

# The Antigluccorticoid RU486 Inhibits Phenobarbital Induction of the Chicken *CYP2H1* Gene in Primary Hepatocytes

BENJAMIN P. DAVIDSON,<sup>1</sup> SATISH C. DOGRA, and BRIAN K. MAY

Department of Molecular Biosciences, University of Adelaide, Adelaide, South Australia, Australia

Received November 6, 2000; accepted May 14, 2001

This paper is available online at <http://molpharm.aspetjournals.org>

## ABSTRACT

The cytochrome P450 gene *CYP2H1* is highly induced by phenobarbital in chick embryo hepatocytes. Recent studies have established that the orphan nuclear receptor CAR plays a critical role in the induction mechanism. Here, we show that a high concentration of the potent glucocorticoid and progesterone receptor antagonist RU486 almost completely blocks phenobarbital-induced accumulation of *CYP2H1* mRNA in hepatocytes yet has no effect on basal expression. In marked contrast, *CYP2H1* mRNA induced by the phenobarbital-type inducers glutethimide and 2-allylisopropylacetamide is not affected by RU486. RU486 inhibition is not mediated through the glucocorticoid or progesterone receptors. Transient transfection studies showed that RU486 does not repress through activation of the orphan receptor PXR and subsequent competition with CAR for

binding to the upstream drug-responsive 556-base-pair enhancer. Additionally, none of the known functional transcription factor binding sites found in the enhancer region was a target of RU486 inhibition. Using an artificial construct containing multiple CAR binding sites, we also established that RU486 has no direct effect on the activity of exogenously expressed CAR. There is no evidence that phenobarbital binds to CAR; we propose that RU486 inhibits phenobarbital induction, either by interfering with a phenobarbital-dependent mechanism responsible for nuclear import of CAR or with the metabolism of phenobarbital to the true inducer. Whether a novel nuclear receptor that binds RU486 at high concentrations plays a role in the inhibitory action of RU486 is an interesting possibility.

The cytochrome P450 (P450) proteins comprise a superfamily of heme-containing enzymes that are involved in the oxidative metabolism of many diverse hydrophilic compounds, including steroids, fatty acids, retinoids, and foreign chemicals, such as pharmaceutical drugs and other xenobiotics (Gonzalez, 1989; Dogra et al., 1998; Waxman, 1999). Transcriptional expression of P450 genes can be modulated both by endogenous compounds and structurally diverse exogenous compounds. Of particular interest is the tissue-selective transcriptional induction of P450 genes in response to xenobiotic inducers. We are studying the molecular mechanism by which phenobarbital, the sedative drug, induces expression of specific chicken P450 genes particularly in the liver (Hansen et al., 1989; Dogra et al., 1998; Dogra et al., 1999).

Xenobiotic-mediated mechanisms of induction of P450 genes have been identified that involve orphan receptors belonging to the nuclear receptor superfamily (Savas et al., 1999; Waxman, 1999). Of particular note is the constitutive

androstane receptor (CAR), which mediates induction of *CYP2B* genes in response to phenobarbital and other chemicals (Honkakoski et al., 1998; Kawamoto et al., 1999) and pregnane X receptor (PXR) that activates *CYP3A* genes in response to many steroids and chemicals, including phenobarbital (Kliewer et al., 1999; Moore et al., 2000). The elegant work from Honkakoski et al. (1998), and more recently Handschin et al. (2000), has defined the central role of CAR in the mechanism underlying phenobarbital induction of rodent *CYP2B* and chicken *CYP2H1* genes. A model of drug induction has been envisaged in which phenobarbital action results in the translocation of CAR into the nucleus, where it interacts with CAR binding sites in the 5' flanking regions of the responsive P450 genes (Honkakoski et al., 1998; Kawamoto et al., 1999; Tzameli et al., 2000). Similarly, *CYP3A* genes are activated in response to phenobarbital through the binding of PXR to the 5' flanking regions (Moore et al., 2000).

Genes from the *CYP2B* and *CYP3A* families can be induced not only by phenobarbital but also by the synthetic glucocorticoid agonist, dexamethasone (Honkakoski and Negishi, 1998; Schuetz et al., 2000). A long-standing question is

<sup>1</sup>Present address: Flinders Cancer Center, Flinders University of South Australia, Bedford Park, SA, Australia.

**ABBREVIATIONS:** P450, cytochrome P-450; CAR, constitutive androstane receptor; PXR, pregnane X receptor; TSA, Trichostatin A; MMTV, mouse mammary tumor virus; kb, kilobase pair(s); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; AIA, 2-allylisopropylacetamide; CAT, chloramphenicol acetyltransferase; bp, base pair(s); SV40, simian virus 40; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene; PB, phenobarbital; Dex, dexamethasone.

whether the glucocorticoid receptor is involved in the dexamethasone-mediated induction of these genes. Recent studies using glucocorticoid receptor-null mice have established that this receptor is absolutely required for dexamethasone induction of *CYP2B* genes but not for *CYP3A* genes, where PXR mediates hormone induction (Schuetz et al., 2000). Because the glucocorticoid/progesterone antagonist RU486 inhibits phenobarbital induction of the rat *CYP2B* genes (Shaw et al., 1993), there is also the issue of whether the glucocorticoid receptor is involved in this induction mechanism in rodents. Interestingly, glucocorticoid receptor is not essential for phenobarbital induction of *CYP2B* and *CYP3A* genes in mice, which proceeds in glucocorticoid receptor null mice, although at diminished levels of induction (Schuetz et al., 2000). These findings are compatible with induction mechanisms involving CAR and PXR together with cooperative interactions from glucocorticoid receptor bound to known glucocorticoid responsive elements in the 5' flanking regions of these genes (Stoltz et al., 1998; Schuetz et al., 2000).

In the present study, we have investigated the chicken *CYP2H1* gene, which is markedly induced by phenobarbital (Hansen et al., 1989; Dogra and May, 1996). Earlier, using transient transfection studies, we and others have characterized an upstream enhancer region that responds to phenobarbital and maximum induction dependent upon the binding of multiple proteins, including CAR (Dogra et al., 1999; Handschin and Meyer, 2000). We now report a novel situation, in which RU486 at high doses inhibits the phenobarbital-induced activation of the *CYP2H1* gene; this inhibition does not seem to be mediated through either glucocorticoid/progesterone or PXR receptor. We discuss these findings in relation to the proposed mechanism of phenobarbital induction of this gene.

## Materials and Methods

**Materials and Plasmids.** Phenobarbital was obtained from Faulding (South Australia), [ $\alpha^{32}$ P]dCTP and oligonucleotides were from GeneWorks Pty Ltd (South Australia). D-Threo-[dichloroacetyl-1- $^{14}$ C]chloroamphenicol (specific activity, 50–60 mCi/mmol) was from Amersham Pharmacia Biotech UK, Ltd. (Little Chalfont, Buckinghamshire, UK). Dexamethasone and cAMP were from Sigma (St. Louis, MO). Medroxyprogesterone acetate was a gift from Dr. Davidson (Flinders University, SA, Australia). RU486 was a kind gift from Roussel Uclaf (Romain Ville, France). Trichostatin A (TSA) was from Wako Pure Chemicals (Tokyo, Japan). Fertile white Leghorn chicken eggs were obtained from Hi-Chick (Gawler, SA, Australia). Construction of p4.8-SVCAT, p556-SVCAT, and pCYP-205CAT has been described previously (Dogra and May, 1997; Dogra et al., 1999). MMTV-luciferase was constructed by digesting pMSG (Pharmacia, Peapack, NJ) with *HindIII*/*SmaI* to release a 1.5 kb MMTV long terminal repeat fragment that was cloned in pBluescript. This construct was then digested with *KpnI*/*SmaI* to release MMTV, which was cloned into the multiple cloning site of pGL3-Basic vector (Promega, Madison, WI). The CAR-SV-luciferase and p556-SV-luciferase reporter plasmids were constructed by cloning four CAR binding sites from the phenobarbital-responsive enhancer (–1637 to –1622) (Handschin and Meyer, 2000) and 556-bp enhancer region of the chicken *CYP2H1* gene into a *SalI* site upstream of SV-luciferase in the pGL3 promoter vector (Promega), respectively. The sequence of the oligonucleotide containing two copies of the CAR binding site used in the construction of the CAR-SV-luciferase is: 5'-TCGAGTGAACCTTCCTTGCCCTTCCTTGAACCTTGAACCTTCCTTGCCCTG-3'.

**Preparation and Treatment of Primary Hepatocytes.** Primary hepatocytes were prepared from 17-day-old chick embryos by a

method described previously (Dogra and May, 1997). Briefly, chick embryos were decapitated and livers cannulated and perfused with 10 ml of EDTA/saline (2 mM EDTA in 0.9% NaCl) and 3 to 4 ml of 0.05% collagenase in Hanks' balanced salt solution. Pooled livers were incubated in fresh collagenase solution for 20 min, cut into small pieces, and further incubated at 37°C for 30 min with gentle pipetting every 5 min. Cells were washed with Hanks' balanced salt solution and treated with Sassa solution to lyse contaminating erythrocytes. Finally, hepatocytes were washed with Williams' E medium and plated at  $6$  to  $8 \times 10^6$  in 60-mm dishes in Williams' E medium supplemented with 10% Serum Supreme (Edward Keller Australia, Hallam, Victoria, Australia). Medium was changed after overnight culture and cells were treated either with solvent only or with RU486 (40–100  $\mu$ M) for 1 h before the following chemicals were added: phenobarbital (500  $\mu$ M), TSA (1  $\mu$ M), dexamethasone (1, 10, or 50  $\mu$ M), or medroxyprogesterone acetate (100 nM) at the final concentrations shown. Hepatocytes were further incubated for 6 h and then were used to prepare total RNA (Chomczynski and Sacchi, 1987).

**Northern Blot Analysis.** Isolated total RNA was fractionated on formaldehyde-agarose gels, blotted onto NYTRAN membrane filters (Schleicher & Schuell), and UV cross-linked. The filters were prehybridized and then hybridized with cDNA probes labeled with [ $\alpha^{32}$ P]dATP by random priming using a DNA labeling kit (Pharmacia). The specific DNA probes were as follows: pCHPB15 for the *CYP2H1* mRNA and a full-length cDNA clone for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (Dogra and May, 1997). Each probe was added at an activity of 0.5 to  $1.0 \times 10^6$  cpm/ml. Filters were washed and quantified as described previously (Dogra and May, 1997).

**Effect of RU486, Glutethimide, and AIA on Transiently Transfected Cells.** Chick embryo primary hepatocytes ( $2 \times 10^7$ ) were transfected by electroporation with 2 pmol of the following vectors: MMTV-Luciferase, p4.8-SVCAT, p556-SVCAT, or pCYP-205CAT. After transfection, each sample was split into two or three equal volumes and these were plated onto 60-mm dishes in Williams' E medium supplemented with 10% Serum Supreme (Edward Keller Australia) and 50  $\mu$ g of gentamicin per ml of medium. Hepatocytes were incubated at 37°C overnight, after which medium was changed and hepatocytes were pretreated for 1 h with solvent only or RU486 (40 or 100  $\mu$ M) before dexamethasone (0.1  $\mu$ M), phenobarbital (500  $\mu$ M), glutethimide (500  $\mu$ M), or AIA (50  $\mu$ g/ml) were added. The cells were further cultured for 48 h and then CAT or luciferase activities were determined.

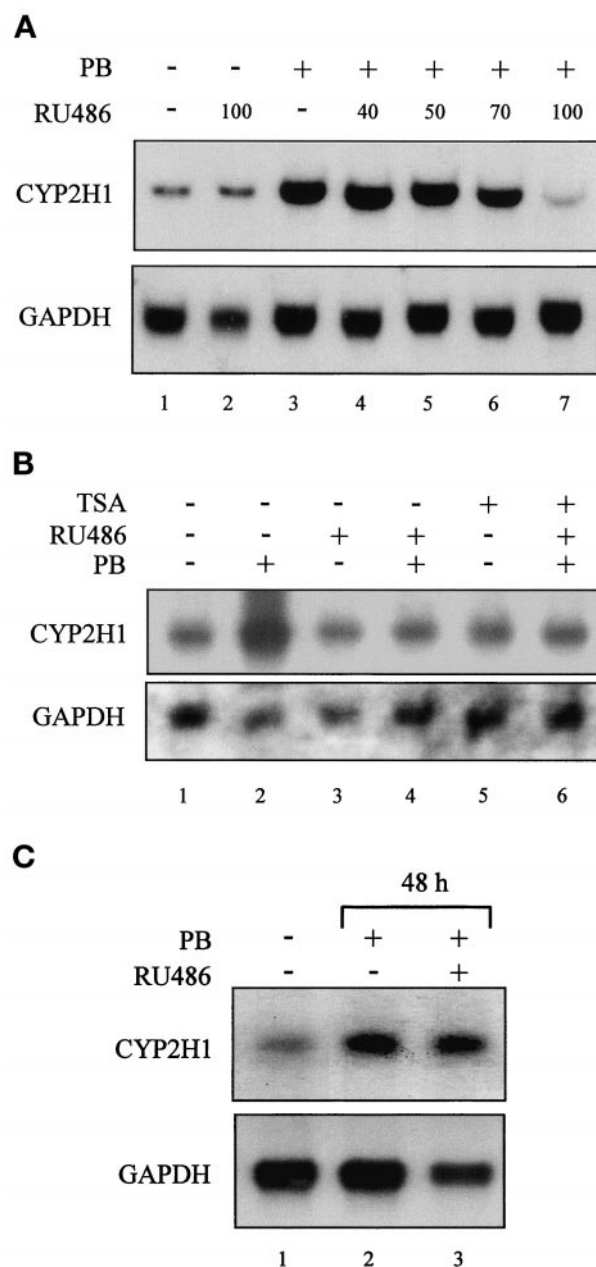
LMH cells were cultured in Williams' E medium supplemented with 10% Serum Supreme and 50  $\mu$ g of gentamicin per ml of medium. Cells were seeded on gelatin-coated 24-well plates at the density of  $6 \times 10^4$  per well 24 h before transfection. The next day, medium was replaced with serum-free Williams' E medium and transfections were performed using CAR $\beta$  (murine CAR1; Choi et al., 1997) expression vector, mPXR expression clone (Moore et al., 2000), CAR-SV-luciferase, p556-SV-luciferase, pRL-SV40 (Promega), and FuGENE6 transfection agent (Roche Molecular Biochemicals, Summitville, NJ). The medium was replaced after 6 h with Williams' E medium containing 10% Serum Supreme with or without RU486 (100  $\mu$ M) or dexamethasone (50  $\mu$ M). After 24 h, cells were lysed and luciferase assays were carried out using the Promega Dual-Luciferase kit.

For the determination of CAT activity, transfected cells were harvested in 40 mM Tris-HCl, pH 7.5, containing 1 mM EDTA and 150 mM NaCl, by scraping with a rubber policeman. The cells were pelleted and resuspended in 50 to 100  $\mu$ l of 250 mM Tris-HCl, pH 7.6, lysed by three cycles of freezing and thawing, and centrifuged for 5 min to remove cell debris. The protein concentration of each cell was determined by protein microassay (Bio-Rad, Hercules, CA). For CAT assays, the cell supernatant was heated at 68°C for 6 to 8 min to remove deacetylase activity and CAT activity was then determined (Gorman et al., 1982). The acetylated products of [ $^{14}$ C]chloramphenicol were separated by thin-layer chromatography. After autoradiog-

raphy, CAT activity was quantified by cutting out the spots from the plate and measuring the radioactivity in a scintillation counter. The results were expressed as a percentage of acetylated chloramphenicol.

## Results

**RU486 Inhibits Phenobarbital-Induced Increase in the Level of CYP2H1 mRNA.** The effect of RU486 on the steady-state levels of mRNA for the *CYP2H1* gene in phenobarbital-induced chick embryo hepatocyte cultures was examined. Hepatocytes were treated with RU486 (40–100  $\mu$ M) for 1 h before the addition of phenobarbital at 500  $\mu$ M. The cells were incubated for a further 6 h and total RNA isolated. The level of CYP2H1 mRNA was determined by Northern blot analysis using a probe specific for the CYP2H1 mRNA (Dogra and May, 1997). The filter was stripped and reprobed for GAPDH mRNA. The results for the autoradiographs of these filters are shown in Fig. 1A. Phenobarbital treatment produced an increase of approximately 9-fold in mRNA expression (lane 3 versus lane 1) as measured 6 h after phenobarbital addition. At concentrations up to 50  $\mu$ M, RU486 had little effect on phenobarbital-induced expression of the CYP2H1 mRNA. However, at 100  $\mu$ M, RU486 almost completely abolished mRNA induction (lane 7). This effect was seen in repeated experiments. RU486 at 100  $\mu$ M had little effect on basal (noninduced) CYP2H1 mRNA levels (lane 2 versus lane 1). In addition, the level of GAPDH mRNA remained unchanged after treatment with phenobarbital or RU486 (up to 100  $\mu$ M) either alone or in combination. As expected, the level of mRNA for the housekeeping form of 5-aminolevulinate synthase, the first enzyme of the heme biosynthetic pathway (May et al., 1995), was also induced by phenobarbital (Dogra and May, 1996), and this induced level was almost totally inhibited by RU486 (data not shown). It was possible that the inhibitory effect of RU486 on the phenobarbital induction could involve the recruitment of a corepressor with histone deacetylase activity (Heinzel et al., 1997; Nagy et al., 1997). However, as shown in Fig. 1B, Trichostatin A, a histone deacetylase inhibitor, did not reverse the inhibitory effect of RU486 on the CYP2H1 mRNA (lane 6 versus lane 2), suggesting that transcriptional silencing by RU486 does not involve a Trichostatin A-sensitive histone deacetylase. To test that the inhibition of phenobarbital induction does not result from RU486-mediated cell toxicity or irreversible cell damage, cells were pre-exposed to RU486 at 100  $\mu$ M for 48 h and then inhibitor was removed by washing the cells. Subsequent induction of CYP2H1 mRNA by phenobarbital addition was identical to that of cells not pretreated with inhibitor (Fig. 1C, lane 3 versus lane 2). Hence, a high concentration of RU486 specifically inhibits phenobarbital induction of at least two genes, *CYP2H1* and 5-aminolevulinate synthase (*ALAS-1*), but not basal expression of these genes, and the inhibitory response cannot be attributed to general cellular damage. In addition, we also examined the effect of RU486 on the induction of the CYP2H1 mRNA by the phenobarbital-like inducers glutethimide and AIA. Glutethimide (500  $\mu$ M) and AIA (50  $\mu$ g/ml) induced the CYP2H1 mRNA level in a Northern blot to about the same level as phenobarbital. However, in marked contrast to the inhibitory action of RU486 on the phenobarbital response, it had no effect on the induction of CYP2H1 mRNA by these chemicals (data not shown).

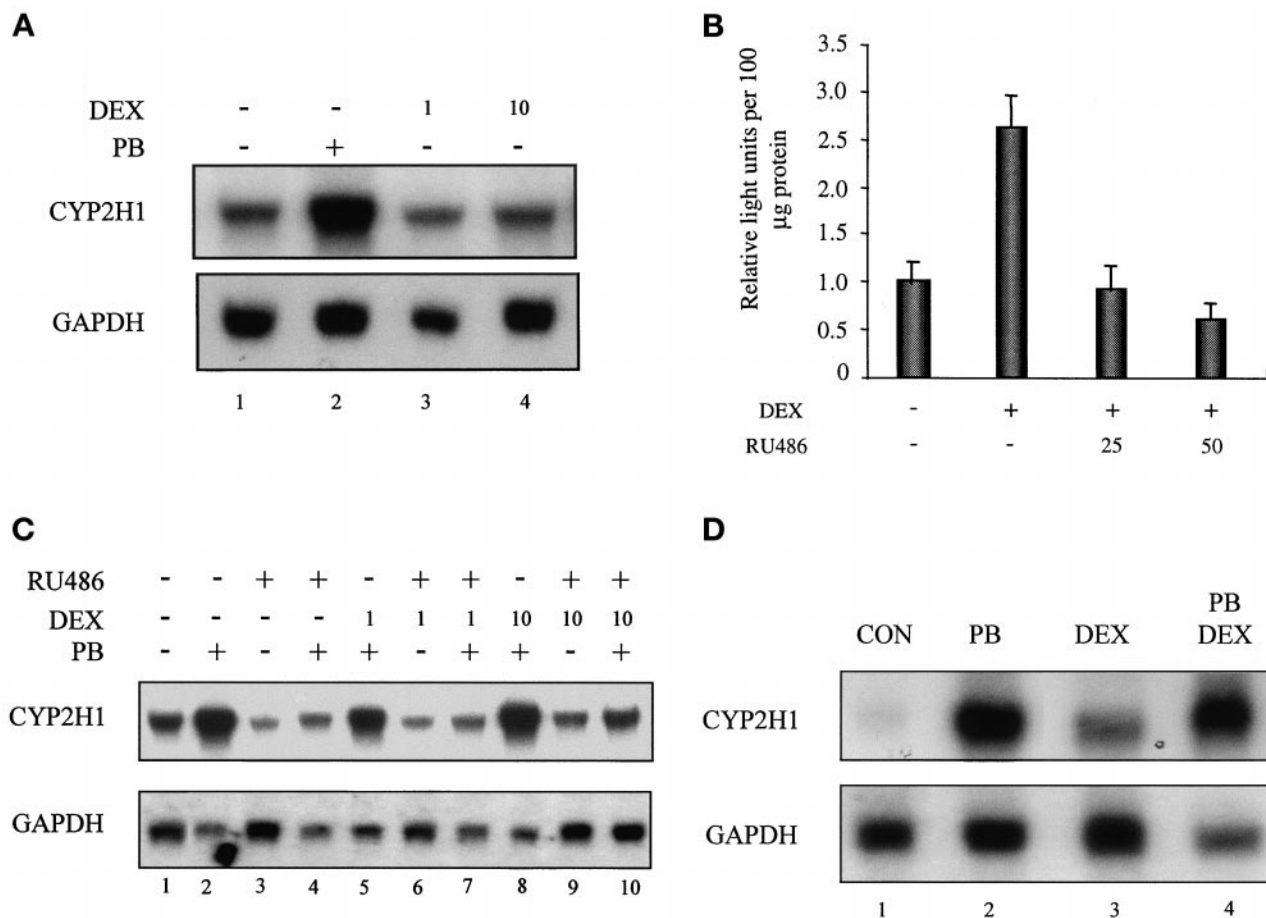


**Fig. 1.** Effect of RU486 on the induced levels of CYP2H1 mRNA. A, representative Northern blot analyses of steady-state levels of mRNAs are shown. Hepatocytes were pretreated with RU486 (40–100  $\mu$ M), 1 h before the addition of PB at 500  $\mu$ M. After a further 6-h incubation, total RNA was isolated and 15  $\mu$ g was analyzed by Northern blotting. The filter was hybridized in turn with  $^{32}$ P-labeled probes specific for *CYP2H1* (pCHB15) and GAPDH (control). Radiolabeled filters were quantified using a PhosphorImager (model 300A; Molecular Dynamics, Sunnyvale, CA), the level of CYP2H1 mRNA was standardized to that of GAPDH mRNA. B, chick embryo hepatocytes were pretreated with 100  $\mu$ M RU486 for 1 h before the addition of PB at 500  $\mu$ M with (lane 6) or without (lane 4) 1  $\mu$ M TSA. Hepatocytes were also treated alone with 500  $\mu$ M PB (lane 2), 100  $\mu$ M RU486 (lane 3), 1  $\mu$ M TSA (lane 5), or were untreated (lane 1). After a 6-h incubation, total RNA was isolated and analyzed for CYP2H1 and GAPDH mRNAs as described above. C, reversal of RU486 inhibition of CYP2H1 mRNA induction was analyzed in hepatocytes. Hepatocytes were untreated (lanes 1 and 2) or treated with RU486 at 100  $\mu$ M for 48 h (lane 3), after which medium was replaced with medium lacking RU486. The cells were left for 18 h, at which time fresh medium without PB (lane 1) or with 500  $\mu$ M PB (lanes 2 and 3) was added and the cells incubated for a further 6 h. Total RNA was isolated and analyzed for CYP2H1 and GAPDH mRNAs as described above.

**Effect of Dexamethasone and Medroxyprogesterone Acetate on CYP2H1 mRNA Expression.** The inhibitory action of RU486 raised the question of whether the glucocorticoid or the progesterone receptor is involved in phenobarbital induction of the *CYP2H1* gene. To investigate this possibility, dexamethasone, a potent synthetic glucocorticoid agonist, was used. Chick embryo primary hepatocytes were treated with either 1 or 10  $\mu$ M dexamethasone. The results of a Northern blot analysis of total RNA extracted from these cultures established that dexamethasone at a concentration of 10  $\mu$ M failed to induce the endogenous gene (Fig. 2A, lane 4 versus lane 1). This finding is in contrast to the rat *CYP2B1/2* genes, which are induced by dexamethasone (Kocarek et al., 1994; Sidhu and Omiecinski, 1995). To confirm that the induction of gene expression by dexamethasone can proceed in chick hepatocytes, a luciferase reporter construct driven by the dexamethasone-responsive MMTV long terminal repeat (MMTV-luciferase) was transfected into hepatocytes. Dexamethasone at a concentration of 0.1  $\mu$ M induced this construct by about 2.7-fold above the control and importantly RU486 even at 25  $\mu$ M significantly repressed induction

(Fig. 2B) supporting the involvement of the glucocorticoid receptor. The effect of 1 or 10  $\mu$ M dexamethasone on RU486-mediated inhibition of phenobarbital induction was examined next (Fig. 2C). It was found that dexamethasone did not reverse the inhibition by 100  $\mu$ M RU486 (lanes 7 and 10), suggesting that RU486 does not suppress phenobarbital induction via a mechanism involving the glucocorticoid receptor. Dexamethasone at a concentration of 30  $\mu$ M or more has been shown to activate orphan receptor PXR, which is known to bind CAR binding motif and thus may influence phenobarbital response. To test this possibility, chick primary hepatocytes were treated with 50  $\mu$ M dexamethasone, alone or in combination with phenobarbital. RNA were prepared and analyzed by Northern Blot analysis. As shown in Fig. 2D, dexamethasone slightly induced CYP2H1 mRNA (which could be caused by activation by PXR), but it had little effect on the induction of CYP2H1 mRNA by phenobarbital (lane 4 versus 2; 18.8-fold versus 21-fold), indicating that activation of PXR does not affect phenobarbital response.

To investigate whether the progesterone receptor was involved in the RU486 mediated inhibitory response, hepato-

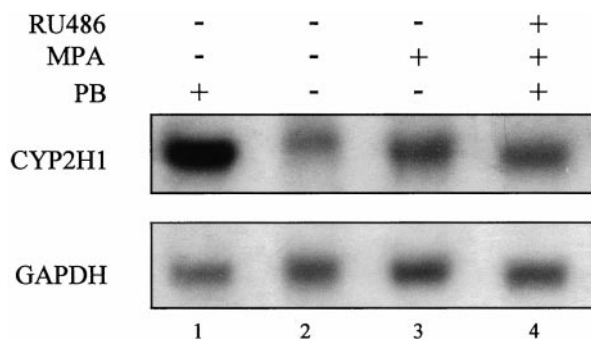


**Fig. 2.** Effect of dexamethasone on the RU486-mediated inhibition of CYP2H1 mRNA induction. **A**, chick embryo primary hepatocytes were treated with either 1 or 10  $\mu$ M Dex (lanes 3 and 4) or 500  $\mu$ M PB (lane 2) for 6 h. Lane 1 is control RNA. After incubation, total RNA was isolated and analyzed for CYP2H1 and GAPDH mRNAs as described in Fig. 1. **B**, an MMTV-luciferase reporter construct was transfected into chick embryo hepatocytes by electroporation, each cuvette was halved, and then either Dex (0.1  $\mu$ M) or Dex and RU486 (25 or 50  $\mu$ M) were added to one dish and solvent was added to the control dish. The mean  $\pm$  S.D. of three independent experiments repeated in duplicate is shown. Luciferase activity is represented as relative light units per 100  $\mu$ g of protein. **C**, chick embryo primary hepatocytes were treated with either 1 or 10  $\mu$ M Dex in combination with 500  $\mu$ M PB or 100  $\mu$ M RU486 or with PB and RU486 together. After an incubation of 6 h, total RNA was isolated and analyzed for CYP2H1 and GAPDH mRNAs as described above. Control cells were treated in the same way but were not initially exposed to Dex or RU486 (lanes 1 and 2) or PB (lanes 1 and 3). **D**, representative Northern blot analyses of steady-state levels of mRNAs are shown. Hepatocytes were treated with 500  $\mu$ M of PB or 50  $\mu$ M of Dex alone or together. After 6 h of incubation, total RNA was isolated and analyzed for CYP2H1 and GAPDH mRNA as described above in Fig. 1.

cytes were treated with the progesterone receptor-specific activator medroxyprogesterone acetate (Smith et al., 1974) at 100 nM, either alone or in combination with RU486 and phenobarbital. Northern blot analysis (Fig. 3) shows that medroxyprogesterone acetate does not induce CYP2H1 mRNA significantly (lane 3) and is unable to reverse the inhibitory effect of RU486 on CYP2H1 mRNA expression (lane 4). There is evidence that cAMP can reverse the antagonistic effect of RU486 on the progesterone (Beck et al., 1993) and glucocorticoid receptors (Nordeen et al., 1993), partly, perhaps, because of a disruption of the interaction between the progesterone or glucocorticoid receptors and the corepressors NCoR and SMRT (Wagner et al., 1998). However, in the present study, cAMP at a concentration of 1 mM had no effect on the RU486-mediated inhibition of phenobarbital induction (result not shown). Overall, the data do not provide any evidence for the involvement of either the glucocorticoid or progesterone receptors in the induction of the *CYP2H1* gene by phenobarbital.

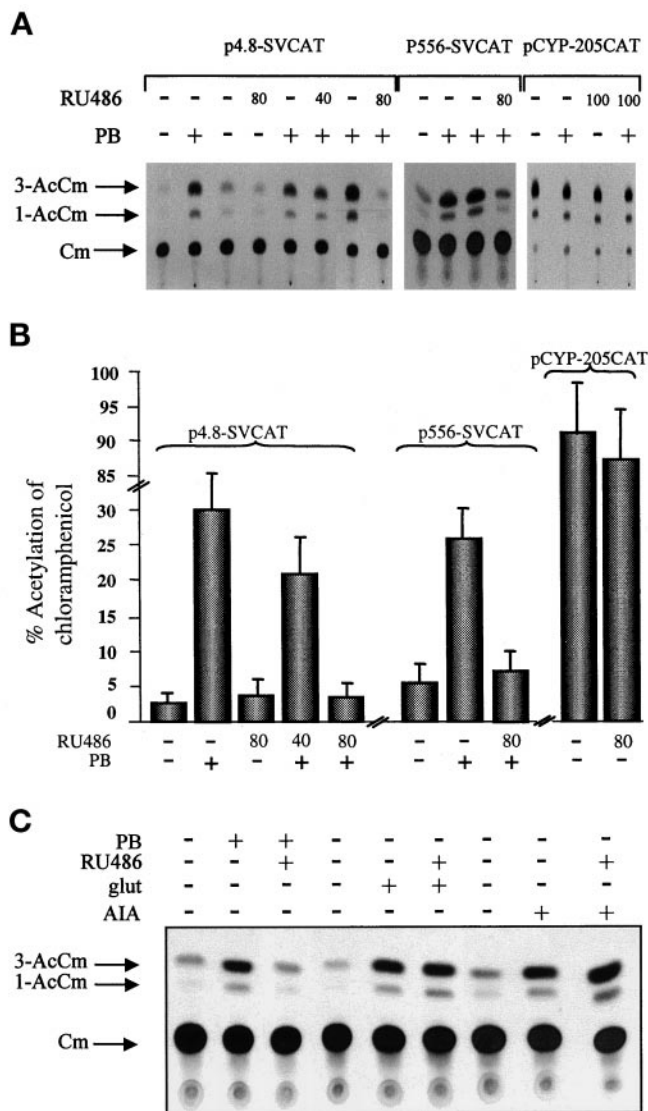
**RU486 Inhibits Phenobarbital-Induction of CYP2H1/CAT Chimeric Constructs in Chick Hepatocytes.** RU486 could inhibit phenobarbital-induced expression of the *CYP2H1* gene through interference with either promoter activity (Dogra and May, 1997) or the activity of the upstream phenobarbital-responsive enhancer domain (Dogra et al., 1999). The effect of RU486 was investigated on the expression of the construct pCYP-205CAT, which contains the first 205 bp of the *CYP2H1* promoter fused to the CAT gene as reporter. This construct has been shown to direct strong basal expression in transient transfection assays but does not respond to phenobarbital (Dogra and May, 1997). It has been postulated that this strong promoter is silenced in the native chromatin and that the drug releases this repression (Dogra and May, 1997). Transient transfection analysis showed that RU486 at 100  $\mu$ M had no effect on the level of expression of pCYP-205CAT (Fig. 4, A and B), a finding that is in keeping with the Northern blot data, in which the basal level of CYP2H1 mRNA remained unaltered by RU486 treatment.

In previous studies, we identified a 4.8-kb *Bam*HI fragment (−5900 to −1100) located in the 5' flanking region of the *CYP2H1* gene which behaved as a drug-responsive enhancer (Dogra et al., 1999). A schematic diagram of the 4.8-kb enhancer region containing a 556-bp domain is given in Fig. 5.



**Fig. 3.** Effect of medroxyprogesterone acetate on CYP2H1 mRNA expression. Representative Northern blot analyses of the steady-state levels of mRNA is shown. Chick embryo hepatocytes were treated either with 500  $\mu$ M of PB (lanes 1), 100 nM medroxyprogesterone acetate (MPA; lanes 3), or with PB plus medroxyprogesterone acetate plus 100  $\mu$ M RU486 (lane 4). Lane 2 is control RNA. After 6 h of incubation, total RNA was isolated and analyzed for CYP2H1 and GAPDH (control) mRNAs as described in Fig. 1.

We investigated whether RU486 could suppress phenobarbital induction from a construct p4.8-SVCAT, where the 4.8-kb phenobarbital-responsive enhancer is fused to the enhancerless SV40 promoter/CAT reporter plasmid. The construct p4.8-SVCAT was transiently expressed in hepatocytes and the effect of RU486 was investigated. The data from one such experiment are shown in Fig. 4A and the average of three independent experiments represented as a percentage conversion of [ $^{14}$ C]chloramphenicol to acetylated product is



**Fig. 4.** Effect of RU486 on the transient expression of p4.8-SVCAT, p556-SVCAT, and pCYP-205CAT. The p4.8-SVCAT, p556-SVCAT, and pCYP-205CAT constructs were transfected into chick embryo hepatocytes, each sample halved, and 500  $\mu$ M PB or 40, 80, or 100  $\mu$ M RU486 was added to one dish and solvent was added to the control dish. When analyzing the effect of PB and RU486 in combination, the control plate received PB (500  $\mu$ M) and the other plate received PB (500  $\mu$ M) and RU486 (40, 80, or 100  $\mu$ M). A, a typical CAT assay of one such experiment is shown. B, the average  $\pm$  S.D. of three independent experiments, repeated in duplicate and represented as a percentage of conversion of [ $^{14}$ C]chloramphenicol to acetylated product is shown. C, the p556-SVCAT construct was transfected into chick embryo hepatocytes and each sample was divided into three equal parts and seeded in 60-mm dishes containing 5 ml of Williams' E medium plus 10% Serum Supreme. In each case, one dish was treated with solvent only for control and other dishes received either 500  $\mu$ M PB or glutethimide (glut) or 50  $\mu$ g/ml AIA with or without 100  $\mu$ M RU486. A typical CAT assay is shown.

shown in Fig. 4B. As expected, the expression of the enhancer construct (p4.8-SVCAT) is increased (9.3-fold) by phenobarbital treatment. Importantly, RU486 at 80  $\mu$ M markedly lowered phenobarbital induction (from 9.3- to 1.3-fold) but did not affect the basal expression. RU486 at 40  $\mu$ M did not antagonize induction by phenobarbital. The results from this experiment parallel those in Fig. 1A, in which RU486 specifically inhibited phenobarbital induction of the endogenous *CYP2H1* gene. To further localize the RU486 sensitive region, the effect of RU486 on a 556-bp phenobarbital enhancer region was investigated (Dogra et al., 1999). This enhancer region lies at the 3' end of the 4.8-kb enhancer and gives level of phenobarbital induction of about 5- to 7-fold in transiently transfected hepatocytes compared with about 9-fold by the 4.8 kb enhancer. The 556-bp enhancer region has been characterized in detail and shown to contain binding sites for several liver-enriched transcription factors (Dogra et al., 1999) together with a site for CAR (Handschin and Meyer, 2000). As shown in Fig. 4A, RU486 markedly lowered phenobarbital induction of this construct (from 5.1- to 1.5-fold). Hence, the 556-bp enhancer region is the inhibitory target of RU486.

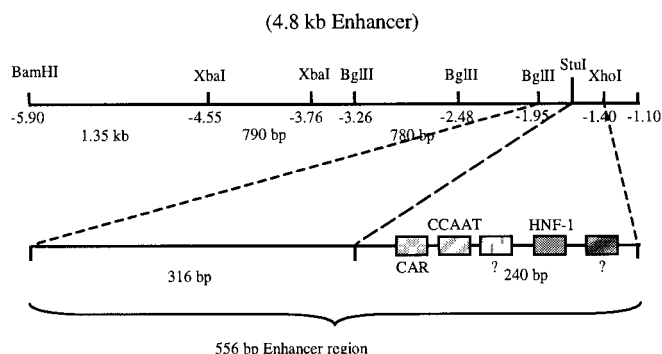
We also investigated the effect of RU486 on the induction of the 556-bp enhancer by either glutethimide or AIA, other phenobarbital-type inducers. Glutethimide and AIA increased expression of the enhancer construct by 7.2- and 5.1-fold, respectively, but RU486 had essentially no effect on this expression (Fig. 4C; one typical CAT experiment). Hence, the RU486 inhibitory response seems to be specific for phenobarbital. We also tested TCPOBOP, which showed very poor induction of the 556-bp enhancer, as has been reported previously for rat *CYP2B* genes (Waxman and Azaroff, 1992) and for its activation of hCAR (Moore et al., 2000).

**RU486 Does Not Act through Any One of the Known Transcription Factors on the 556 bp Enhancer.** In earlier work, we identified four transcription factor binding sites important for 556-bp enhancer activity, including HNF-1, CCAAT/enhancer-binding protein, and two unknown (Dogra et al., 1999). Mutagenesis of each site results in a lowering of phenobarbital induction by about 30%, whereas mutagenesis of multiple sites together almost completely abolishes induction (Dogra et al., 1999). To investigate whether repression of

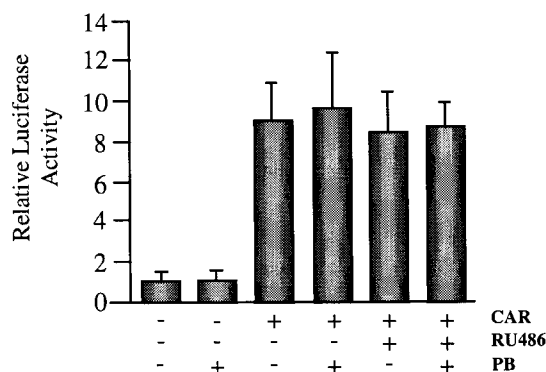
phenobarbital enhancer by RU486 is mediated through one of these binding sites, transient transfection assays, in which one of the four transcription factor binding-sites was mutated, were carried out in chick embryo primary hepatocytes using the 556-bp enhancer reporter constructs. In these assays, phenobarbital induction of the mutated enhancer constructs could still be inhibited by RU486 (data not shown). This suggested that RU486-mediated inhibition cannot be assigned to a single protein binding site in 556-bp enhancer.

The 556-bp enhancer region has recently been shown to contain a binding site for CAR, mutagenesis of which interferes with phenobarbital induction of this enhancer (Handschin and Meyer, 2000). Therefore, to test whether RU486 could interfere with phenobarbital induction by deactivation of CAR, the effect of RU486 on the induction of a CAR-SV-luciferase construct by exogenously expressed CAR $\beta$  in the chicken hepatoma LMH cells was investigated. As shown in Fig. 6, exogenously expressed CAR $\beta$  *trans*-activated the CAR-SV-luciferase construct (8- to 10-fold). Treatment with phenobarbital did not further induce this construct, and the addition of RU486 at 100  $\mu$ M did not alter CAR $\beta$ -activated expression with or without phenobarbital (Fig. 6). No effect of exogenously expressed CAR $\beta$  or RU486 on the control enhancerless SV40 promoter construct was observed.

**RU486 Could Affect Inhibition through Activation of PXR.** A number of recent studies show that orphan nuclear receptor CAR plays a key role in mediating induction of genes by phenobarbital (Honkakoski et al., 1998; Sueyoshi et al., 1999). It has also been demonstrated that a CAR-related orphan receptor PXR can compete with CAR for binding to CAR binding sites on DNA (Xie et al., 2000). Therefore, RU486 could activate the orphan receptor PXR (Moore et al., 2000), which then competes with the binding of CAR (Xie et al., 2000) on the enhancer to abrogate the phenobarbital response. In an attempt to further evaluate this hypothesis, we investigated CAR-mediated expression of the 556-bp enhancer in transfected LMH cells in the presence of either RU486 (100  $\mu$ M) or dexamethasone (50  $\mu$ M), which are known to activate mPXR (Moore et al., 2000). As shown in Fig. 7, CAR $\beta$  activates the enhancer about 14-fold, and mPXR on its own has little effect (1.4-fold), whereas CAR $\beta$  and mPXR together activates this enhancer at a level about 65% of the activation with CAR $\beta$  alone. In the presence of



**Fig. 5.** Schematic diagram of the 4.8-kb phenobarbital enhancer of *CYP2H1* gene. Schematic diagram of the 4.8-kb phenobarbital enhancer region of *CYP2H1* gene is shown. The 556-bp enhancer domain lies in the *BglIII/XhoI* restriction fragment at the 3' end of 4.8-kb enhancer region. Within the 556-bp enhancer domain, the 316-bp *BglIII/StuI* region helps the phenobarbital-induced enhancer activity of the 240-bp region. Various transcription factor binding sites including CAR, CCAAT/enhancer-binding protein, HNF-1, and two unknown sites are shown.



**Fig. 6.** Effect of RU-486 on the *trans*-activation by CAR $\beta$ . A luciferase reporter construct containing four copies of the CAR site upstream of the enhancerless SV40 promoter was cotransfected into LMH cells together with 50 ng of CAR $\beta$  expression vector and pRL-SV40 as an internal transfection control. Relative activity is expressed as a ratio of test to the control and as mean  $\pm$  S.D. of three independent assays.

dexamethasone or RU486, no significant effect on the CAR $\beta$  mediated activation of enhancer construct was observed. Also, induction of enhancer activity by CAR $\beta$  and mPXR together was of the same extent as with CAR $\beta$  alone. Induction of enhancer by mPXR alone in the presence of dexamethasone or RU486 was about 16-fold and 7.5-fold, respectively. From these data, we conclude that activation of mPXR by RU486 (or by dexamethasone) may not be responsible for the inhibitory action of RU486 on the CAR-mediated phenobarbital response.

## Discussion

The most important finding in the present work is that RU486 strongly inhibits, in a dose-dependent fashion, the phenobarbital-induced expression of the endogenous hepatic *CYP2H1* gene in chick embryo hepatocytes but does not affect basal expression of this gene (similar results were seen with the phenobarbital-induced ALAS1 mRNA). In this regard, our experiments with Trichostatin A indicated that chromatin remodeling is not a prerequisite for the inhibitory action of RU486. In marked contrast to phenobarbital effect, induction of the *CYP2H1* mRNA by either of the two phenobarbital-type inducers, glutethimide or 2-allylisopropylacetamide, was unaffected by RU486, providing evidence that RU486 inhibitory action is drug selective.

Our Northern blot studies showed that dexamethasone was unable to antagonize the inhibitory effect of RU486 on drug induction of the *CYP2H1* gene and, in addition, had no effect on either basal or phenobarbital-induced expression of the gene. The progesterone receptor specific activator (Smith et al., 1974) was also ineffective. These results demonstrate that the ability of RU486 to inhibit induction of the *CYP2H1* gene is not caused by its antagonist activity toward the glucocorticoid or progesterone receptors. This is also further supported by the fact that no consensus glucocorticoid or progesterone receptor response elements are present in the 556-bp enhancer region. This situation is somewhat different from that of the rodent phenobarbital inducible *CYP2B* genes, in which functional glucocorticoid receptor elements

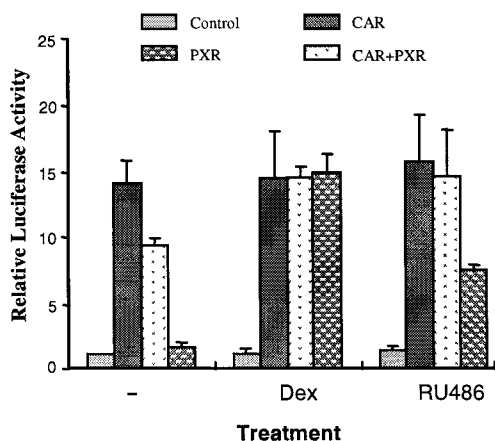
have been located in the 5' flanking regions of the genes and contribute to the level of induced response (Stoltz et al., 1998; Schuetz et al., 2000).

Transient expression assays in transfected chick embryo hepatocytes demonstrated that the inhibitory target for RU486 lies in the upstream 556-bp drug-responsive enhancer region of the *CYP2H1* gene and not in the proximal promoter, which is unresponsive to drug (Dogra and May, 1997). This finding agrees with the Northern blot data that basal expression driven by the promoter is not inhibited by RU486. Transcription factor binding-sites that participate in the phenobarbital-induced activation of 556-bp enhancer have been identified (Dogra et al., 1999). The differential effects of RU486 on the induction of 556-bp enhancer by phenobarbital and phenobarbital-type inducers (glutethimide and AIA) may be because one of these transcription factors is specifically activated by phenobarbital (not by glutethimide and AIA), and this activation is RU486-sensitive. Alternatively, phenobarbital, but not the phenobarbital-type inducers, could increase enhancer activity specifically through one of the enhancer proteins with RU486 affecting inhibition by promoting the binding of a repressor to this protein. However, this possibility seems unlikely, because RU486 does not alter the activity of any single protein, as demonstrated by analysis of phenobarbital-induced 556-bp enhancer constructs with mutations in each binding site.

The orphan receptor CAR binding plays a critical role in drug induction (Honkakoski et al., 1998; Handschin and Meyer, 2000) and a CAR binding site has been identified in the 556-bp enhancer region (Handschin and Meyer, 2000) that binds chicken xenobiotic-sensing orphan nuclear receptor, a homolog of CAR $\beta$  (Handschin et al., 2000). It was possible that RU486 inhibited phenobarbital induction through interference with CAR. An orphan receptor similar to CAR, PXR, is known to bind RU486 (Moore et al., 2000) and to compete with CAR for binding to CAR binding site (Xie et al., 2000). Hence, PXR activated by RU486 could prevent CAR binding to the enhancer. However, such a scenario did not explain why RU486 inhibits the action of phenobarbital and not that of the phenobarbital-type inducers (glutethimide and AIA), which presumably function through CAR. Also, as shown in Fig. 2D, treatment of chick primary hepatocytes with 50  $\mu$ M dexamethasone, a concentration at which it is known to activate mPXR (Moore et al., 2000), did not affect induction of *CYP2H1* mRNA by phenobarbital. These data suggested that activation of PXR does not seem to interfere with phenobarbital response.

Subsequent transient expression data in LMH cells on the induction of the 556-bp enhancer by exogenous CAR $\beta$  in the presence of mPXR, and neither of the two mPXR activators, RU486 and dexamethasone, supported the hypothesis of competition between these receptors. In fact, activated mPXR was a strong inducer of the enhancer. In other experiments with an artificial promoter containing multiple CAR binding sites, exogenous CAR $\beta$ -induced activation of this construct was not affected by RU486. We conclude from this work that RU486 inhibits phenobarbital induction at a step before enhancer activation by nuclear CAR.

Although CAR does not bind phenobarbital, it has been shown to bind TCPOBOP, a phenobarbital-type inducer of *CYP2B* genes (Tzamelis et al., 2000). In the present system, TCPOBOP was not an inducer of the *CYP2H1* gene. There is



**Fig. 7.** Effect of PXR activation on the CAR induced induction of the 556-bp enhancer. LMH cells were transfected with p556-SV-luciferase reporter with or without 50 ng of CAR $\beta$ , 50 ng of mPXR expression clones alone or together, and pRL-SV40 as internal control. Transfected cells were treated with 50  $\mu$ M Dex or 100  $\mu$ M RU486. Cell extracts were assayed for luciferase activity. Relative activity is expressed as a ratio of test to the control and as a mean  $\pm$  S.D. of three independent assays

no information on the binding of glutethimide and AIA to CAR. One possibility is that RU486 inhibits a phenobarbital-dependent modification that is essential for nuclear import of CAR. For example, phenobarbital can activate a phosphatase that removes a phosphate group from CAR and allows nuclear import of CAR (Kawamoto et al., 1999) or it could activate a kinase (Dogra and May, 1996) that phosphorylates CAR to allow its nuclear import, with RU486 preventing phenobarbital action. Another possibility is that phenobarbital, but not glutethimide or AIA, must be metabolized to the true inducer (Moore et al., 2000), which then binds to CAR. RU486 could then inhibit the metabolism of phenobarbital to a true inducer. How RU486 could interfere with phosphorylation events or with the metabolism of phenobarbital is unclear.

A novel member of the steroid hormone receptor family could be involved in the inhibitory action of RU486. The requirement for a high concentration of RU486 may indicate weak binding to such a putative receptor; alternatively, RU486 may compete with a high-affinity endogenous ligand. What role such a receptor would play in the RU486 inhibitory action (e.g., by interfering with CAR phosphorylation events or the metabolism of phenobarbital) remains unclear. Nevertheless, our data provide evidence that although CAR may be central to the induction response, the mechanism by which the various phenobarbital-type inducers provoke CAR activation deserves further attention.

## References

- Beck CA, Weigel NL, Moyer ML, Nordeen SK and Edwards DP (1993) The progesterone antagonist RU486 acquires agonist activity upon stimulation of cAMP signaling pathways. *Proc Natl Acad Sci USA* **90**:4441–4445.
- Choi HS, Chung M, Tzamelis I, Simha D, Lee YK, Seol W and Moore DD (1997) Differential transactivation by two isoforms of the orphan nuclear receptor CAR. *J Biol Chem* **272**:23565–23571.
- Chomczynski P and Sacchi N (1987) Single step method of RNA isolation by acid guanidine thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**:156–159.
- Dogra SC, Davidson BP and May BK (1999) Analysis of a phenobarbital-responsive enhancer region located in the 5' flanking region of the chicken *CYP2H1* gene: identification and characterisation of functional protein binding sites. *Mol Pharmacol* **55**:14–22.
- Dogra SC and May BK (1996) Phenobarbital-induced activation of *CYP2H1* and 5-aminolevulinic synthase genes in chick embryo hepatocytes is blocked by an inhibitor of protein phosphorylation. *Arch Biochem Biophys* **327**:271–278.
- Dogra SC and May BK (1997) Liver-enriched transcription factors HNF-1, HNF-3 and C/EBP are major contributors to the strong activity of the chicken *CYP2H1* promoter in chick embryo hepatocytes. *DNA Cell Biol* **16**:1407–1418.
- Dogra SC, Whitelaw ML and May BK (1998) Transcriptional activation of cytochrome P450 genes by different classes of chemical inducers. *Clin Exp Pharmacol Physiol* **25**:1–9.
- Gonzalez FJ (1989) The molecular biology of cytochrome P450s. *Pharmacol Rev* **40**:243–288.
- Gorman CM, Moffat LF and Howard BH (1982) Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol Cell Biol* **2**:1044–1051.
- Handschin C and Meyer UA (2000) A conserved nuclear receptor consensus sequence (DR-4) mediates transcriptional activation of the chicken *CYP2H1* gene by phenobarbital in hepatoma cell line. *J Biol Chem* **275**:13362–13369.
- Handschin C, Podvinec M and Meyer UA (2000) CXR, a chicken xenobiotic-sensing orphan nuclear receptor, is related to both mammalian pregnane X receptor (PXR) and constitutive androstane receptor (CAR). *Proc Natl Acad Sci USA* **97**:10769–10774.
- Hansen AJ, May BK and Elferink LA (1989) Sequence of a chicken phenobarbital-inducible cytochrome P450 cDNA: regulation of two P450 mRNAs transcribed from different genes [published erratum appears in *DNA* **9**:301]. *DNA* **8**:179–191.
- Heinzel T, Lavinsky RM, Mullen TM, Sonderstrom M, Laherty CD, Trochia J, Yang WM, Brard G, Ngo SD, Davie JR, et al. (1997) A complex containing N-CoR, mSin3 and histone deacetylase mediate transcriptional repression. *Nature (Lond)* **387**:43–48.
- Honkakoski P and Negishi M (1998) Protein serine/threonine phosphatase inhibitors suppress phenobarbital-induced *cyp2b10* gene transcription in mouse primary hepatocytes. *Biochem J* **330**:889–895.
- Honkakoski P, Zelko I, Sueyoshi T and Negishi M (1998) The nuclear orphan receptor CAR-retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the *CYP2B* gene. *Mol Cell Biol* **18**:5652–5658.
- Kawamoto T, Sueyoshi T, Zelko I, Moore R, Washburn K and Negishi M (1999) Phenobarbital-responsive nuclear translocation of the receptor CAR in the induction of the *CYP2B* gene. *Mol Cell Biol* **19**:6318–6322.
- Kliewer SA, Lehmann JM and Willson TM (1999) Orphan nuclear receptors: shifting endocrinology into reverse. *Science (Wash DC)* **284**:757–760.
- Kocarek TA, Schuetz EG and Guzelian PS (1994) Biphasic regulation of cytochrome P450 2B1/2 mRNA expression by dexamethasone in primary cultures of adult rat hepatocytes maintained on matrigel. *Biochem Pharmacol* **48**:1815–1822.
- May BK, Dogra SC, Sadlon TJ, Bhasker CR, Cox TC and Bottomley SS (1995) Molecular regulation of heme biosynthesis in higher vertebrates. *Prog Nucleic Acid Res Mol Biol* **51**:1–51.
- Moore LB, Parks DJ, Jones SA, Bledsoe RK, Consler TG, Stimmel JB, Goodwin B, Liddle C, Blanchard SG, Wilson TM, et al. (2000) Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. *J Biol Chem* **275**:15122–15125.
- Nagy L, Kao HY, Chakravarti D, Lin RJ, Hassig CA, Ayer DE, Schreiber SL and Evans RM (1997) Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. *Cell* **89**:373–380.
- Nordeen SK, Bona BJ and Moyer ML (1993) Latent agonist activity of the steroid antagonist, RU486, is unmasked in cells treated with activators of protein kinase-A. *Mol Endocrinol* **7**:731–742.
- Savas U, Griffin KJ and Johnson (1999) Molecular mechanisms of cytochrome P450 induction by xenobiotics: an expanded role for nuclear hormone receptors. *Mol Pharmacol* **56**:851–857.
- Schuetz EG, Schmid W, Schutz G, Brimer C, Yasuda K, Kamataki T, Bornheim L, Myles K and Cole TJ (2000) The glucocorticoid receptor is essential for induction of cytochrome P-450B by steroids but not for drug or steroid induction of *CYP3A* or P-450 reductase in mouse liver. *Drug Metab Dispos* **28**:268–278.
- Shaw PM, Adesnick M, Weiss MC and Corcos L (1993) The phenobarbital-induced transcriptional activation of cytochrome P450 genes is blocked by the glucocorticoid-progesterone antagonist RU486. *Mol Pharmacol* **44**:775–783.
- Sidhu JS and Omiecinski CJ (1995) Modulation of xenobiotic-inducible cytochrome P450 gene expression by dexamethasone in primary rat hepatocytes. *Pharmacogenetics* **5**:24–36.
- Smith HE, Smith RG, Toft DO, Neergaard JR, Burrows EP and O'Malley BW (1974) Binding of steroids to progesterone receptor proteins in chick oviduct and human uterus. *J Biol Chem* **249**:5924–5932.
- Stoltz C, Vachon M-H, Trotter E, Dubois S, Paquet Y and Anderson A (1998) The *CYP2B2* phenobarbital responsive unit contains an accessory factor element and a putative glucocorticoid response element essential for conferring maximal phenobarbital responsiveness. *J Biol Chem* **273**:8528–8536.
- Sueyoshi T, Kawamoto T, Zelko I, Honkakoski P and Negishi M (1999) The repressed nuclear receptor CAR responds to phenobarbital in activating the human *CYP2B6* gene. *J Biol Chem* **274**:6043–6046.
- Tzamelis I, Pissios P, Schuetz EG and Moore DD (2000) The xenobiotic compound 1,4-bis[2-(3,5-dichloropropylidyl)oxy]benzene is an agonist ligand for the nuclear receptor CAR. *Mol Cell Biol* **20**:2951–2958.
- Wagner BL, Norris JD, Knotts TA, Weigel NL and McDonnell DP (1998) The nuclear corepressors NCOR and SMRT are key regulators of both ligand- and 8-bromocyclic AMP-dependent transcriptional activity of the human progesterone receptor. *Mol Cell Biol* **18**:1369–1378.
- Waxman DJ (1999) P450 gene induction by structurally diverse xenobiotics: central role of nuclear receptors CAR, PXR, and PPAR. *Arch Biochem Biophys* **369**:11–23.
- Waxman DJ and Azaroff L (1992) Phenobarbital induction of cytochrome P-450 gene expression. *Biochem J* **281**:577–592.
- Xie W, Barwick JL, Simon CM, Pierce AM, Safe S, Blumberg B, Guzelian PS and Evans M (2000) Reciprocal activation of xenobiotic response genes by nuclear receptors SXR/PXR and CAR. *Genes Dev* **14**:3014–3023.

**Address correspondence to:** Dr. Brian K. May, Department of Molecular Biosciences, University of Adelaide, Adelaide, SA 5005, Australia. E-mail: brian.may@adelaide.edu.au