articles

molecular pharmaceutics

Activation of Human CYP2C9 Promoter and Regulation by CAR and PXR in Mouse Liver

Mohammed S. Al-Dosari,† Joseph E. Knapp, and Dexi Liu*

Department of Pharmaceutical Sciences, School of Pharmacy, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

Received September 30, 2005

Abstract: The activity of various genomic segments at the 5′-flanking region of the human CYP2C9 gene in driving gene expression and their involvement in pregnane X receptor (PXR) and constitutive androstane receptor (CAR) mediated activation were evaluated in mouse hepatocytes. Using the genomic sequence of human CYP2C9 as a template, segments covering different regions of CYP2C9 5′-flanking sequences starting from the translation start site were amplified by PCR and inserted into a pGL-3 luciferase vector. Plasmid DNA containing the 0.2K, 1K, 2K, 3K, 5K, or 10K upstream sequences of the CYP2C9 gene were transfected into mouse liver by hydrodynamic delivery, and the activity of each fragment in driving reporter gene expression was assessed. With the exception of the 10K fragment, the level of luciferase activity in transfected mouse liver was similar among the constructs examined. Cotransfection of these reporter constructs with the pCMX-PXR or pCMX-CAR plasmids resulted in a slight increase in luciferase gene expression that could be significantly enhanced by chemical inducers. In mice cotransfected with pCMX-PXR, pregnenolone-16 α-carbonitrile (PCN) induced a 20-fold increase in the luciferase level compared to a 70-fold increase induced by rifampicin. Similarly, when animals were cotransfected with the pCMX-CAR plasmid, phenobarbital and 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene enhanced luciferase gene expression by 10- and 57-fold, respectively. The element responsible for PXR- and CAR-mediated activation of luciferase gene expression by chemical inducers was found to reside in the −2000 to −1000 bp region of the 5′-flanking sequence of the CYP2C9 gene. These results prove that PXR and CAR are transcription factors regulating CYP2C9 gene expression.

Keywords: Drug metabolism; CYP2C9; cytochrome P450; hydrodynamic gene delivery

Introduction

The human CYP2C subfamily consists of four members, CYP2C8, CYP2C9, CYP2C18, and CYP2C19, whose genes are located in chromosome 10[1−3] and predominantly expressed in the liver.[4−6] The total CYP2C enzymes account for about 18% of total adult liver CYP proteins[7] with CYP2C9 as the predominant isoform.[4] CYP2C9 metabolizes about 16% of clinically important drugs,[8] including the hypoglycemic tolbutamide,[9] the anticonvulsant phenytoin,[10] and the anticoagulant *S*-warfarin,[11] known for their narrow therapeutic indices. In principle, changes in the level of

* Author to whom correspondence should be addressed. Mailing address: 527 Salk Hall, Department of Pharmaceutical Sciences, University of Pittsburgh School of Pharmacy, Pittsburgh, PA 15261. Tel: (412) 648-8553. Fax: (412) 383−7436. E-mail: dliu@pitt.edu.

† Current address: Department of Pharmacognosy, King Saud University School of Pharmacy, P.O. Box 2457, Riyadh 11451, Saudi Arabia.

(1) Gray, I. C.; Nobile, C.; Muresu, R.; Ford, S.; Spurr, N. K. A 2.4-megabase physical map spanning the CYP2C gene cluster on chromosome 10q24. *Genomics* **1995**, *28*, 328−332.

(2) Inoue, K.; Inazawa, J.; Suzuki, Y.; Shimada, T.; Yamazaki, H.; Guengerich, F. P.; Abe, T. Fluorescence in situ hybridization analysis of chromosomal localization of three human cytochrome P450 2C genes (CYP2C8, 2C9, and 2C10) at 10q24.1. *Jpn. J. Hum. Genet.* **1994**, *39*, 337−343.

10.1021/mp0500824 CCC: $33.50
Published on Web 03/03/2006

functional CYP2C9 enzyme could have significant pharmacological and toxicological consequences for many drug-based treatments.

Control of the CYP2C9 enzyme level is carried out at the transcriptional level. As demonstrated by induction studies using primary culture and human hepatoma cell lines, regulation of CYP2C9 gene expression has been reported to involve the pregnane X receptor (PXR) and constitutive androstane receptor (CAR).[12] Vitamin D receptor,[13] glucocorticoid receptor,[14] hepatic nuclear factor 4α (HNF4α),[15] HNF3γ,[16] and CCAAT/enhancer binding protein α (C/EBPα),[17] all have been shown to up-regulate the expression of CYP2C9. Furthermore, four different regulatory elements in the 5′-flanking region of CYP2C9 were identified and functionally studied, including the HNF4α binding site (−155 bp),[15] glucocorticoid receptor-responsive element (−1675 bp),[16] proximal CAR-responsive element (−1839 bp),[14] and distal CAR-responsive element (−2899 bp).[18] However, the results from some of these in vitro studies are controversial. For example, using human hepatocytes, Runge et al.[19] reported that rifampicin and phenobarbital have no inductive role in the expression of CYP2C9 while Raucy et al.[20] demonstrated the opposite. The reasons for the discrepancy are currently unknown, but could be due to the biology of the systems employed since these studies were conducted in vitro using primary culture or established cell lines. Although widely used and the best system available, these in vitro systems often suffer from the lack of many crucial liver specific transcription factors that might have been lost during the culturing.[21] To overcome this problem we employed the newly developed hydrodynamics-based procedure[22] to transfect mice liver cells and evaluate the activity of human CYP2C9 5′-flanking region in driving gene expression in whole animals.[23] This procedure has been used to evaluate gene regulation of other CYP genes in vivo.[24−27] The experiments described in this report were designed to determine the functional role of CYP2C9 5′-flanking region,

(3) Meehan, R. R.; Gosden, J. R.; Rout, D.; Hastie, N. D.; Friedberg, T.; Adesnik, M.; Buckland, R.; van Heyningen, V.; Fletcher, J.; Spurr, N. K. Human cytochrome P-450 PB-1: a multigene family involved in mephenytoin and steroid oxidations that maps to chromosome 10. *Am. J. Hum. Genet.* **1988**, *42*, 26−37.

(4) Goldstein, J. A.; de Morais, S. M. Biochemistry and molecular biology of the human CYP2C subfamily. *Pharmacogenetics* **1994**, *4*, 285−299.

(5) Richardson, T. H.; Griffin, K. J.; Jung, F.; Raucy, J. L.; Johnson, E. F. Targeted antipeptide antibodies to cytochrome P450 2C18 based on epitope mapping of an inhibitory monoclonal antibody to P450 2C51. *Arch. Biochem. Biophys.* **1997,** *338*, 157−164.

(6) Wrighton, S. A.; Stevens, J. C. The human hepatic cytochromes P450 involved in drug metabolism. *Crit. Rev. Toxicol.* **1992,** *22*, 1−2.

(7) Shimada, T.; Yamazaki, H.; Mimura, M.; Inui, Y.; Guengerich, F. P. Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *J. Pharmacol. Exp. Ther.* **1994**, *270*, 414−423.

(8) Schwarz, U. I. Clinical relevance of genetic polymorphisms in the human CYP2C9 gene. *Eur. J. Clin. Invest.* **2003**, *33* (Suppl. 2), 23−30.

(9) Relling, M. V.; Aoyama, T.; Gonzalez, F. J.; Meyer, U. A. Tolbutamide and mephenytoin hydroxylation by human cytochrome P450s in the CYP2C subfamily. *J. Pharmacol. Exp. Ther.* **1990**, *252*, 442−447.

(10) Veronese, M. E.; Mackenzie, P. I.; Doecke, C. J.; McManus, M. E.; Miners, J. O.; Birkett, D. J. Tolbutamide and phenytoin hydroxylations by cDNA-expressed human liver cytochrome P4502C9. *Biochem. Biophys. Res. Commun.* **1991**,*175*, 1112−1118.

(11) Rettie, A. E.; Korzekwa, K. R.; Kunze, K. L.; Lawrence, R. F.; Eddy, A. C.; Aoyama, T.; Gelboin, H. V.; Gonzalez, F. J.; Trager, W. F. Hydroxylation of warfarin by human cDNA-expressed cytochrome P-450: a role for P-4502C9 in the etiology of (S)-warfarin-drug interactions. *Chem. Res. Toxicol.* **1992,** *5*, 54−59.

(12) Gerbal-Chaloin, S.; Pascussi, J. M.; Pichard-Garcia, L.; Daujat, M.; Waechter, F.; Fabre, J. M.; Carrere, N.; Maurel, P. Induction of CYP2C genes in human hepatocytes in primary culture. *Drug Metab. Dispos.* **2001,** *29*, 242−251.

(13) Drocourt, L.; Ourlin, J. C.; Pascussi, J. M.; Maurel, P.; Vilarem, M. J. Expression of CYP3A4, CYP2B6, and CYP2C9 is regulated by the vitamin D receptor pathway in primary human hepatocytes. *J. Biol. Chem.* **2002**, *277*, 25125−25132.

(14) Gerbal-Chaloin, S.; Daujat, M.; Pascussi, J. M.; Pichard-Garcia, L.; Vilarem, M. J.; Maurel, P. Transcriptional regulation of CYP2C9 gene. Role of glucocorticoid receptor and constitutive androstane receptor. *J. Biol. Chem.* **2002**, *277*, 209−217.

(15) Ibeanu, G. C.; Goldstein, J. A. Transcriptional regulation of human CYP2C genes: functional comparison of CYP2C9 and CYP2C18 promoter regions. *Biochemistry* **1995**, *34*, 8028−8036.

(16) Bort, R.; Gomez-Lechon, M. J.; Castell, J. V. Jover, R. Role of hepatocyte nuclear factor 3 gamma in the expression of human CYP2C genes. *Arch. Biochem. Biophys.* **2004**, *426*, 63−72.

(17) Jover, R.; Bort, R.; Gomez-Lechon, M. J.; Castell, J. V. Re-expression of C/EBP alpha induces CYP2B6, CYP2C9 and CYP2D6 genes in HepG2 cells. *FEBS. Lett.* **1998**, *431* (2), 227−230.

(18) Ferguson, S. S.; LeCluyse, E. L.; Negishi, M.; Goldstein, J. A. Regulation of human CYP2C9 by the constitutive androstane receptor: discovery of a new distal binding site. *Mol. Pharmacol.* **2002**, *62*, 737−746.

(19) Runge, D.; Kohler, C.; Kostrubsky, V. E.; Jager, D.; Lehmann, T.; Runge, D. M.; May, U.; Stolz, D. B.; Strom, S. C.; Fleig, W. E.; Michalopoulos G. K. Induction of cytochrome P450 (CYP)-1A1, CYP1A2, and CYP3A4 but not of CYP2C9, CYP2C19, multidrug resistance (MDR-1) and multidrug resistance associated protein (MRP-1) by prototypical inducers in human hepatocytes. *Biochem. Biophys. Res. Commun.* **2000**, *273*, 333−341.

(20) Raucy, J. L.; Mueller, L.; Duan, K.; Allen, S. W.; Strom, S.; Lasker, J. M. Expression and induction of CYP2C P450 enzymes in primary cultures of human hepatocytes. *J. Pharmacol. Exp. Ther.* **2002**, *302*, 475−482.

(21) Chen, Y.; Ferguson, S. S.; Negishi, M.; Goldstein, J. A. Induction of human CYP2C9 by rifampicin, hyperforin, and phenobarbital is mediated by the pregnane X receptor. *J. Pharmacol. Exp. Ther.* **2004**, *308*, 495−501.

(22) Liu, F.; Song, Y.; Liu, D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther.* **1999**, *6*, 1258−1266.

(23) Zhang, G.; Gao, X.; Song, Y. K.; Vollmer, R.; Stolz, D. B.; Gasiorowski, J. Z.; Dean, D. A.; Liu, D. Hydroporation as the mechanism of hydrodynamic delivery. *Gene Ther.* **2004**, 11, 675−682.

Table 1. Primer Sequences for PCR Amplification[a]

fragment name	sequence range	primer sequences
0.2K	−211/+25	FP: ATTGAGGTACCAGTGGACAATGGAACGAAGG
		RP: AATTACTCGAGGCACAAGGACCACAAGAGAATC
1K	−1011/+25	FP: ATTGAGGTACCCACTGAGCGTTTCACTTCTGC
		RP: AATTACTCGAGGCACAAGGACCACAAGAGAATC
2K	−2145/+2	FP: GGTACCGATCTCAGATATCCCTTCTATC
		RP: ACGCGTATTGTTGCCTTCTCTTCTTGAC
3K	−3024/+25	FP: ATTGAGGTACCAAGGAAGGGAGAGAGAACACG
		RP: AATTACTCGAGGCACAAGGACCACAAGAGAATC
5K	−5470/+25	FP: ATTGAGGTACCCAGCGAACTAAGAATAGAGGAGG
		RP: AATTACTCGAGGCACAAGGACCACAAGAGAATC
6K	−6437/+25	FP: TAGTGAAAGCAGTGGTTAGAGGG
		RP: AATTACTCGAGGCACAAGGACCACAAGAGAATC
4K	−9900/−5485	FP: ATTGAGGTACCTTGTGGAGGAAGTGAGTCCC
		RP: GAATGTGTGCTGGATTTAGGC

[a] Ligation of 6K and 4K fragments together to make a 10K fragment. FP: forward primer sequence. RP: reverse primer sequence.

PXR, and CAR in CYP2C9 induction. Using a series of plasmid constructs containing a reporter gene and various lengths of 5′-flanking region inserted at the 5′-end of the coding sequence of reporter gene, here we demonstrate that both PXR and CAR are essential CYP2C9 inducing factors. We also point out that the critical responsive element(s) required for both PXR and CAR resides within the −1K and −2K region of CYP2C9 5′-flanking sequences.

Materials. PCR kits were purchased from Epicentre (Madison, WI). The RP11-208C17 BAC clone containing the full genomic sequence of human CYP2C9 was obtained from CHORI (Oakland, CA). PCR primers were synthesized at IDT (Coralville, IA). Rifampicin (RIF), phenobarbital (PHB), 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (BOP), pregnenolone-16 α-carbonitrile (PCN), and DMSO were obtained from Sigma-Aldrich (St. Louis, MO). Luciferase assay kits were purchased from Promega (Madison, WI). Protein assay reagent was from Bio-Rad (Hercules, CA). All other chemicals were obtained from Sigma-Aldrich (Saint Louis, MO). CD-1 mice (female, 18−20 g) were obtained from Charles River (Wilmington, MA).

Plasmid Construction. Various fragments of CYP2C9 5′-flanking sequences were cloned into pGL3-basic vector (Promega, Madison, WI). The primer sequences for PCR amplification are listed in Table 1, and the cloning procedures are illustrated schematically in Figure 1. The primer sequences were synthesized with a *Kpn*I site attached to the 5′-end of the forward and an *Xho*I site to the 5′-end of the reverse primers. The reverse primer for the 2K fragment was synthesized with an *Mlu*I site attached to the 5′-end. The 10K fragment was ligated from two PCR products (4K, 6K) after *Xba*I digestion. BAC clone was used as PCR template for all generated CYP2C9 fragments (with exception of the 2K, for which we used genomic DNA of human liver as template). To clone a CYP2C9 fragment into the pGL3-basic vector, the PCR product was separated on an agarose gel and the proper fragment isolated. After digestion with the proper restriction enzymes, PCR fragments and linearized pGL3-basic vector were ligated and transformed into *Escherichia coli* (DH-5α). Each sequence insertion was confirmed by PCR and restriction enzyme digestion. The resulting plasmids were named on the basis of the length of the inserted CYP2C9 fragment. The p2C9-4K-Luc plasmid which contains the sequence from −5485 to −9900 without promoter sequence was constructed as the negative control. The expression vectors for mouse CAR (pCMX-CAR) and human PXR (pCMX-PXR) were kindly provided by Dr. Wen Xie (Center for Pharmacogenetics, University of Pittsburgh School of Pharmacy).

Plasmid DNA Preparation. Plasmid DNA was purified from transformed *E. coli* (DH-5α) using cesium chloride gradient centrifugation and kept in saline. DNA purity was confirmed by spectroscopy and gel electrophoresis.

Hydrodynamic Transfection of Animals. CD-1 mice were injected via the tail vein within 4−5 s with 1.8 mL of saline solution containing different CYP2C9 plasmid constructs with or without pCMX-PXR or pCMX-CAR. To investigate the effects of drug treatment, the animals were intraperitoneally (ip) treated with 100 μL of DMSO contain-

(24) Rivera-Rivera, I.; Kim, J.; Kemper, B. Transcriptional analysis in vivo of the hepatic genes, Cyp2b9 and Cyp2b10, by intravenous administration of plasmid DNA in mice. *Biochim. Biophys. Acta* **2003**, *1619*, 254−262.

(25) Schuetz, E.; Lan, L.; Yasuda, K.; Kim, R,; Kocarek, T. A.; Schuetz, J.; Strom, S. Development of a real-time in vivo transcription assay: application reveals pregnane X receptor-mediated induction of CYP3A4 by cancer chemotherapeutic agents. *Mol. Pharmacol.* **2002**, *62*, 439−445.

(26) Tirona, R. G.; Lee, W.; Leake, B. F.; Lan, L. B.; Cline, C. B.; Lamba, V.; Parviz, F.; Duncan, S. A.; Inoue, Y.; Gonzalez, F. J.; Schuetz, E. G.; Kim, R. B. The orphan nuclear receptor HNF4alpha determines PXR- and CAR-mediated xenobiotic induction of CYP3A4. *Nat. Med.* **2003**, *9*, 220−224.

(27) Zhang, W.; Purchio, A.; Chen, K.; Burns, S. M.; Contag, C. H.; Contag, P. R. In vivo activation of the human CYP3A4 promoter in mouse liver and regulation by pregnane X receptors. *Biochem. Pharmacol.* **2003**, *65*, 1889−1896.

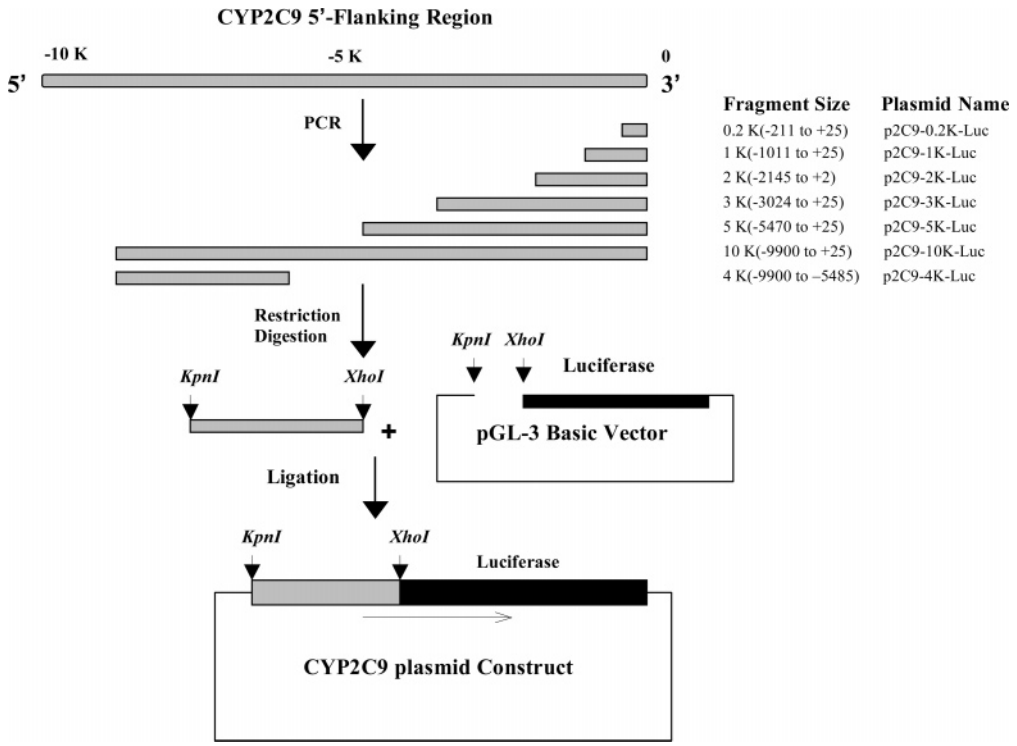

Figure 1. Generation of CYP2C9 plasmid constructs. Various DNA fragments of the 5′-flanking region of CYP2C9 genome sequences were PCR amplified using selected primers listed in Table 1. After restriction enzyme digestion and to create the necessary complimentary ends, the PCR product was then ligated to the same restriction digestion site of pGL3-basic vector to generate CYP2C9 plasmid constructs.

ing RIF (200 mg/kg), BOP (10 mg/kg), PHB (200 mg/kg), or PCN (200 mg/kg) 2 h posttransfection. Animals were sacrificed 24 h after hydrodynamic injection and the livers obtained. Individual tissue samples of approximately 200 mg were homogenized in 1 mL of lysis buffer (0.1 M Tris-HCl, 2 mM EDTA, 0.1% Triton X-100, pH 7.8), followed by centrifugation in a microcentrifuge (12 000 rpm, 10 min, 4 °C). Protein concentration and luciferase activity in the supernatant were determined using assay reagents from Bio-Rad and Promega, respectively.

Luciferase Assay. Ten microliters of supernatant of tissue homogenate were added to 100 μL of substrate solution provided as part of the luciferase assay kit. Luciferase activity was measured in a luminometer (Autolumat LB953, EG & G, Berthhold, Germany) using a 10 s measurement time. Luciferase activity in each sample was normalized to relative light units per milligram of extracted protein.

Statistical Analysis. A one-way ANOVA test was performed between the testing and control groups. A P value at ≤ 0.05 represents a significant difference.

Results

Basal Activity of 5′-Flanking Sequence of CYP2C9 in Driving Reporter Gene Expression. To systematically evaluate the function of various segments of the 5′-flanking sequence of CYP2C9, we generated a series of plasmid constructs containing different portions of the 5′-flanking sequence of CYP2C9 ranging from the translation start site to −9900 bp. Figure 1 illustrates the strategy and procedure used for generation of the plasmids. The activities of the inserted 5′-flanking sequences of CYP2C9 in driving luciferase gene expression were assessed in animals after hydrodynamic gene delivery. Figure 2 shows that the level of luciferase expression was very similar among the plasmid constructs, with the exception of p2C9-10K-Luc. This construct exhibited a level of luciferase activity lower even than that of promoterless construct (p2C9-4K-Luc).

Effect of DNA Dose on the Level of Transgene Expression. The amount of DNA injected into each animal in Figure 2 was 20 μg. To rule out the possibility that low activity seen in p2C9-10K-Luc transfected animals was caused by a relatively low copy number of the injected plasmid, we transfected mice with increasing amounts of plasmid DNA. Three constructs (p2C9-1K-Luc, p2C9-5K-Luc, and p2C9-10K-Luc) were selected and the injection dose was adjusted to give equal molar amounts to each animal. The data presented in Figure 3 show an increase in luciferase gene expression with increasing amounts of p2C9-1K-Luc and p2C9-5K-Luc. However, the level of luciferase gene expression resulting from injection of an identical molar amount of p2C9-10K-Luc remained significantly low.

Enhancement of the Basal CYP2C9 Activity by CAR and PXR. When the p2C9-5K-Luc was transfected into animals, cotransfection with pCMX-PXR increased luciferase activity by 2-fold, while treatment of the animals with PCN or RIF brought the level of increase to 18- and 70-fold,

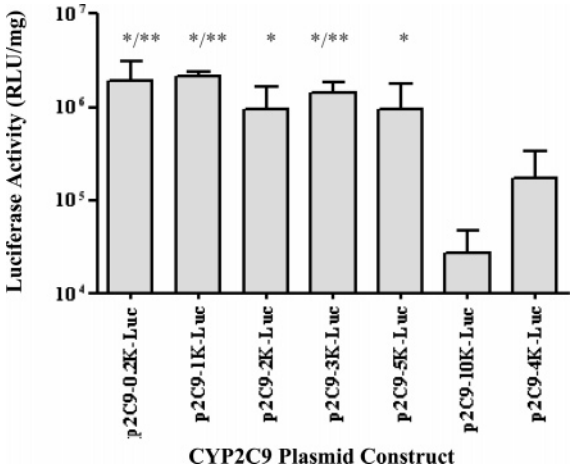

Figure 2. Basal activity of 5′-flanking sequence of CYP2C9 in driving reporter gene expression. Each animal received 20 μg of plasmid by the hydrodynamics-based procedure, and luciferase activity was analyzed 24 h posttransfection. Values represent the mean ± SD of 3 independent transfections. $P < 0.05$ compared with p2C9-10K-Luc (∗), and $P < 0.05$ compared to p2C9-4K-Luc (∗∗).

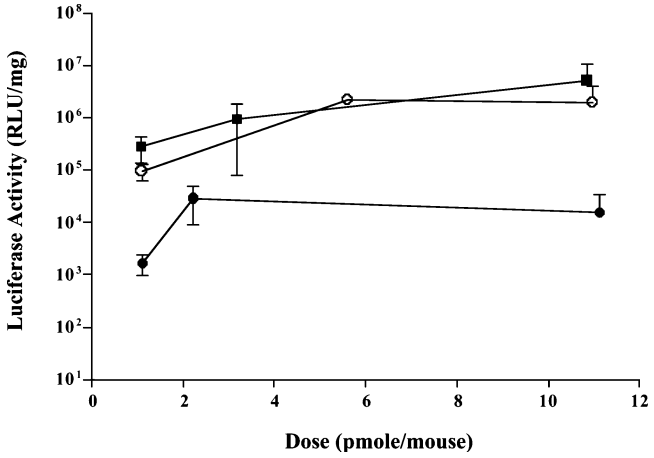

Figure 3. Dose dependent luciferase gene expression. Mice were hydrodynamically transfected with different amounts of p2C9-10K-Luc (●), p2C9-5K-Luc (■), or p2C9-1K-Luc (○). Twenty-four hours post transfection, luciferase activities in liver were determined. Values represent the mean ± SD of 3 independent transfections.

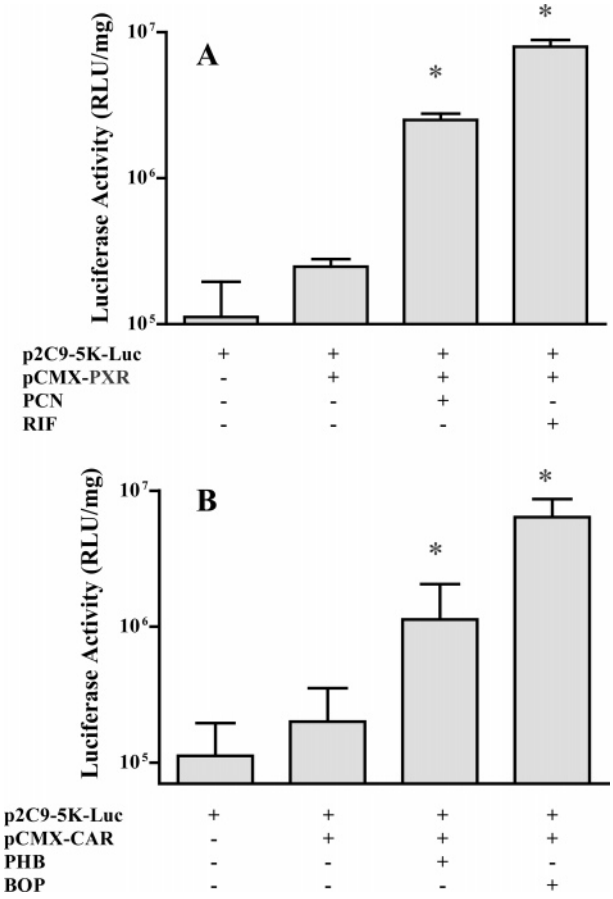

Figure 4. Enhancement of the basal CYP2C9 activity by PXR and CAR. Mice were cotransfected with 2 μg of p2C9-5K-Luc and 5 μg of pCMX-PXR (A) or pCMX-CAR (B). Two hours post transfection, the animals cotransfected with pCMX-PXR were treated with 100 μL of DMSO with or without PCN (200 mg/kg) or RIF (200 mg/kg). Similarly, animals cotransfected with pCMX-CAR were treated with 100 μL of DMSO with or without PHB (200 mg/kg) or BOP (10 mg/kg). Luciferase gene expression was assessed 22 h after drug treatment. Values represent the mean ± SD ($n = 3$). The asterisk symbol (*) indicates $P < 0.05$ compared to control groups without drug treatment.

respectively (Figure 4A). Under our experimental conditions, RIF appears to be a stronger inducer than PCN. Similarly, Figure 4B shows that cotransfection with pCMX-CAR increased luciferase activity by approximately 2-fold, while treatment of the animals with PHB or BOP generated further enhancement by a total of 10- and 56-fold, respectively.

Time Response Curve of CAR/PXR-Induced Enhancement of CYP2C9 Activity. Two groups of animals were cotransfected with 2 μg of p2C9-5K-Luc and 5 μg of either pCMX-PXR or pCMX-CAR. The groups of animals cotransfected with p2C9-5K-Luc/pCMX-PXR were treated with RIF (200 mg/kg), and those cotransfected with p2C9-5K-Luc/pCMX-CAR were treated with BOP (10 mg/kg). Figure 5 shows that PXR, upon activation by RIF, was able to induce a transient luciferase gene expression that peaked at 22 h and declined to background level after 48 h. The same pattern was seen in animals treated with BOP (CAR-mediated activation), reaching peak level in 1 day and returning to background level in about 4 days. Although significantly lower in magnitude, a second dose of RIF produced a 6-fold increase in p2C9-5K-Luc activity, while a second dose of BOP led to a 2-fold increase. These data suggest that drug induced enhancement of luciferase gene expression is transient.

Identification of the Functional Elements in the 5′-Flanking Region of CYP2C9 Responsible for PXR- and CAR-Mediated Activation. To identify the sequence element responsible for PXR- and CAR-mediated activation of CYP2C9, a cotransfection procedure identical to those

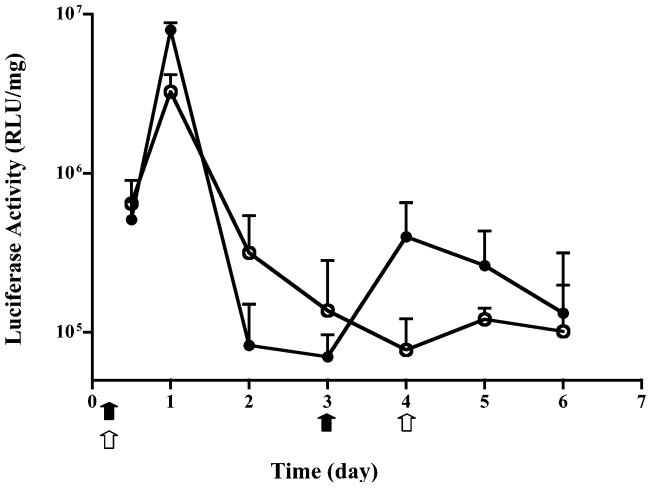

Figure 5. Time response curve of drug-induced enhancement of CYP2C9 activity. Two groups of animals were cotransfected on day zero with either p2C9-5K-Luc/pCMX-PXR (2 μg/5 μg per mouse) (●) or p2C9-5K-Luc/pCMX-CAR (2 μg/5 μg per mouse) (○). Each group was further divided into two subgroups. For animals cotransfected with p2C9-5K-Luc and pCMX-PXR, all animals were injected 2 h posttransfection with RIF (200 mg/kg). The first subgroup was sacrificed at the indicated time point after drug treatment. The second subgroup of the animals received a second equivalent dose of RIF on day 3, and animals were subsequently sacrificed at the indicated time point. An identical experimental procedure was used for animals cotransfected with p2C9-5K-Luc and pCMX-CAR with the exception that animals were treated with BOP (10 mg/kg) and the second dose was given on day 4. Arrows indicate the time of drug treatment. Values represent the mean ± SD ($n = 3$).

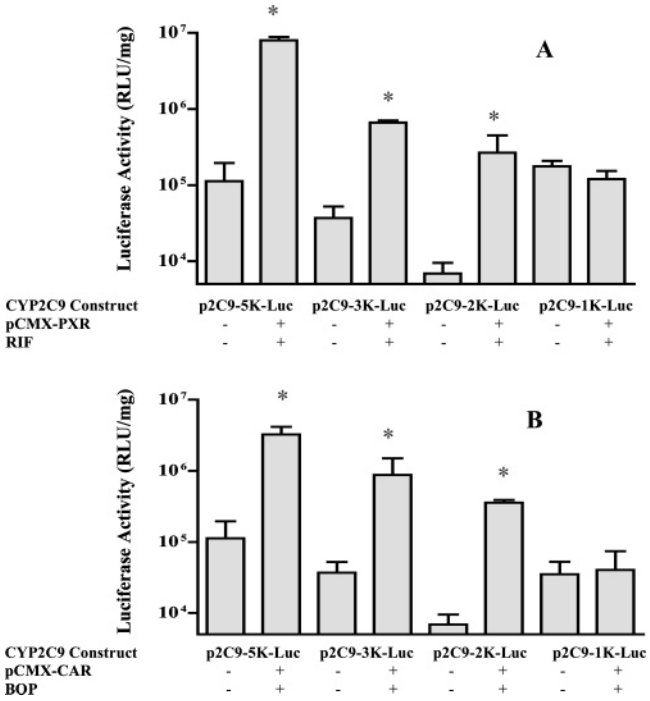

Figure 6. Identification of the responsive elements in the 5′-flanking region of CYP2C9 responsible for PXR- and CAR-mediated activation. Animals were cotransfected with 2 μg of various CYP2C9 plasmid constructs and 5 μg of pCMX-PXR (A) or pCMX-CAR (B). Drug treatments were performed 2 h transfection at the dose of 200 mg/kg for RIF and 10 mg/kg for BOP. Luciferase activity in the liver was assessed 22 h after the treatment. Values represent the mean ± SD ($n = 3$). The asterisk symbol (*) indicates $P < 0.05$ compared to group without drug treatment.

summarized in Figure 5 was performed using different CYP2C9 plasmid constructs. Figure 6A shows that RIF, in the presence of PXR, enhanced the p2C9-5K-Luc activity by 71-fold. It also enhanced the p2C9-3K-Luc activity by 18-fold and the p2C9-2K-Luc by 39-fold. This significant activation was not observed when p2C9-1K-Luc was used. In animals cotransfected with pCMX-CAR, BOP enhanced the activity of p2C9-5K-Luc, p2C9-3K-Luc, and p2C9-2K-Luc by 57-, 23-, and 52-fold, respectively. Again, no enhancement was seen in animals transfected with the p2C9-1K-Luc plasmid (Figure 6B).

Discussion

In the current study we have evaluated the activity of the CYP2C9 5′-flanking sequence in driving reporter gene expression in mouse liver. We also investigated the role of different nuclear receptors and transcription factors in regulation of CYP2C9 promoter activity and identified the elements responsible for PXR- and CAR-mediated activation.

Results in Figure 2 show that all cloned promoter containing 5′-flanking sequences of CYP2C9 were active in driving transgene expression in mouse liver with the exception of p2C9-10K-Luc that exhibited background luciferase activity. We attributed such weak activity of the 10K fragment to possible existence of effective repressor sequences in the upper flanking region (−5K to −10K). A similar observation was reported by Schuetz et al.,[25] who demonstrated significantly lower activity of the 10K fragment than that of shorter fragments derived from the same 5′-flanking region of CYP3A4. Figure 3 demonstrates that the difference in plasmid size and DNA amount did not cause the observed low activity of p2C9-10K-Luc, particularly when we know that efficient transfection by hydrodynamic delivery of a much larger construct (157K) has been reported.[28]

Under physiological conditions, we found that PXR significantly enhanced luciferase gene expression of p2C9-5K-Luc, especially in the presence of xenobiotics. Rifampicin, known for its in vivo and in vitro induction of CYP2C9,[29,30] was found to be the most potent inducer for

(28) Magin-Lachmann, C.; Kotzamanis, G.; D'Aiuto, L.; Cooke, H.; Huxley, C.; Wagner, E. In vitro and in vivo delivery of intact BAC DNA—comparison of different methods. *J. Gene Med.* **2004,** *6,*195−209.

(29) Gerbal-Chaloin, S.; Pascussi, J. M.; Pichard-Garcia, L.; Daujat, M.; Waechter, F.; Fabre, J. M.; Carrere, N.; Maurel, P. Induction of CYP2C genes in human hepatocytes in primary culture. *Drug Metab. Dispos*. **2001**, *29,* 242−251.

CYP2C9 via PXR activation (Figure 4A). In mouse liver, RIF treatment in the absence of PXR did not exhibit any enhancement (data not shown), suggesting a crucial role of PXR in promoting CYP2C9 induction. The fact that PCN (Figure 4A), a selective rodent PXR activator, was also able to induce the PXR-dependent CYP2C9 activity proves the same point. This PCN activity was found to be mainly due to activation of the injected PXR (data not shown). Our results disagree with the findings of Gebral-Chaloin et al.[14] but agree with the recent findings of Chen et al.[21]

In mouse liver, we also found that CAR is another potent factor required for CYP2C9 induction. BOP, a potent selective mouse CAR activator, was able to significantly enhance the activity of CYP2C9 (Figure 4B). On the other hand, BOP was able to partially induce the activity of CYP2C9 even in the absence of CAR cotransfection (data not shown), indicating the potential involvement of the endogenous mouse CAR in the activation. PHB, known for its inductive activity toward CYP2C9,[20,31] also enhanced the activity of CYP2C9 but to a lesser extent (Figure 4B). These results agree with the findings of Ferguston et al.[18] and Gerbal-Chaloin et al.[12] with regard to the essential role of CAR in CYP2C9 induction.

As shown in Figure 5, the induction mediated by CAR/PXR in CYP2C9 activity is transient, peaking at 22 h after drug treatment and returning to the basal level within 48−72 h. Although drug metabolism is likely the main cause of activity declining, promoter silencing through methylation is also likely to be involved. CpG based silencing has been demonstrated to be the cause for transient transgene expression in mice,[32] and could happen for both CYP2C9 plasmid construct, PXR, or CAR expression vectors. Nevertheless, statistically significant enhancement induced by a second dose of drug treatment suggests that these plasmids remain functional after 2−4 days posttransfection.

After we found that PXR and CAR are essential factors for CYP2C9 induction, we proceeded in exploring the mechanisms underlying CYP2C9 induction by aiming at identification of the responsive elements in the CYP2C9 5′-flanking region required for PXR and CAR activation mechanism. Similar to p2C9-5K-Luc, Figure 6 shows that p2C9-3K-Luc and p2C9-2K-Luc, but not p2C9-1K-Luc, all contain these elements. Collectively, these findings indicate that the PXR and CAR responsive elements are located within the −2K and −1K region. This is consistent with the findings of Gerbal-Chaloin et al.[12] and Chen et al.,[21] who proposed the responsive element located at −1839 bp as the primary element required for PXR and CAR activation. A slightly greater enhancement shown by p2C9-3K-Luc compared to that of p2C9-2K-Luc suggests that elements at −2988 bp suggested by Ferguson et al.[18] are also involved.

While the data presented in this report support the conclusions of some of the previously published studies, our results are characterized by their significant and clear induction profiles. For example, Chen et al.[21] showed, using HepG2 cells, that RIF enhanced CYP2C9 activity by 3−5-fold in the presence of PXR. In the current study, the extent of CYP2C9 induction was 18-fold (p2C9-3K-Luc), 37-fold (p2C9-2K-Luc), and 70-fold (p2C9-5K-Luc) (Figure 6). The high sensitivity demonstrated in the current and previous studies[24−27] proves that transfection of mouse liver by hydrodynamic delivery is a valuable system for induction studies.

Furthermore, unlike most of the previously conducted studies that investigated regulation of CYP genes using an artificial promoter with a minimal TK or CMV sequences adjacent to a short fragment derived from the CYP 5′-flanking region, all plasmid constructs used in the current study contain intact genomic 5′-flanking sequences of CYP2C9. In principle, the sequence in its original alignment in the chromosome may reflect the real behavior of CYP gene expression in vivo. This is particularly relevant in view of recent findings revealing that nuclear receptor binding to the target of the target gene responsive element is genomic context dependent.[33]

In summary, here we provide direct evidence in support of the conclusion that PXR and CAR are responsible for xenobiotic-mediated CYP2C9 induction. We further demonstrate that the responsive element, required for both nuclear receptors, resides within the −2K and −1K in-between area of the CYP2C9 5′-flanking region. Greater response in reporter gene expression upon drug treatment proves that hydrodynamic gene delivery is a valuable tool for studying regulation of CYP gene expression under physiological conditions.

Acknowledgment. This work was supported in part by the National Institutes of Health through Grants RO1 HL075542 and RO1 EB002946.

MP0500824

(30) Zilly, W.; Breimer, D. D.; Richter, E. Stimulation of drug metabolism by rifampicin in patients with cirrhosis or cholestasis measured by increased hexobarbital and tolbutamide clearance. *Eur. J. Clin. Pharmacol.* **1977**, *11*, 287−293.

(31) Madan, A.; Graham, R. A.; Carroll, K. M.; Mudra, D. R.; Burton, L. A.; Krueger, L. A.; Downey, A. D.; Czerwinski, M.; Forster, J.; Ribadeneira, M. D.; Gan, L. S.; LeCluyse, E. L.; Zech, K.; Robertson, P., Jr., Koch, P.; Antonian, L.; Wagner, G.; Yu, L.; Parkinson, A. Effects of prototypical microsomal enzyme inducers on cytochrome P450 expression in cultured human hepatocytes. *Drug Metab. Dispos.* **2003**, *31*, 421−431.

(32) Yew, N. S.; Zhao, H.; Przybylska, M.; Wu, I. H.; Tousignant, J. D.; Scheule, R. K.; Cheng S. H. CpG-deleted plasmid DNA vectors with enhanced safety and long-term gene expression in vivo. *Mol. Ther.* **2002**, 5, 731−738.

(33) Song, X.; Xie, M.; Zhang, H.; Li, Y.; Sachdeva, K.; Yan, B. The pregnane X receptor binds to response elements in a genomic context-dependent manner, and PXR activator rifampicin selectively alters the binding among target genes. *Drug Metab. Dispos.* **2004**, *32*, 35−42.