

Role of Specificity Protein 1, Hepatocyte Nuclear Factor 1 α , and Pregnane X Receptor in the Basal and Rifampicin-Induced Transcriptional Regulation of Porcine Cytochrome P450 3A46[§]

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ABSTRACT

Cytochrome P450 (CYP) 3A46, one of human CYP3A4 homologs, functions as a key enzyme in the metabolism of xenobiotics in pigs. However, the regulatory mechanism for the transcriptional activation of CYP3A46 in porcine liver remains unknown. In this study, we confirmed that CYP3A46 is constitutively expressed in porcine primary hepatocytes, and its expression was significantly induced by rifampicin (RIF) instead of dexamethasone. We further found that a proximal GC box and a distal hepatocyte nuclear factor 1 (HNF1) binding site within the 5'-flanking region of CYP3A46 are the important *cis*-regulatory elements involved in regulating the constitutive expression of CYP3A46, via recruiting specificity protein 1 (Sp1) and HNF1 α , respectively.

Furthermore, we revealed that HNF1 α and pregnane X receptor (PXR) activate the RIF-mediated transcription of CYP3A46 by binding to the distal HNF1 binding site and the proximal direct repeats of AGGTCA separated by 4 bases motif, respectively. Meanwhile, HNF1 α is also involved in regulating RIF-induced expression of CYP3A4 through a novel distal HNF1 binding site identified in the xenobiotic-responsive enhancer module. In summary, our data demonstrate that several transcription factors, including Sp1, HNF1 α , and PXR, function in the basal and RIF-mediated transcriptional regulation of CYP3A46 by binding to their related *cis*-regulatory elements in the proximal promoter and distal enhancer.

Introduction

Cytochrome P450 (CYP) enzymes are a large family of heme-thiolate-containing proteins that play important roles in the oxidative metabolism of xenobiotics, including therapeutic drugs, environmental procarcinogens, and toxins (Nelson et al., 1996). CYP3A4, as one of the most important CYPs in humans, is mainly expressed in the liver and small intestine and responsible for the metabolism of approximately 50% of currently used drugs (Guengerich, 1999). However, the expression level and activity of CYP3A4 in human differentiate greatly between individuals due to genetic variations and environmental stimulations (Plant, 2007). The expression of CYP3A4 is remarkably induced by commonly used drugs such as the glucocorticoid dexamethasone (DEX), the antibiotic rifampicin (RIF), and the antimycotic clotrimazole, which tends to cause a high risk of adverse drug-drug interactions in patients undergoing combination drug therapy (Goodwin et al., 2002b; Zanger and Schwab, 2013).

The molecular regulatory mechanisms for the transcriptional activation of human CYP3A4 in the liver have been extensively studied. The liver-

enriched transcription factors (LETFs) such as hepatocyte nuclear factor (HNF)1, HNF3, HNF4, CCAAT/enhancer-binding protein (C/EBP), and D-element-binding protein, as well as ubiquitous transcription factors [e.g., specificity protein 1 (Sp1), upstream stimulatory factor-1, and activating protein-1] coordinately regulate the basal expression of CYP3A4 by binding to their *cis*-regulatory elements within both proximal promoter and distal enhancer [e.g., the constitutive liver enhancer module (CLEM)] (Ourlin et al., 1997; Rodriguez-Antona et al., 2003; Bombail et al., 2004; Matsumura et al., 2004; Martinez-Jimenez et al., 2005). Besides, several nuclear receptors, such as pregnane X receptor (PXR) (Lehmann et al., 1998), constitutive androstane receptor (CAR) (Goodwin et al., 2002a), glucocorticoid receptor (GR) (Pascucci et al., 2003), and vitamin D receptor (Drocourt et al., 2002), play decisive roles in the xenobiotic activation of CYP3A4. As an example, PXR forms heterodimer with 9-*cis* retinoic receptor (RXR) α under stimulations of xenobiotics such as RIF and clotrimazole, and then activates the expression of CYP3A4 by binding to its cognate response elements within the regulatory region (Honkakoski et al., 2003). Several binding sites of PXR have been identified, including the everted repeats of AGGTCA separated by six bases (ER6) in the proximal promoter (Lehmann et al., 1998), the dNR1, dNR2 as well as eNR3A4 in the xenobiotic-responsive enhancer module (XREM) (Goodwin et al., 1999; Toriyabe et al., 2009), and the ER6 in the CLEM (Liu et al., 2008).

Pigs, one of the most important livestock in the world, are frequently and inevitably exposed to toxins, veterinary chemicals, and other pollutants. Understanding the metabolic pathways of xenobiotics

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ABBREVIATIONS: ANOVA, analysis of variance; CAR, constitutive androstane receptor; C/EBP, CCAAT/enhancer-binding protein; CLEM, constitutive liver enhancer module; CYP, cytochrome P450; DEX, dexamethasone; DMSO, dimethylsulfoxide; DR3, direct repeats of AGGTCA separated by 3 bases; DR4, direct repeats of AGGTCA separated by 4 bases; EMSA, electrophoretic mobility shift assay; ER6, everted repeats of AGGTCA separated by six bases; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GR, glucocorticoid receptor; HNF, hepatocyte nuclear factor; LETF, liver-enriched transcription factor; MT, mutant; PCR, polymerase chain reaction; PXR, pregnane X receptor; RIF, rifampicin; RXR, 9-*cis* retinoic receptor; Sp1, specificity protein 1; WT, wild-type; XREM, xenobiotic-responsive enhancer module.

and the potential drug residues in pigs is particularly important for the safety of food productions and the health of human beings. We previously reported that *CYP3A46*, a porcine *CYP3A* isoform, structurally and functionally related to human *CYP3A4* (Jiang et al., 2011), catalyzes T-2 toxin (a highly toxic mycotoxin produced by *Fusarium* species) to form 3'-hydroxy-T-2 toxin (a less toxic metabolite) (Wang et al., 2011). Both studies evidence *CYP3A46* as a key enzyme in the metabolism of xenobiotics. *CYP3A46* is mainly expressed in the liver, and its expression can be induced by RIF (Nannelli et al., 2008). Nevertheless, the regulatory mechanism is yet to be clarified for the expression of *CYP3A46* in comparison with the well-studied human *CYP3A4* in the basal and drug-mediated regulations.

In this study, we unveiled the regulatory mechanisms for both the basal and RIF-mediated transcriptional activation of *CYP3A46* in porcine liver. We found that Sp1 and HNF1 α are involved in regulating the constitutive expression of *CYP3A46* by binding to the proximal GC box and the distal HNF1 binding site. However, under the induction of RIF, which was used as the representative of xenobiotics, the transcription factors HNF1 α and PXR bind to the distal HNF1 binding site and the proximal direct repeats of AGGTCA separated by 4 bases (DR4) motif, respectively, and then mediate the transcriptional activation of *CYP3A46*.

Materials and Methods

Ethics Statement. All related experiments were performed in accordance with the recommendations in the Regulations for the Administration of Affairs Concerning Experimental Animals of Guangdong Province, China. All efforts were made to minimize suffering.

Animals and Cell Culture. Danish Landrace \times Yorkshire \times Duroc cross-breed pigs (3 days old; male) were purchased from the College of Veterinary Medicine, South China Agricultural University. Hepatocytes were isolated by modified two-step in situ collagenase perfusion described previously (Wang et al., 2011). Porcine primary hepatocytes were then cultured in William's E medium (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin, 10^{-6} mM insulin, and 10^{-6} mM DEX (Sigma-Aldrich). For induction, porcine primary hepatocytes were treated with different concentrations of DEX or RIF (Sigma-Aldrich) for 24 or 48 hours, and then the cells were collected for RNA extraction. The control cells were incubated with 0.1% dimethylsulfoxide (DMSO).

HepG2 cells (American Type Culture Collection, Manassas, VA; HB-8065) and COS-7 cells (American Type Culture Collection; CRL-1651) were maintained at 37°C in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin (all from Invitrogen, Carlsbad, CA).

Plasmid Construction. Due to incomplete genomic information, the 5'-upstream sequence of porcine *CYP3A46* was first amplified by a genome walking kit (TaKaRa, Qingdao, China), using the following primers: sp1 (5'-ATAGAGGAGCACCAGGCTGGTAGC-3'), sp2 (5'-CTGTGGAAAAGCC-TGGGATCAGGT-3'), and sp3 (5'-CACTGTCTCCGTGATTCTCTCTC-3'), designed based on the mRNA sequence of *CYP3A46* (NM_001134824). The sequence of the polymerase chain reaction (PCR) product was almost identical (98%) to the porcine genome sequence NW_003540597.1, which is an incomplete sequence yet. Then a 7.3-kb fragment, corresponding to bases -7235 to +85 (+1 stands for the transcriptional start site) of *CYP3A46*, was obtained and ligated into pMD 19-T (TaKaRa) using the following primers: -7.3k_F (5'-CTCCCACCCTCACATTAGACTTTG-3') and -7.3k_R (5'-GGCCACTGTCTCCGTGATTCTCT-3') designed based on the sequence of NW_003540597.1. The sequence has been submitted to the GenBank database under the accession number KP670896.

For promoter activity analyses, the 5'-flanking region (-7235~+85) of *CYP3A46* was cloned and inserted into MluI/NheI sites of the pGL3-Basic vector (Promega, Madison, WI) to generate the (-7235~+85)-luc construct. A series of 5'-truncated constructs were then made on the basis of the (-7235~+85)-luc construct, including (-6221~+85)-luc, (-4504~+85)-luc, (-2707~

+85)-luc, (-2128~+85)-luc, (-565~+85)-luc, (-295~+85)-luc, (-165~+85)-luc, and (-34~+85)-luc. The primers used for the synthesis of above DNA fragments are as follows: (-7235~+85)-luc-F (5'-CGACGC-GTCTCCCACCCTCACATTAGACTTTG-3'), (-6221~+85)-luc-F (5'-CGAC-GCGTAACCTCTGTAGCGTGGTGCTCATCC-3'), (-4504~+85)-luc-F (5'-CGACGCGTAGTGTGACCTCAGGCAGCTGTTGCTC-3'), (-2707~+85)-luc-F (5'-CGACGCGTCTCCATAGTTTCCCCTTGCAGATC-3'), (-2128~+85)-luc-F (5'-CGACGCGTCCGCATTTCAGAGCAGCAGTAGACT-3'), (-565~+85)-luc-F (5'-CGACGCGTGTGCCAAGCTCTCCATCTGTAAG-3'), (-295~+85)-luc-F (5'-GGAAGATCTAAGTATTTGGAGTGCAGGCAGCTT-3'), (-165~+85)-luc-F (5'-CGACGCGTAGCACTGGTGTATTTTCACTGGCTG-3'), (-34~+85)-luc-F (5'-GGAAGATCTCCCAGCATATAAATCTTTCAGCCTC-3'), and (-7235~+85)-luc-R (5'-CTAGCTAGCGGCCACTGTCTCCGTGATTCTCT-3'). To further analyze the region from bases -7235 to -6220, the region itself and its serial deletions were inserted into KpnI/MluI sites of the (-165~+85)-luc construct to generate the following luciferase reporter constructs: (-7235~-6220)-(-165~+85)-luc, (-6638~-6220)-(-165~+85)-luc, (-6507~-6220)-(-165~+85)-luc, (-6426~-6220)-(-165~+85)-luc, (-6507~-6276)-(-165~+85)-luc, (-6287~-6220)-(-165~+85)-luc, (-6279~-6220)-(-165~+85)-luc, and (-6257~-6220)-(-165~+85)-luc. The primers used for the synthesis of above DNA fragments are as follows: (-7235~-6220)-(-165~+85)-luc-F (5'-CGGGGTACCTCC-CACCCCTCACATTAGACTTTG-3'), (-6638~-6220)-(-165~+85)-luc-F (5'-CGGGGTACCACTAATGAGGCAAACACTGCCATG-3'), (-6507~-6220)-(-165~+85)-luc-F (5'-CGGGGTACCTCACAAAGTAAGCCTGGGGAAG-CAG-3'), (-6426~-6220)-(-165~+85)-luc-F (5'-CGGGGTACCTCCCTCCCTCAAATGCTC-3'), (-6507~-6276)-(-165~+85)-luc-F (5'-CGGGGTACCTCACAAAGTAAGCCTGGGGAAGCAG-3'), (-6287~-6220)-(-165~+85)-luc-F (5'-CGGGGTACCGGCAGGCAGCATAGTCAACAATGAT-3'), (-6279~-6220)-(-165~+85)-luc-F (5'-CGGGGTACCCATAGTCAACAATG-ATTAATTCAGG-3'), (-6257~-6220)-(-165~+85)-luc-F (5'-CGGGGTAC-CAGGATTCCTCCCTCTGTGAC-3'), (-7235~-6220)-(-165~+85)-luc-R (5'-CGACGCGTACCACGCTACAGAAAGTATCAGC-3'), and (-6507~-6276)-(-165~+85)-luc-R (5'-CGACGCGTTATGGTCTGC-CTCTGCATCTTTG-3'). Using the same methods, the 5'-flanking region (-362~+53) of human *CYP3A4* was cloned and inserted into BglII/HindIII sites of the pGL3-Basic vector (Promega) to generate the (-362~+53)-luc construct. Then the region from bases -7836 to -7208 was inserted into KpnI/BglII sites of the (-362~+53)-luc construct to generate the XREM-(-362~+53)-luc construct. The primers used are as follows: *CYP3A4* distal-F (5'-CGGGGTACCATGCTGGTTGCTGGTTTATTCTAG-3'), *CYP3A4* distal-R (5'-GGAAGATCTGAATGGTTATAAGATCATCTCAATGG-3'), *CYP3A4* proximal-F (5'-GGAAGGTAAGATCTGTAGGTGTGGCTTG-3'), and *CYP3A4* proximal-R (5'-CCCAAGCTTCTTTGCTGGGCTATGTG-CATG-3'). All mutated and internal inserted luciferase reporter constructs were prepared on the basis of the constructs above with the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Primers used for site-directed mutagenesis are listed in Supplemental Table 1.

For overexpression, the open reading frame region of porcine Sp1 (XM_003355406.1), HNF1 α (NM_001032388.1), PXR (NM_001038005.1), and RXR α (GACC01000108.1), as well as human HNF1 α (NM_000545.5) and PXR (NM_003889.3), was cloned and inserted into the pcDNA3.1/myc-His(-) A vector (Invitrogen) to generate pcDNA-pSp1, pcDNA-pHNF1 α , pcDNA-pPXR, pcDNA-pRXR α , pcDNA-hHNF1 α , and pcDNA-hPXR plasmids, respectively. The corresponding restriction sites used are XhoI/HindIII for pcDNA-pSp1 and BamHI/HindIII for pcDNA-pHNF1 α , pcDNA-pPXR, pcDNA-hHNF1 α , and pcDNA-hPXR. The primers used are as follows: pSp1-F (5'-CCGCTCGA-GATGAGCGACCAAGATCACTCCATGG-3'), pSp1-R (5'-CCCAAGCTTTCAG-AAGCCATTGCCACTGATATTA-3'), pHNF1 α -F (5'-CGCGGATCCGCCAT-GGTCTCCAAGCTGAGC-3'), pHNF1 α -R (5'-CCCAAGCTTCTGGGAGGATG-AGGCCATCTG-3'), pPXR-F (5'-CGCGGATCCATGCAATGCAATGAA-ACAGACTCC-3'), pPXR-R (5'-CCCAAGCTTGTCTTCTGTGATGCTGAA-TAACTC-3'), pRXR α -F (5'-CCGCTCGAGATGGACACCAAAACATTT-CTGC-3'), pRXR α -R (5'-CCCAAGCTTGGTCATTTGGTGGGGCCCT-3'), hHNF1 α -F (5'-CGCGGATCCGCCATGGTTTCTAAACTGAGCCAGC-3'), hHNF1 α -R (5'-CCCAAGCTTCTGGGAGGAAGAGGCCATCTGG-3'), hPXR-F (5'-CGCGGATCCATGGAGGTGAGACCCAAAGAAAG-3'), and hPXR-R (5'-CCCAAGCTTGTACTCTGTGATGCCGAACAAC-3').

For preparation of all the plasmids, KOD-plus DNA polymerase (Toyobo, Osaka, Japan) was used. All plasmids were verified by sequencing and prepared by the Endo-Free Plasmid Kit (Omega, Norcross, GA).

Transfection and Luciferase Activity Detection. HepG2 cells were seeded into 24-well plates at a density of 5×10^4 cells/well before transfection. For each well, the cells were transfected with 0.6 μg corresponding luciferase reporter construct, 0.1 μg pRL-TK control vector (Promega), and 0.1 μg expression vector (if necessary) by using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. After 24 hours, the cells were lysed by Passive Lysis Buffer (Promega). The luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) on a GLOMAX 20/20 luminometer (Promega), and the firefly luciferase activity for each construct was normalized to the *Renilla* luciferase activity. The results shown are representative of at least three independent experiments (each performed in triplicate).

For the drug-induction experiments, HepG2 cells were transfected as described above and subsequently cultured in serum-free Dulbecco's modified Eagle's medium with 10 μM RIF or 0.1% DMSO for another 24 hours. Then the cells were lysed, and the luciferase activities were measured, as mentioned above.

For the electrophoretic mobility shift assays (EMSA), COS-7 cells were first transfected with the corresponding expression vectors and then collected to extract the nuclear proteins.

RNA Isolation and Real-Time PCR. The total RNA was extracted from porcine primary hepatocytes using TRIzol Reagent (Invitrogen), and the first-strand cDNA was synthesized using oligo(dT)₁₈ primers (TaKaRa) and Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega). The primers used in real-time PCR were designed to span exon-exon junctions, to prevent the potential genomic DNA contamination. The sequences of the primer pairs are as follows: CYP3A46-F (5'-GCGACTTCCCAATAAG-3'), CYP3A46-R (5'-AGCGCAAAGATTGGCACC-3'), glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-F (5'-GTCGGTTGTGGATCTGAC-3'), and GAPDH-R (5'-TGGTCGTTGAGGGCAATG-3'). The specificities of the primers were confirmed by sequencing the PCR products. Real-time PCR was performed on a Bio-Rad CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA) under the following steps: initial denaturation at 94°C for 3 minutes, then 40 cycles of 94°C/20 seconds, 58°C/20 seconds, and 72°C/20 seconds. Reaction was performed in a 20 μL mixture containing Premix Taq (TaKaRa) and SYBR (Invitrogen). The GAPDH was chosen as an internal control and used for normalization. Fold differences in the expression levels were calculated using the $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen, 2001). The results shown are representative of at least three independent experiments (each performed in triplicate).

EMSA. The nuclear extracts from COS-7 cells transfected with different transcription factors were prepared with the Nuclear Extraction Kit (Beyotime, Haimen, China). Probes used were generated by the EMSA Probe Biotin Labeling Kit (Beyotime), according to the manufacturer's recommendation. The EMSAs were performed with the EMSA Assay Kit (Beyotime). Briefly, binding buffer and nuclear extracts (6 μg) in a total volume of 10 μL were incubated at 22°C for 10 minutes to prevent the nonspecific binding. Then double-stranded biotinylated probes (0.1 pmol) were added in the reaction, and the mixtures were further incubated at 22°C for 20 minutes. For the competition assays, a 100-fold molar excess of unlabeled competitors was added to the mixture and incubated at 22°C for 10 minutes before adding biotinylated probes. For the supershift assays, 1 μg polyclonal antibody against Sp1 (AB61137a; Sangon Biotech, Shanghai, China), polyclonal antibody against HNF1 α (AB60769a; Sangon Biotech), or rabbit IgG (as a control) was added to the mixture and incubated at 22°C for 10 minutes before adding biotinylated probes. The protein-DNA complexes and the unbound free probes were separated on 6% nondenaturing polyacrylamide [acrylamide/bisacrylamide 29:1 (v/v)] gels and detected using the chemiluminescence (Millipore, Bedford, MA).

Statistical Analysis. All experiments were performed independently at least three times, and all data are expressed as mean \pm S.D. Data were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Significance was defined as *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$. GraphPad Prism 5.00 software (San Diego, CA) was used for statistical analysis.

Results

RIF Significantly Induces the Expression of CYP3A46 in Porcine Primary Hepatocytes. To investigate the basal and inducible

expression of porcine *CYP3A46*, its mRNA level was measured by real-time PCR in porcine primary hepatocytes with or without drug treatment. The expression of *CYP3A46* was significantly induced by RIF, a typical CYP3A inducer, up to five fold when 10 μM RIF was used to treat cells for 24 hours, in comparison with the expression level of *CYP3A46* in control group (DMSO-treated cells) (Fig. 1). However, we did not observe any apparent inductions when DEX, another representative CYP3A inducer, was used (Fig. 1).

The Proximal GC Box and Distal HNF1 Binding Site in the 5'-Flanking Region Regulate the Constitutive Expression of CYP3A46. Due to incomplete genomic information, we amplified the 5'-upstream sequence of porcine *CYP3A46* by genome walking, as described in *Materials and Methods*. A unique unpublished genomic DNA sequence (7.2 kb in length) containing the 5'-flanking region of *CYP3A46* has been confirmed by sequencing and uploaded in GenBank (accession number KP670896).

According to the sequence information, truncated mutations covering the region from bases -7235 to $+85$ of *CYP3A46* driving a luciferase reporter gene were constructed and transfected into HepG2 cells, to identify the possible *cis*-acting elements responsible for the basal expression of *CYP3A46* (Fig. 2A). The ($-7235\sim+85$)-luc construct-transfected group exhibited a 76-fold increment of the luciferase activity compared with the pGL3-Basic vector-transfected group (Fig. 2A). The deletion of the fragment from bases -7235 to -6220 attenuated the luciferase activity significantly, suggesting the existence of distal enhancers in the region (Fig. 2A). Further deletions to bases -4504 , -2707 , -2128 , -565 , and -295 , respectively, resulted in gradual increment of the luciferase activities from 36-fold to 172-fold compared with the pGL3-Basic vector-transfected group, indicating that negative regulatory elements may locate within these regions (Fig. 2A). Among the truncated constructs, the ($-295\sim+85$)-luc construct showed the highest promoter activity, accounting for the most transcription activation. However, the removal of the region $-165\sim-35$ remarkably decreased the luciferase activity of the ($-165\sim+85$)-luc construct to 2.1%, strongly indicating that there are *cis*-acting elements responsible for the constitutive expression of *CYP3A46* in the region from bases -165 to -35 (Fig. 2A).

Therefore, the DNA sequence from bases -165 to $+85$ was further analyzed by the online software MatInspector to predict the *cis*-acting

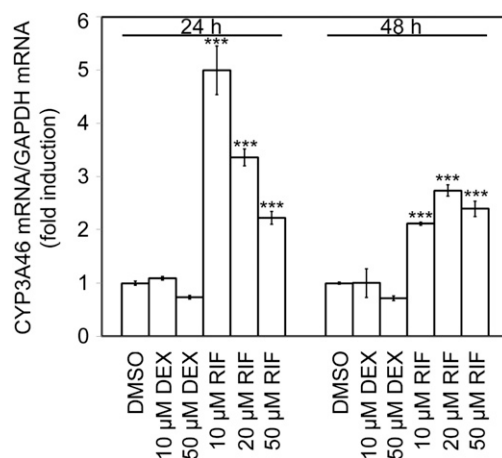


Fig. 1. RIF significantly induces the expression of *CYP3A46* in porcine primary hepatocytes. Porcine primary hepatocytes were treated with different concentrations of DEX and RIF for 24 hours or 48 hours, as described in *Materials and Methods*. Real-time PCR was used to detect the mRNA levels of *CYP3A46*. The ratio of *CYP3A46* to GAPDH in 0.1% DMSO-treated group was set to 1, and the values of other groups were normalized relative to this value. The experiment was performed three times independently, and similar results were obtained. The data shown are derived from a representative experiment expressed as the mean \pm S.D. Statistical significance was calculated by one-way ANOVA, and significance was defined as *** $P < 0.001$.

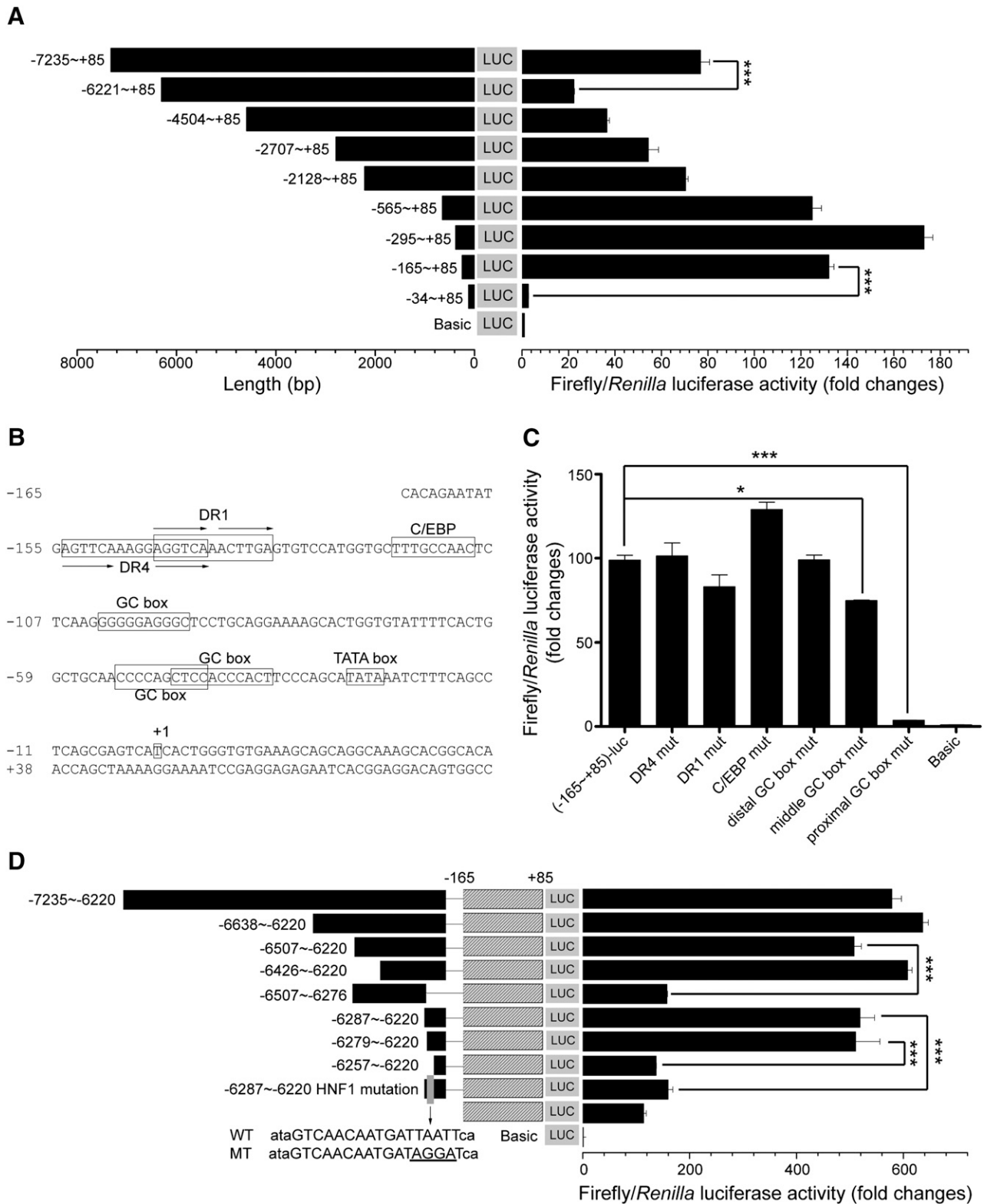


Fig. 2. The proximal GC box and distal HNF1 binding site in the 5'-flanking region regulate the constitutive expression of *CYP3A46*. (A) Deletion analysis of the *CYP3A46* 5'-flanking region. A series of deletion mutants were generated and transfected into HepG2 cells, as described in *Materials and Methods*. Schematic representations of the deletion constructs are shown on the left, and the indicated positions are relative to the transcriptional start site. The luciferase activities were measured after transfection using the Dual-Luciferase Reporter Assay System, and the firefly luciferase activity for each construct was normalized to the *Renilla* luciferase activity. The results, illustrated on the right, represent the fold change of each deleted construct relative to the pGL3-Basic plasmid (Basic). The experiment was performed four times independently, and similar results were obtained. The data shown are derived from a representative experiment expressed as the mean \pm S.D. Statistical significance was calculated by one-way ANOVA, and significance was defined as $***P < 0.001$ or $*P < 0.05$. (B) Schematic diagram showing the putative transcription factor binding sites in the proximal promoter (bases -165 to +85) region of *CYP3A46*. The transcriptional start site is designated as +1. The transcription factor binding sites, predicted by the online software MatInspector, are indicated with boxes. (C) Mutation analysis of the *CYP3A46* proximal promoter. The putative transcription factor binding sites in the proximal promoter (bases -165 to +85) region of *CYP3A46* were mutated one by one, as described in *Materials and Methods*.

elements in this region. Several *cis*-acting elements for the binding of liver-enriched and ubiquitous transcription factors are identified in this region, including one DR4 motif (usually recognized by PXR), one DR1 motif (usually recognized by HNF4 α), one C/EBP binding site (usually recognized by C/EBP α), and three GC boxes (usually recognized by Sp1) (Fig. 2B). In addition, a putative TATA box is also found in bases -27 to -24 (Fig. 2B).

To confirm the functional importance of these *cis*-acting elements in the transcriptional activation of *CYP3A46*, different mutations that destroyed the recruitment of the transcription factors, respectively, were introduced into the region of -165 to $+85$ based on the ($-165\sim+85$)-luc construct (Supplemental Table 1). The newly generated constructs were then transfected into HepG2 cells, and their effects on promoter activities were examined by dual luciferase assay. As shown in Fig. 2C, the mutation of the proximal GC box caused a significant and drastic decrease of the relative luciferase activity, only 4% of the wild-type control, which means that the promoter activity of *CYP3A46* is strongly inhibited by the dysfunction of the proximal GC box. Other mutations only caused the minimal effects on the luciferase activity. It strongly evidences that the proximal GC box is the pivotal regulatory element in the proximal promoter of *CYP3A46*. A slight decrease of the promoter activity was detected when the middle GC box was mutated, which may be due to the partial overlap with the proximal GC box (Fig. 2C).

To further investigate the function of the region from bases -7235 to -6220 for the transcriptional activation of *CYP3A46*, we artificially fused this region or serial deletions to the proximal promoter (bases -165 to $+85$) of *CYP3A46*. The region from bases -7235 to -6220 acts as an enhancer, which increased the luciferase activity of the *CYP3A46* proximal promoter ($-165\sim+85$) by five fold (Fig. 2D). Deletions to bases -6638 , -6507 , -6426 , -6287 , and -6279 , respectively, only caused the minimal effects on the luciferase activity, whereas the internal deletion of the region from bases $-6277\sim-6220$ led to 70% reduction of the luciferase activity relative to that of the ($-6507\sim-6220$)-($-165\sim+85$)-luc construct (Fig. 2D). Moreover, further deletion of the region from bases -6279 to -6258 [the ($-6257\sim-6220$)-($-165\sim+85$)-luc construct] remarkably decreased the luciferase activity of ($-6279\sim-6220$)-($-165\sim+85$)-luc construct to 25% and suggested that there are important regulatory elements responsible for the constitutive expression of *CYP3A46* in the region from bases -6279 to -6258 (Fig. 2D). Using the online software JASPAR to analyze the region from bases -6279 to -6258 , only one consensus regulatory element was identified as HNF1 binding site. Mutation of this HNF1 binding site drastically decreased the activity of ($-6287\sim-6220$)-($-165\sim+85$)-luc construct to 30% (Fig. 2D), proving that the HNF1 binding site is the key functional regulatory element within the distal enhancer responsible for the basal expression of *CYP3A46*.

Sp1 and HNF1 α Bind to the Proximal GC Box and the Distal HNF1 Binding Site, Respectively. The GC box and the HNF1 binding site are known to be recognized by the transcription factors Sp1 and HNF1 α , respectively. To determine whether Sp1 and HNF1 α bind to the proximal GC box and the distal HNF1 binding site in the 5'-flanking region of *CYP3A46*, EMSAs were carried out with the nuclear extracts from Sp1- or HNF1 α -overexpressed COS-7 cells. When the nuclear extracts from Sp1-transfected COS-7 cells were incubated with the labeled wild-type (WT) probe corresponding to the

putative GC box, two shifted bands named shifts 1 and 2 were detected (Fig. 3A, lane 2). The formation of shift 1 was competitively disrupted by the addition of a 100-fold excess of the unlabeled WT competitor (Fig. 3A, lane 3), but not the unlabeled mutant (MT) competitor (Fig. 3A, lane 4). Moreover, shift 1 was supershifted by the incubation with the antibody against Sp1 in the reaction (Fig. 3A, lane 5). These results indicate that Sp1 specifically binds to the proximal GC box in the 5'-flanking region of *CYP3A46*. Additionally, the band of shift 2 disappeared in the competition assays, but not the supershift assays, suggesting the existence of an unknown protein specifically binding to the flanking sequences of the GC box within the WT probe (Fig. 3A). Using the same method, we further confirmed the binding of HNF1 α to the distal HNF1 binding site. As shown in Fig. 3B (lane 2), adding nuclear extracts from HNF1 α -overexpressed COS-7 cells in the reaction led to the formation of a single protein-DNA complex with the labeled WT probe corresponding to the putative HNF1 binding site. This shifted band was disappeared by the addition of unlabeled WT competitor (Fig. 3B, lane 3), whereas the MT competitor possessing four mutations within the HNF1 binding site was unable to abolish the formation of the complex (Fig. 3B, lane 4). Moreover, a supershifted complex was observed when a specific antibody against HNF1 α was added in the binding reaction (Fig. 3B, lane 5). These results show that HNF1 α specifically binds to the distal HNF1 binding site in the 5'-flanking region of *CYP3A46*.

HNF1 α and PXR Activate the RIF-Mediated Transcription of *CYP3A46*. To analyze the regulatory mechanisms for RIF-induced expression of *CYP3A46*, HepG2 cells were transfected with the deletion constructs used in Fig. 2A and treated with 10 μ M RIF or DMSO for 24 hours. Compared with the DMSO-treated cells, no induction was observed in the RIF-treated cells, which were transfected with the ($-7235\sim+85$)-luc construct only (data not shown). However, cotransfection of pcDNA-PXR and the ($-7235\sim+85$)-luc construct caused a 1.4-fold induction of the luciferase activity in the RIF-treated cells, compared with the DMSO-treated cells (Fig. 4A). The removal of bases -7235 to -6220 abolished the inductive effect of RIF on the luciferase activity, suggesting that the region from bases -7235 to -6220 is required for RIF-induced expression of *CYP3A46* (Fig. 4A).

To further analyze the region from bases -7235 to -6220 , a second set of deletion mutants used in Fig. 2D was cotransfected with pcDNA-PXR into HepG2 cells, and then the cells were treated with 10 μ M RIF or 0.1% DMSO for 24 hours. As shown in Fig. 4B, cotransfection of pcDNA-PXR and the ($-7235\sim-6220$)-($-165\sim+85$)-luc construct resulted in a two fold induction of the luciferase activity in the RIF-treated cells, compared with the DMSO-treated cells. Progressive deletions to base -6279 did not influence the inductive effect of RIF on the luciferase activity, whereas the internal deletion of the region from bases $-6277\sim-6220$ abolished the inductive effect of RIF. Moreover, further deletion of the region from bases -6279 to -6258 [the ($-6257\sim-6220$)-($-165\sim+85$)-luc construct] showed almost no response to RIF induction, compared with the ($-6279\sim-6220$)-($-165\sim+85$)-luc construct, strongly indicating that there are important regulatory elements responsible for the RIF-induced expression of *CYP3A46* in the region from bases -6279 to -6258 . As presented in Fig. 2D, the region from bases -6279

and their activities were analyzed by Dual-Luciferase Reporter Assay System. The data were analyzed and shown, as described in (A). (D) Deletion and mutation analyses of the *CYP3A46* distal 5'-upstream sequence. Deleted and mutated fragments of the region from bases -7235 to -6220 were prepared, as outlined in *Materials and Methods*. All fragments were then ligated to the proximal promoter (bases -165 to $+85$) of *CYP3A46* to form the different chimeric *CYP3A46*-luciferase reporter gene constructs, as shown on the left. The constructs were transfected into HepG2 cells, the activities of which were detected, as described in *Materials and Methods*. The data shown on the right were analyzed and shown, as described in (A). The gray box indicates mutated HNF1 element. Its sequence is shown below with the mutated bases underlining.

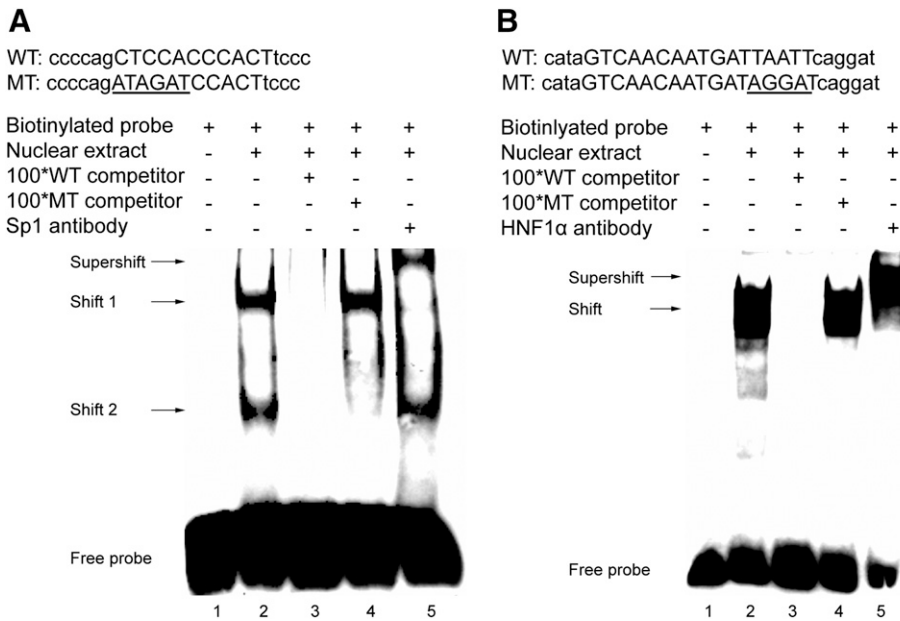


Fig. 3. Sp1 and HNF1 α bind to the proximal GC box and the distal HNF1 binding site, respectively. The ability of the potential GC box and the HNF1 binding site to be recognized by Sp1 and HNF1 α , respectively, was investigated using EMSAs, as described in *Materials and Methods*. The nuclear extracts from COS-7 cells transfected with Sp1 (A) or HNF1 α (B) were used. The sequences of the WT probes/competitors and MT competitors used in each experiment are shown above the gel. In the competition assays, unlabeled WT or MT competitors were added to the binding reaction 100-fold molar excess. In the supershift assays, specific antibody against Sp1 or HNF1 α was added to the mixture and incubated before adding biotinylated probes.

to -6258 contains a HNF1 binding site. Mutation of the HNF1 binding site destroyed the inductive effect of RIF on the luciferase activity of the (-6287~-6220)-(-165~+85)-luc construct (Fig. 4B). These results with the EMSA results shown in Fig. 3B indicate that HNF1 α mediates the RIF-induced expression of *CYP3A46* by binding to the distal HNF1 binding site.

The DR4 motif is known to be recognized by PXR. Because the inductive effect of RIF on *CYP3A46* requires PXR, we further mutated the putative DR4 motif identified in Fig. 2B to confirm whether the DR4 motif is the functional *cis*-acting element involved in regulating the RIF-induced expression of *CYP3A46*. As expected, mutation to the DR4 motif eliminated the inductive effect of RIF on the luciferase activity of (-6287~-6220)-(-165~+85)-luc construct (Fig. 4B). Whether the DR4 motif is recognized by the PXR/RXR α heterodimer was further investigated by EMSAs. As shown in Fig. 4C, a shift band was detected, when the nuclear extracts from PXR- and RXR α -cotransfected COS-7 cells were incubated with the labeled WT probe corresponding to the DR4 motif. The formation of the shift was competitively disrupted by the addition of a 100-fold excess of the unlabeled WT competitor, but not the unlabeled MT competitor, suggesting that PXR/RXR α complex specifically binds to the DR4 motif. No shift band was formed when nuclear extracts from PXR- or RXR α -transfected COS-7 cells were used, indicating that PXR and RXR α need to form heterodimer to bind to the DR4 motif. In short, our results show that PXR is also involved in regulating the RIF-induced expression of *CYP3A46* by binding to the proximal DR4 motif.

HNF1 α Is Also Necessary for the Transcriptional Activation of Human *CYP3A4* Induced by RIF. To investigate whether HNF1 α is also involved in regulating the transcription of human *CYP3A4* induced by RIF, we scanned the XREM of human *CYP3A4* and found a putative HNF1 binding site adjacent to the reported PXR binding site eNR3A4 (Toriyabe et al., 2009). To elucidate the role of the HNF1 binding site in the RIF-induced transcriptional activation of human *CYP3A4*, we performed transient transfection assays with the XREM(-362~+53)-luc construct and the HNF1 binding site mutated construct. Consistent with the previous report (Goodwin et al., 1999), cotransfection of XREM(-362~+53)-luc construct and pcDNA-hPXR caused a 11-fold induction of the luciferase activity

in the RIF-treated cells, compared with the DMSO-treated cells (Fig. 5A). However, mutations to the HNF1 binding site in the XREM(-362~+53)-luc construct led to a remarkable decrease of the activity of XREM(-362~+53)-luc construct (Fig. 5A). These results imply that the HNF1 binding site is a key regulatory element responsible for the RIF-induced transactivation of human *CYP3A4*. Furthermore, we performed EMSAs to examine the binding of HNF1 α to the HNF1 binding site. As expected, nuclear extracts from HNF1 α -overexpressed COS-7 cells formed a specific shift band with the labeled WT probes corresponding to the putative HNF1 binding site (Fig. 5B). Its binding specificity was further confirmed with the competition and supershift assays (Fig. 5B). Our results reveal that HNF1 α is also necessary for the RIF-induced transcriptional activation of human *CYP3A4* by binding to the distal HNF1 binding site.

Discussion

In the previous study, we demonstrated *CYP3A46*, like its homolog (*CYP3A4*) in humans, is an important drug-metabolizing enzyme in pigs. In this study, we focused on the regulatory mechanism of its transcriptional activation and identified the related *cis*-acting elements and *trans*-acting factors. To our knowledge, this is the first report comprehensively uncovering the molecular regulatory mechanisms for the constitutive and inducible expression of *CYP3A46* in porcine liver.

Sp1, one of the Sp family transcription factors, is ubiquitously expressed in different tissues and binds to GC box to transactivate various kinds of genes (Li et al., 2004). One putative binding site for Sp1 has been identified in the proximal promoter of *CYP3A4* (Bombail et al., 2004). This element only functioned in regulating the metyrapone- or phenobarbital-mediated transcriptional activation of *CYP3A4*, but had no effect on the basal transcription of *CYP3A4* (Bombail et al., 2004). In our study, three putative Sp1 binding sites (GC box) located in the proximal promoter of *CYP3A46* have been identified; only the proximal GC box is the prerequisite for the basal transcription of *CYP3A46*, which is recognized by Sp1. In contrast to the study of *CYP3A4*, the proximal GC box has no regulatory effect on the RIF-induced expression of *CYP3A46*.

HNF1 α is a liver-enriched transcription factor, but is also expressed in other tissues, such as kidney, intestine, stomach, and pancreas

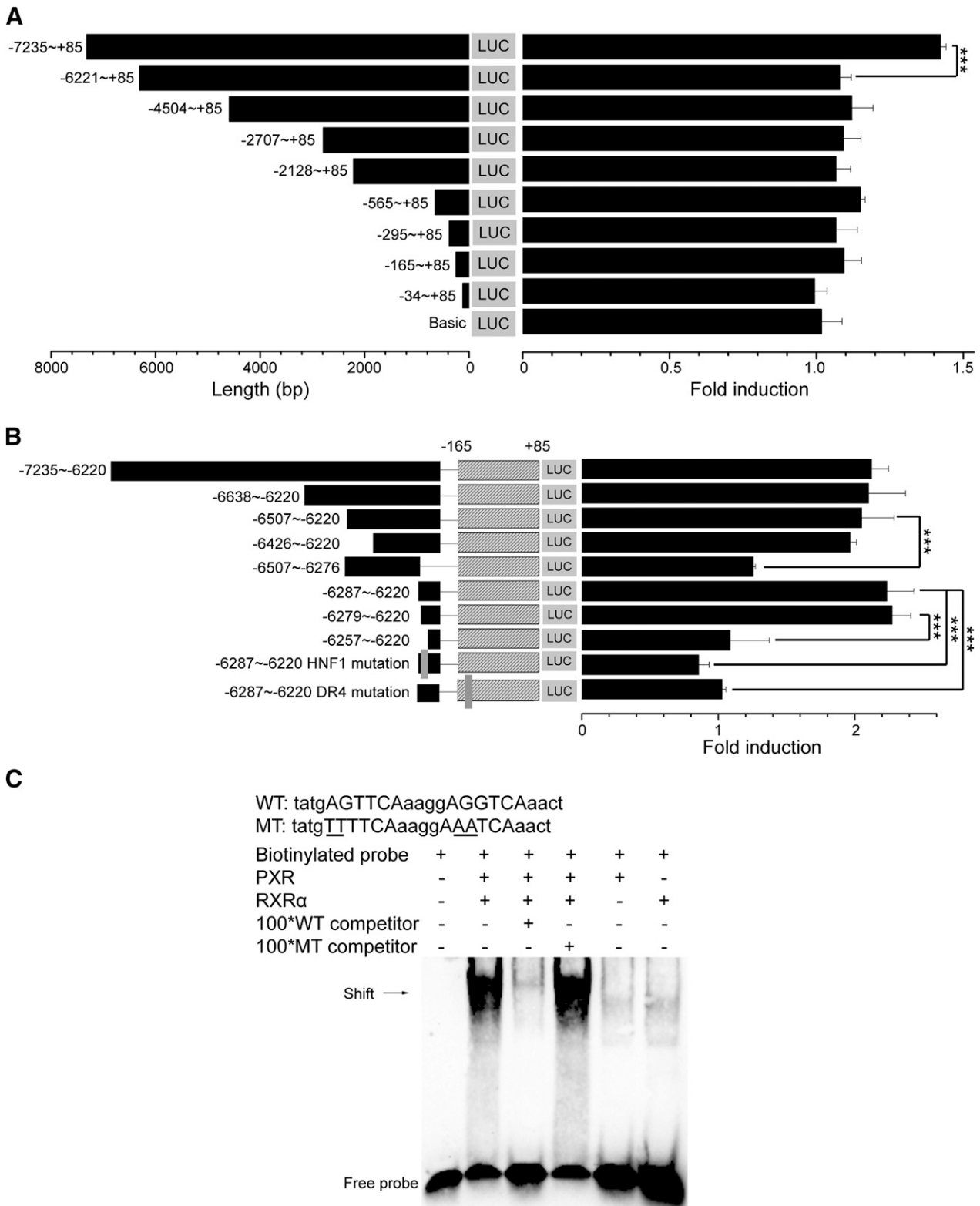


Fig. 4. HNF1 α and PXR mediate the RIF-induced transcriptional activation of *CYP3A46*. (A) Identification of the RIF-responsive region in the 5'-upstream sequence of *CYP3A46*. Different deletion constructs of *CYP3A46* and pcDNA-pPXR were cotransfected into HepG2 cells, and the cells were subsequently cultured in the presence of RIF (10 μ M) or DMSO (0.1%) for 24 hours. The luciferase activities were detected and normalized, as described in the legend of Fig. 2A. Each column represents the fold induction of the normalized luciferase activities in the RIF-treated cells relative to the DMSO-treated cells. The experiment was performed four times independently, and similar results were obtained. The data shown are derived from a representative experiment expressed as the mean \pm S.D. Statistical significance was calculated by one-way ANOVA, and significance was defined as *** P < 0.001. (B) Further deletion and mutation analysis of the RIF-responsive region in the 5'-upstream sequence of *CYP3A46*. Deletion and mutation constructs were cotransfected with pcDNA-pPXR into HepG2 cells, and the luciferase activities were detected and analyzed, as described in (A). (C) Binding of pPXR/pRXR α to the proximal DR4 motif in EMSAs. EMSAs were performed with nuclear extracts from pcDNA-pPXR/pRXR α -overexpressed COS-7 cells and biotinylated oligonucleotides corresponding to the proximal DR4 motif. The sequences of the WT probe/competitor and MT competitor used in the experiment are shown above the gel. The consensus binding sites are shown in capital, and the mutated nucleotides are underlined. In the competition assays, unlabeled competitors were added to the binding reaction 100-fold molar excess.

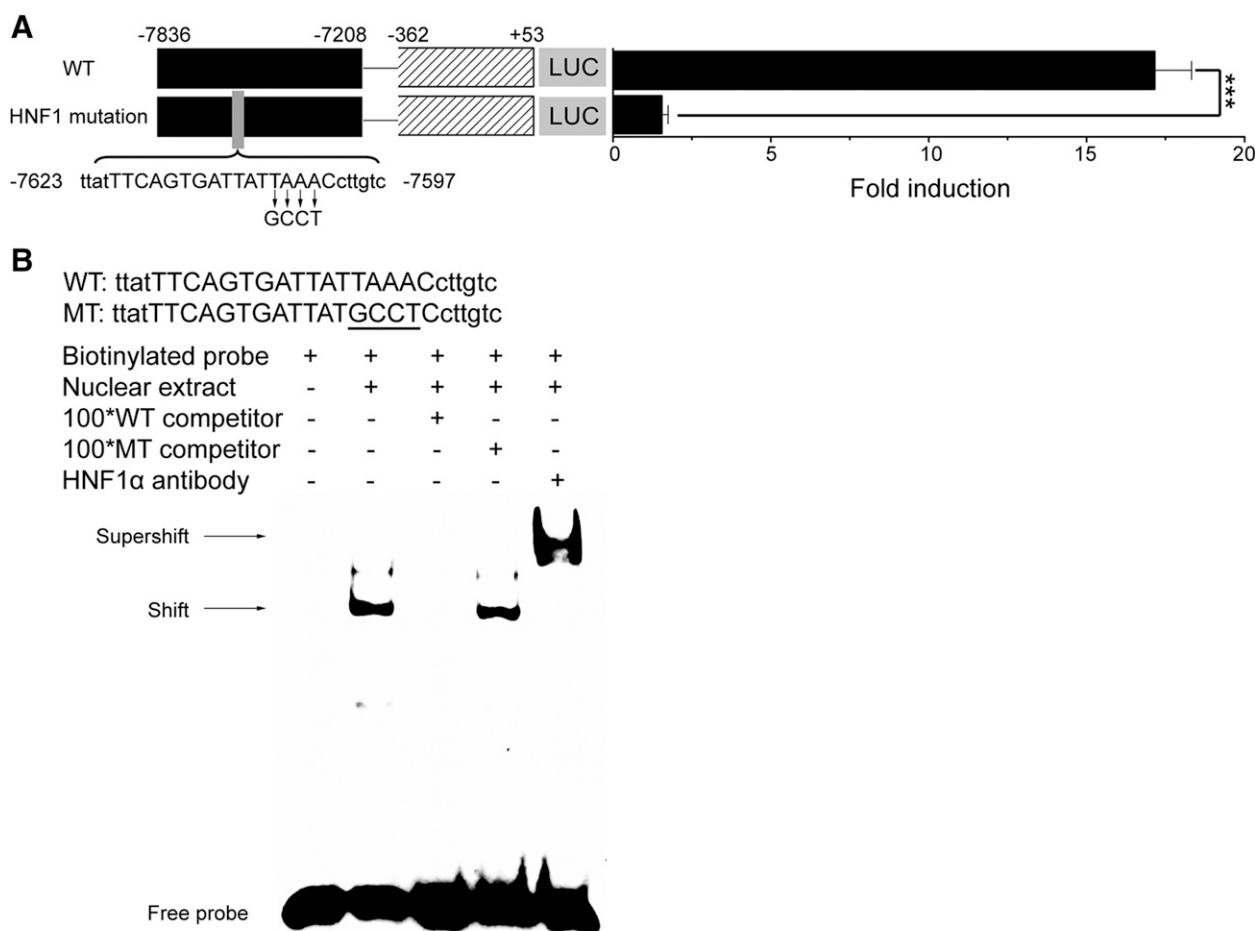


Fig. 5. HNF1 α is also necessary for the transcriptional activation of human *CYP3A4* induced by RIF. (A) Mutation analysis of the putative HNF1 binding site in the XREM of human *CYP3A4*. The sequence of the putative HNF1 binding site is shown, and the mutated bases are indicated. The XREM(-362~+53)-luc construct or HNF1 binding site mutated construct was cotransfected with pcDNA-hPXR. Transfected HepG2 cells were then cultured in the presence of RIF (10 μ M) or DMSO (0.1%) for 24 hours before harvest. The luciferase activities were detected and normalized, as described in the legend of Fig. 2A. Each column represents the fold induction of the normalized luciferase activities in the RIF-treated cells relative to the DMSO-treated cells. The experiment was performed four times independently, and similar results were obtained. The data shown are derived from a representative experiment expressed as the mean \pm S.D. Statistical significance was calculated by one-way ANOVA, and significance was defined as *** P < 0.001. (B) Binding of HNF1 α to the HNF1 binding site in EMSAs. EMSAs were performed with nuclear extracts from HNF1 α -overexpressed COS-7 cells and biotinylated oligonucleotides corresponding to the HNF1 binding site. The sequences of the WT probe/competitor and MT competitor used in the experiment are shown above the gel. The consensus binding sites are shown in capital, and the mutated nucleotides are underlined. In the competition assays, unlabeled competitors were added to the binding reaction 100-fold molar excess. In the supershift assays, specific antibody against HNF1 α was added to the mixture and incubated before adding biotinylated probes.

(Cheung et al., 2003). It specifically binds to a consensus 13-bp DNA sequence 5'-GTTAATNATTANC-3' and functions as a key transcription factor to regulate the expression of many liver-enriched genes (Küstaki et al., 1997; Cheung et al., 2003). HNF1 α regulates the basal expression of *CYP3A4* by binding to the distal HNF-1 consensus site in the CLEM (-11.4 kb to -10.5 kb) (Matsumura et al., 2004). In agreement with that, we identified a HNF-1 consensus site (from bases -6279 to -6258) in the distal enhancer of *CYP3A46*, and this element was specifically recognized by HNF1 α and involved in regulating the constitutive expression of *CYP3A46*. More interestingly, we found that HNF1 α also mediates the RIF-induced transcriptional activation of *CYP3A46* through this distal HNF1 binding site. Meanwhile, we identified a novel HNF1 consensus site (from bases -7622 to -7596) within XREM, which can be recognized by HNF1 α and involved in RIF-induced expression of *CYP3A4*. These results confirmed that HNF1 α plays evolutionary conserved but versatile roles in the regulations of both basal and inducible expression of *CYP3A46* and *CYP3A4*.

PXR belongs to the nuclear receptor superfamily, and is mainly expressed in the liver and intestine, to a less extent in the kidney and

lung (LeCluyse, 2001). It usually forms a heterodimer with RXR α and binds to direct repeats of AGGTCA separated by three base (DR3), DR4, or ER6 to transactivate a large set of phase I/II drug-metabolizing genes and drug transporter genes (Tolson and Wang, 2010). Numerous studies have demonstrated that PXR mediates the RIF-induced transactivation of *CYP3A4* by binding to multiple interacting *cis*-regulatory elements, including the ER6 in the proximal promoter (Lehmann et al., 1998); the dNR1, dNR2, and eNR3A4 in the distal XREM (Goodwin et al., 1999; Toriyabe et al., 2009); as well as the ER6 in the CLEM (Liu et al., 2008). In the present study, we also found that PXR is involved in regulating the inducible expression of *CYP3A46* by RIF. However, only one functional DR4 element in the proximal promoter was identified.

LETfs are well known to be important regulators in the coordination of PXR- and CAR-mediated response to xenobiotics. For example, HNF4 α , which binds to the DR1 element adjacent to the PXR response element in the distal module, is critically involved in the PXR-mediated transcriptional activation of *CYP3A4* by interacting with PXR (Tirona et al., 2003; Li and Chiang, 2006; Liu et al., 2008).

In this study, we found another LETF HNF1 α , which is required for the PXR-mediated transcriptional activation of *CYP3A46* under RIF induction. We further identified a novel HNF1 binding site adjacent to the reported PXR binding site eNR3A4, which is also crucial for PXR-mediated transcriptional activation of *CYP3A4*. In fact, it has been reported that HNF1 α can modestly alter PXR-mediated activation of the *CYP3A4* promoter (Tirona et al., 2003). Several studies suggested that HNF1 α can facilitate chromatin remodeling (Rollini and Fournier, 1999; Viollet et al., 2001). Whether HNF1 α regulates the RIF-induced transactivation of *CYP3A4* and *CYP3A46* through facilitating chromatin remodeling or interacting with PXR is still unknown and needs to be further studied.

Our data showed that the maximal induction of *CYP3A46* was observed at 24 hours, when 10 μ M RIF was used to treat porcine primary hepatocytes. Higher doses of RIF or longer time of RIF treatment did not produce further increases in the *CYP3A46* mRNA expression. In fact, similar dose- and time-dependent inductions of *CYP3A4* were observed when RIF was used to treat both human primary hepatocytes and HepG2 cells (Goodwin et al., 1999; Raucy, 2003). The difference is that the maximal induction of *CYP3A4* was observed at 48 hours when 10 μ M RIF was used (Raucy, 2003). Higher doses of RIF or longer time of RIF treatment may tend to increase cellular toxicity and ultimately weaken its inductive effect on the expression of CYP3As.

In human primary hepatocytes, RIF treatment induced the expression of *CYP3A4* up to 100-fold (Li and Chiang, 2006). However, RIF only induced the expression of *CYP3A46* for four times in the porcine liver (Nannelli et al., 2008). Our studies drew similar results that the expression of *CYP3A46* at the mRNA level was induced by RIF only up to five fold. It has been suggested that the number of interacting *cis*-regulatory elements correlates to the drug-induction effect on the target gene (Toriyabe et al., 2009). Given that several PXR binding sites presented in the *CYP3A4* promoter, whereas only one functional PXR binding site presented in the proximal promoter of *CYP3A46*, we believe that the different numbers of PXR binding sites may account for the different inductive effects of RIF on the expression of *CYP3A46* and *CYP3A4*. In fact, the insertion of the PXR binding site eNR3A4 of human *CYP3A4* adjacent to the HNF1 binding site in the *CYP3A46* reporter (−6287~−6220)−(−165~+85)-luc greatly enhanced the response of the reporter to RIF from two fold to five fold, whereas the abilities of RIF to activate hPXR or pPXR were the same (Supplemental Fig. 1).

As reported, DEX is a representative inducer of *CYP3A4*, but not a potent inducer of porcine CYP3A (Monshouwer et al., 1998). In the present paper, we observed the similar phenomenon that RIF rather than DEX induced the expression of *CYP3A46*. In contrast to the other xenobiotic inducers of *CYP3A4*, DEX plays a dual role in *CYP3A4* expression, consisting of a low-DEX component of low amplitude and a high-DEX component of high amplitude (Pascussi et al., 2001). The low-DEX component induces the expression of *CYP3A4* through GR-mediated activation of PXR and CAR, whereas the high-DEX component transactivates *CYP3A4* directly through the regulation of PXR (Pascussi et al., 2001). This dual role of DEX did not work on the inducible expression of *CYP3A46*, because a high concentration of DEX (50 μ M) still had no inductive effect on the expression of *CYP3A46*. The regulatory effects of GR, PXR, and CAR may be different between humans and pigs under the stimulation of DEX.

Similar to human CYP3A subfamily, porcine CYP3As consist of four genes, as follows: *CYP3A22*, *CYP3A29*, *CYP3A39*, and *CYP3A46*. Their expression patterns and inducible aspects are different, indicating the underlying of different regulatory mechanisms (Nannelli et al., 2008; Yao et al., 2012; Shang et al., 2013). However, related research is limited. Recently, Li et al. (2014, 2015) reported

that PXR was required for the IFN γ - and IFN α -mediated inductive effects on porcine CYP3A29, but they did not observe the binding of PXR to any *cis*-regulatory elements. In this work, we not only found PXR accounting for the inducible effect on the expression of *CYP3A46* by RIF, but also confirmed the binding of PXR to the DR4 element in the proximal promoter of *CYP3A46*. Further studies are still required to clarify the regulatory mechanisms for the transcriptional activation of different porcine CYP3A isoforms.

In summary, the present paper systematically studied the regulatory mechanisms for the basal and inducible expression of *CYP3A46*, and clearly demonstrated that several key *trans*-acting factors, including Sp1, HNF1 α , and PXR, function in the basal and RIF-mediated regulation of *CYP3A46*.

Authorship Contributions

Participated in research design: Dong, Jiang, Deng.

Conducted experiments: Dong, Chen, Liu.

Performed data analysis: Dong, Jiang, Deng.

Wrote or contributed to the writing of the manuscript: Dong, Wen, Jiang, Deng.

References

- Bombail V, Taylor K, Gibson GG, and Plant N (2004) Role of Sp1, C/EBP α , HNF3, and PXR in the basal- and xenobiotic-mediated regulation of the CYP3A4 gene. *Drug Metab Dispos* **32**:525–535.
- Burk O, Koch I, Raucy J, Hustert E, Eichelbaum M, Brockmöller J, Zanger UM, and Wojnowski L (2004) The induction of cytochrome P450 3A5 (CYP3A5) in the human liver and intestine is mediated by the xenobiotic sensors pregnane X receptor (PXR) and constitutively activated receptor (CAR). *J Biol Chem* **279**:38379–38385.
- Cheung C, Akiyama TE, Kudo G, and Gonzalez FJ (2003) Hepatic expression of cytochrome P450s in hepatocyte nuclear factor 1-alpha (HNF1alpha)-deficient mice. *Biochem Pharmacol* **66**:2011–2020.
- Drocourt L, Ourlin JC, Pascussi JM, Maurel P, and Vilarem MJ (2002) Expression of CYP3A4, CYP2B6, and CYP2C9 is regulated by the vitamin D receptor pathway in primary human hepatocytes. *J Biol Chem* **277**:25125–25132.
- Goodwin B, Hodgson E, D'Costa DJ, Robertson GR, and Liddle C (2002a) Transcriptional regulation of the human CYP3A4 gene by the constitutive androstane receptor. *Mol Pharmacol* **62**:359–365.
- Goodwin B, Hodgson E, and Liddle C (1999) The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. *Mol Pharmacol* **56**:1329–1339.
- Goodwin B, Redinbo MR, and Klierer SA (2002b) Regulation of cyp3a gene transcription by the pregnane X receptor. *Annu Rev Pharmacol Toxicol* **42**:1–23.
- Guengerich FP (1999) Cytochrome P-450 3A4: regulation and role in drug metabolism. *Annu Rev Pharmacol Toxicol* **39**:1–17.
- Honkakoski P, Sueyoshi T, and Negishi M (2003) Drug-activated nuclear receptors CAR and PXR. *Ann Med* **35**:172–182.
- Jiang J, Wang J, Cai H, Li K, and Deng Y (2011) CYP3As catalyze nifedipine oxidation in pig liver microsomes: enzyme kinetics, inhibition and functional expression. *Catal Commun* **12**:694–697.
- Kitstaki E and Talianidis I (1997) Modulation of hepatic gene expression by hepatocyte nuclear factor 1. *Science* **277**:109–112.
- LeCluyse EL (2001) Pregnane X receptor: molecular basis for species differences in CYP3A induction by xenobiotics. *Chem Biol Interact* **134**:283–289.
- Lehmann JM, McKee DD, Watson MA, Wilson TM, Moore JT, and Klierer SA (1998) The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J Clin Invest* **102**:1016–1023.
- Li L, He S, Sun J-M, and Davie JR (2004) Gene regulation by Sp1 and Sp3. *Biochem Cell Biol* **82**:460–471.
- Li T and Chiang JY (2006) Rifampicin induction of CYP3A4 requires pregnane X receptor cross talk with hepatocyte nuclear factor 4alpha and coactivators, and suppression of small heterodimer partner gene expression. *Drug Metab Dispos* **34**:756–764.
- Li X, Hu X, Jin X, Zhou X, Wang X, Shi D, and Bi D (2015) IFN-gamma regulates cytochrome 3A29 through pregnane X receptor in pigs. *Xenobiotica* **45**:373–379.
- Li X, Jin X, Zhou X, Wang X, Shi D, Xiao Y, and Bi D (2014) Pregnane X receptor is required for IFN- α -mediated CYP3A29 expression in pigs. *Biochem Biophys Res Commun* **445**:469–474.
- Liu FJ, Song X, Yang D, Deng R, and Yan B (2008) The far and distal enhancers in the CYP3A4 gene co-ordinate the proximal promoter in responding similarly to the pregnane X receptor but differentially to hepatocyte nuclear factor-4alpha. *Biochem J* **409**:243–250.
- Livak KJ and Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-($\Delta\Delta$ C_T)} Method. *Methods* **25**:402–408.
- Martínez-Jiménez CP, Gómez-Lechón MJ, Castell JV, and Jover R (2005) Transcriptional regulation of the human hepatic CYP3A4: identification of a new distal enhancer region responsive to CCAAT/enhancer-binding protein beta isoforms (liver activating protein and liver inhibitory protein). *Mol Pharmacol* **67**:2088–2101.
- Matsumura K, Saito T, Takahashi Y, Ozeki T, Kiyotani K, Fujieda M, Yamazaki H, Kunitoh H, and Kamataki T (2004) Identification of a novel polymorphic enhancer of the human CYP3A4 gene. *Mol Pharmacol* **65**:326–334.
- Monshouwer M, Van't Klooster GA, Nijmeijer SM, Witkamp RF, and van Miert AS (1998) Characterization of cytochrome P450 isoenzymes in primary cultures of pig hepatocytes. *Toxicol In Vitro* **12**:715–723.

- Nannelli A, Chirulli V, Longo V, and Gervasi PG (2008) Expression and induction by rifampicin of CAR- and PXR-regulated CYP2B and CYP3A in liver, kidney and airways of pig. *Toxicology* **252**:105–112.
- Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Waterman MR, Gotoh O, Coon MJ, and Estabrook RW, et al. (1996) P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* **6**:1–42.
- Ourlin JC, Jounaïdi Y, Maurel P, and Vilarem MJ (1997) Role of the liver-enriched transcription factors C/EBP alpha and DBP in the expression of human CYP3A4 and CYP3A7. *J Hepatol* **26** (Suppl 2):54–62.
- Pascussi JM, Drocourt L, Gerbal-Chaloin S, Fabre JM, Maurel P, and Vilarem MJ (2001) Dual effect of dexamethasone on CYP3A4 gene expression in human hepatocytes: sequential role of glucocorticoid receptor and pregnane X receptor. *Eur J Biochem* **268**:6346–6358.
- Pascussi JM, Gerbal-Chaloin S, Drocourt L, Maurel P, and Vilarem MJ (2003) The expression of CYP2B6, CYP2C9 and CYP3A4 genes: a tangle of networks of nuclear and steroid receptors. *Biochim Biophys Acta* **1619**:243–253.
- Plant N (2007) The human cytochrome P450 sub-family: transcriptional regulation, inter-individual variation and interaction networks. *Biochim Biophys Acta* **1770**:478–488.
- Raucy JL (2003) Regulation of CYP3A4 expression in human hepatocytes by pharmaceuticals and natural products. *Drug Metab Dispos* **31**:533–539.
- Rodríguez-Antona C, Bort R, Jover R, Tindberg N, Ingelman-Sundberg M, Gómez-Lechón MJ, and Castell JV (2003) Transcriptional regulation of human CYP3A4 basal expression by CCAAT enhancer-binding protein alpha and hepatocyte nuclear factor-3 gamma. *Mol Pharmacol* **63**:1180–1189.
- Rollini P and Fournier RE (1999) The HNF-4/HNF-1alpha transactivation cascade regulates gene activity and chromatin structure of the human serine protease inhibitor gene cluster at 14q32.1. *Proc Natl Acad Sci USA* **96**:10308–10313.
- Shang H, Guo K, Liu Y, Yang J, and Wei H (2013) Constitutive expression of CYP3A mRNA in Bama miniature pig tissues. *Gene* **524**:261–267.
- Tirona RG, Lee W, Leake BF, Lan LB, Cline CB, Lamba V, Parviz F, Duncan SA, Inoue Y, and Gonzalez FJ, et al. (2003) The orphan nuclear receptor HNF4alpha determines PXR- and CAR-mediated xenobiotic induction of CYP3A4. *Nat Med* **9**:220–224.
- Tolson AH and Wang H (2010) Regulation of drug-metabolizing enzymes by xenobiotic receptors: PXR and CAR. *Adv Drug Deliv Rev* **62**:1238–1249.
- Toriyabe T, Nagata K, Takada T, Aratsu Y, Matsubara T, Yoshinari K, and Yamazoe Y (2009) Unveiling a new essential cis element for the transactivation of the CYP3A4 gene by xenobiotics. *Mol Pharmacol* **75**:677–684.
- Viollet B, Yaniv M, and Pontoglio M (2001) Embryonic but not postnatal reexpression of hepatocyte nuclear factor 1alpha (HNF1alpha) can reactivate the silent phenylalanine hydroxylase gene in HNF1alpha-deficient hepatocytes. *Mol Cell Biol* **21**:3662–3670.
- Wang J, Jiang J, Zhang H, Wang J, Cai H, Li C, Li K, Liu J, Guo X, and Zou G (2011) Integrated transcriptional and proteomic analysis with in vitro biochemical assay reveal the important role of CYP3A46 in T-2 toxin hydroxylation in porcine primary hepatocytes. *Mol Cell Proteomics* **10**:M111.008748.
- Yao M, Dai M, Liu Z, Cui W, Li D, Zhang H, Li J, Liu Y, and Yuan Z (2012) mRNA expression profiles of P450 3A enzymes in the liver and small intestine of the domestic pig. *Res Vet Sci* **93**:360–365.
- Zanger UM and Schwab M (2013) Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacol Ther* **138**:103–141.

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