the low level of ^{35}S-incorporation into Mrp2 in pregnant rats is consistent with a decreased rate of synthesis. The increased

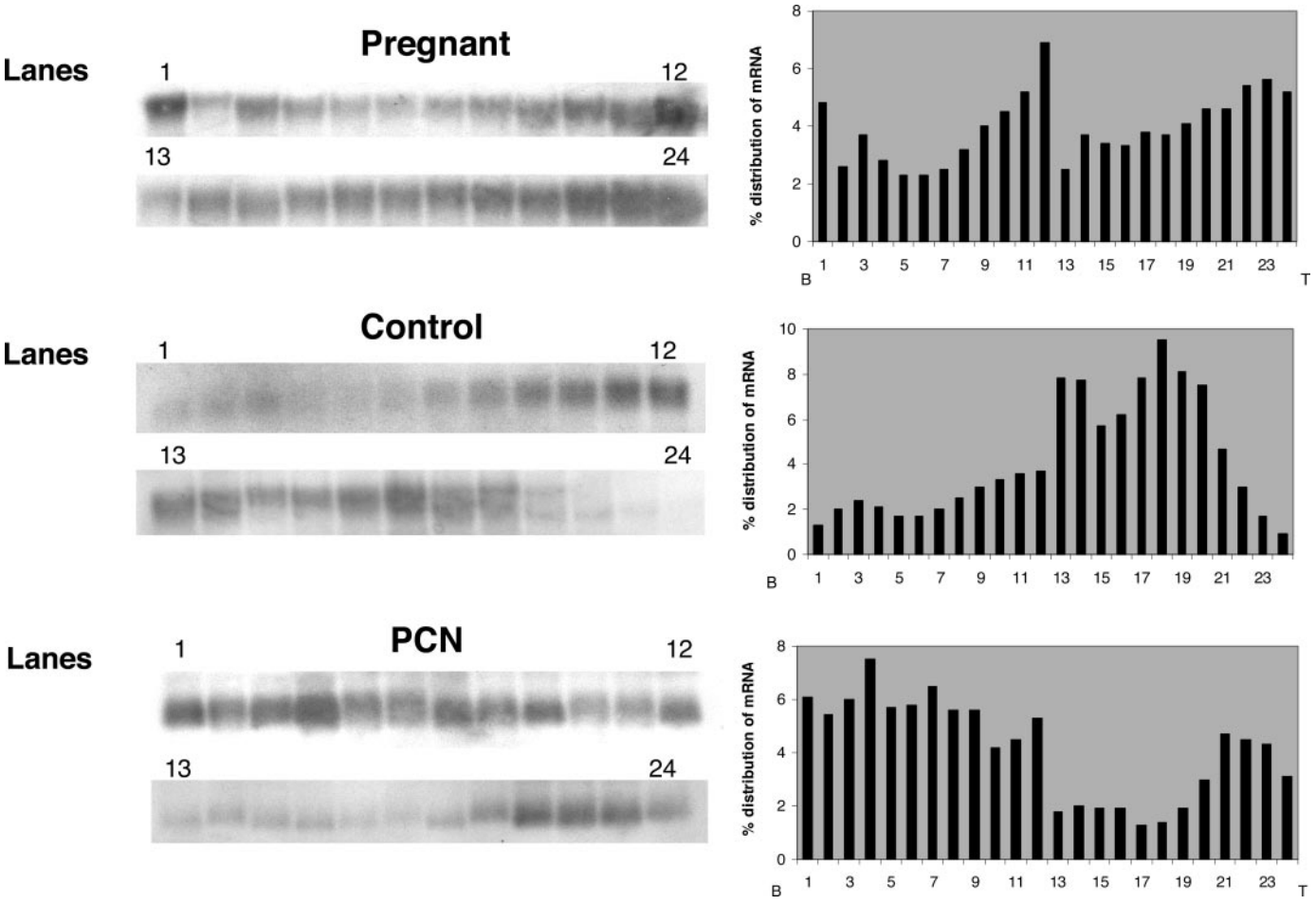

Fig. 5. Polysomal distribution analysis of control, 19-day pregnant, and PCN-treated female rats. Left, the Northern analysis of fractions collected from sucrose gradient fractionation of ribosomal-bound Mrp2 mRNA. Lane 1 represents the bottom of the gradient and lane 24 the top of the gradient. Right, the percentage of total Mrp2 mRNA in each fraction. B, bottom; T, top of the gradient. Data are typical of at least five analyses per group.

association of Mrp2 mRNA with monosomes at the top of the gradient of the polysomal distribution analysis in liver from pregnant rats is also consistent with decreased protein synthesis. The decreased expression of Mrp2 protein in pregnancy is believed to be caused by increased levels of estrogens, because treatment with high doses of ethinylestradiol also decreases Mrp2 protein with no alteration in Mrp2 mRNA expression (Trauner et al., 1997). We are currently developing an estrogen-treatment model to investigate more fully the effects on Mrp2 protein synthesis.

To investigate further the basis for changes in Mrp2 protein synthesis, we examined the potential transcription start site for Mrp2. We identified multiple transcription-initiation sites, with the major site located 98 nt upstream from the AUG initiation codon (Fig. 6). No differences were detected in the overall pattern of use of initiation sites between any of the groups examined. The locations of the initiation sites in rat liver RNA were confirmed using 5′-RACE (Table 1). The different transcription-initiation sites do not reflect altered splicing, because the sequence of Mrp2 mRNA is the same as that of the gene (data not shown). The rat Mrp2 promoter, which resembles a housekeeping gene promoter with its high G + C content, lack of a TATA box, and the presence of multiple potential binding sites for transcriptional factors, also exhibits numerous upstream open reading frames (Tanaka et al., 1999; Geier et al., 2003). We are currently investigating their role in the translational regulation of Mrp2.

In summary, our studies represent the first definitive measurement of Mrp2 protein half-life in rats. The half-life of 27 h indicates a faster turnover of Mrp2 than that suggested in earlier studies for other canalicular membrane proteins. We have confirmed that the post-transcriptional regulation of rat Mrp2 is complex and does not involve altered rates of degradation of the protein. Rather, PCN treatment increases the rate of synthesis of Mrp2 protein as determined by the rate of incorporation of [^{35}S]methionine/cysteine into Mrp2 protein and polysomal distribution analyses. The low incorporation of [^{35}S]methionine/cysteine in Mrp2 protein in pregnancy is also consistent with decreased Mrp2 protein synthesis. We did not detect any differences in the use of transcription-initiation sites between groups; however, the presence of multiple transcription-initiation sites indicates a further level of control of Mrp2 expression. The importance of the three different mRNA transcripts reflecting distinct 3′-UTRs has not been explored but may represent an additional site of regulation of Mrp2 protein expression. Although transcriptional regulation confers the ability to regulate expression over longer periods of time, translational regulation enables a more rapid response to physiological, pharmacological, or toxicological stress. Our findings, which demonstrate multiple sites of post-transcriptional regulation of Mrp2 activity, reinforce the concept that the function of this protein is critically important for the cell and organism.

TABLE 1

Analysis of the major Mrp2 transcription initiation sites in rat liver

A cDNA cloning strategy was implemented to analyze the size and frequency of transcription initiation sites in control rats. The transcript leader sequence of rat Mrp2 cDNA was isolated by a 5′-rapid amplification of cDNA ends procedure (GeneRacer; Invitrogen). Thirty-three clones were sequenced as described in the text.

Transcription Initiation Site	Frequency
	clones/total clones
−213 nt	1/33
−163 nt	2/33
−132 nt	1/33
−98 nt	15/33
−71 nt	2/33

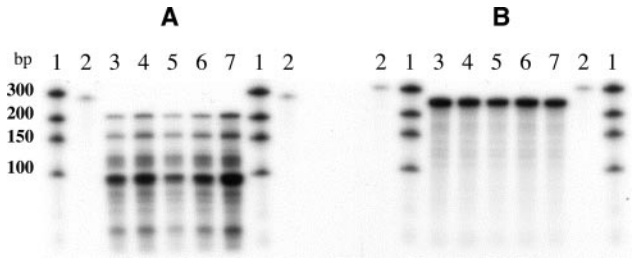

Fig. 6. Ribonuclease protection assay analysis of the major Mrp2 transcription initiation sites in male and female, 19-day pregnant, and PCN-treated female rats. Total RNA (20 μg) was loaded into each lane and hybridized with ^{32}P-labeled Mrp2 (A) and GAPDH (B) cDNA probes. A, lane 1, molecular weight marker; lane 2, rat Mrp2 RPA probe; lane 3, control male rat liver RNA; lane 4, control female rat liver RNA; lane 5, 19-day pregnant rat liver RNA; lane 6, corn oil-treated rat liver RNA; lane 7, PCN-treated rat liver RNA. B, the lanes correspond as outlined for A, except lane 2 contains rat GAPDH RPA probe. The data are representative of similar analyses in three rats per group.

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