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Human *CYP2C8* is Transcriptionally Regulated by the Nuclear Receptors CAR, PXR, GR, and
HNF4 α

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Running Title: Regulation of the human CYP2C8 gene

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Number of text pages: from 3-36 (34 pages)

Number of tables: 0

Number of figures: 11

Number of references: 35

Number of words in abstract: 235

Number of words in introduction: 743

Number of words in discussion: 1488

Nonstandard abbreviations: CYP, Cytochrome P450 protein; hCAR, human constitutive androstane receptor; hPXR, human pregnane X receptor; GR, glucocorticoid receptor; HNF4 α , hepatic nuclear factor 4 α ; EETs, Epoxyeicosatrienoic acids, CITCO, 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime.

Abstract

Cytochrome P450 (CYP) enzymes play important roles in the metabolism of endogenous and xenobiotic substrates in humans. CYP2C8 is an important member of the CYP2C subfamily which metabolizes both endogenous compounds (i.e. arachidonic acids and retinoic acid) and xenobiotics (e.g. paclitaxel). Induction of CYP enzymes by drugs can result in tolerance as well as drug-drug interactions. CYP2C8 is the most strongly inducible member of the CYP2C subfamily in human hepatocytes, but the mechanism of induction by xenobiotics has not been delineated. To determine the mechanisms controlling the regulation of this important CYP, we cloned the 5'-flanking region of *CYP2C8* and investigated its transcriptional regulation by nuclear factors such as the pregnane X receptor (PXR), constitutive androstane receptor (CAR), glucocorticoid receptor (GR) and hepatic nuclear factor 4 (HNF4 α) that are known to be involved in the induction of other CYP enzymes using both cell lines and primary hepatocyte models. First, we identified a distal PXR/CAR binding site in the *CYP2C8* promoter that confers inducibility of *CYP2C8* via the PXR agonist/ligand rifampicin, and the CAR agonist/ligand CITCO. A glucocorticoid responsive element was identified that mediates dexamethasone induction via the GR. Finally, we identify an HNF4 α binding site within the *CYP2C8* basal promoter region that is cis-activated by cotransfected HNF4 α . In summary, the present studies show that CAR, PXR, GR, and HNF4 α can regulate *CYP2C8* expression and identify specific cis-elements within the promoter that control these regulatory pathways.

Introduction

The CYP2C subfamily of cytochrome P450 enzymes is an important class of drug metabolizing enzymes responsible for the metabolism of approximately 20% of all clinically prescribed therapeutics (Goldstein, 2001). In humans, this subfamily is composed of four members: *CYP2C8*, *CYP2C9*, *CYP2C18*, and *CYP2C19* that are localized within a single gene locus on Chromosome 10. Significant progress has been made in elucidating the pharmacological and physiological function of these enzymes in human liver; however, only recently have reports emerged describing factors that influence their transcriptional regulation (Bort et al., 2004; Chen et al., 2004; Ferguson et al., 2002; Pascussi et al., 2000). Of the four CYP2C isozymes, *CYP2C9* and *CYP2C8* proteins are expressed at the highest levels in human liver (Goldstein and de Morais, 1994; Inoue et al., 1994), while *CYP2C9*>*CYP2C19*>*CYP2C8* are expressed in small intestine (Lapple et al., 2003). *CYP2C8* expression has also been reported in kidney, lung, nasal mucosa, and heart (Ding and Kaminsky, 2003; Klose et al., 1999).

Our laboratory has recently examined the regulation of CYP2C gene expression by clinically prescribed drugs such as phenobarbital, rifampicin, and dexamethasone (Chen et al., 2003; Chen et al., 2004; Ferguson et al., 2002). We have identified transcription factor binding sites within the 5'-flanking regions of the *CYP2C9* and *CYP2C19* genes that are essential for these responses. In this report, we turn our focus to the most strongly inducible member of the human CYP2C subfamily, *CYP2C8*. *CYP2C8* is the principle enzyme that metabolizes a number of clinically prescribed therapeutics such as: the antidiabetic drugs rosiglitazone and repaglinide, anti-cancer drug paclitaxel, the cholesterol lowering drug cerivastatin, and the anti-malarials amodiaquine and chloroquine (Totah and Rettie, 2005). *CYP2C8* is also known to

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metabolize endogenous molecules retinoic acid and arachidonic acid. Due to the significant expression of *CYP2C8* in various extrahepatic tissues, this enzyme may play an important role in endogenous signaling. In fact, *CYP2C8/9* have been proposed as endothelial derived hyperpolarizing factor (EDHF) synthetases, and are thought to be located in arteries (Fisslthaler et al., 1999). Therefore, understanding the factors that control *CYP2C8* basal expression and induction by drugs is important to understand its biological interactions and to better predict adverse drug-drug interactions.

The current consensus is that *CYP2C8*>*CYP2C9*>*CYP2C19* are induced by phenobarbital and rifampicin in liver and primary human hepatocytes (Gerbai-Chaloin et al., 2001; Madan et al., 2003; Raucy et al., 2002). These prototypical inducers are also known to strongly induce *CYP2B* and *CYP3A* expression and play significant roles in drug induced drug-drug interactions. Significant progress has been made over the past few years in unraveling the mechanism of these induction processes (Goodwin et al., 1999; Sueyoshi and Negishi, 2001; Wang et al., 2004). At present, it is thought that both the constitutive androstane receptor (NR1I3) and pregnane X receptor (NR1I2) are important for phenobarbital-mediated drug induction in humans (Wei et al., 2002). Rifampicin, and other inducers such as hyperforin (active ingredient in St. John's Wort) and paclitaxel are thought to predominantly activate gene expression as ligands to PXR. Our laboratory, among others, has extended these studies to the *CYP2C* subfamily. *CYP2C9* and *CYP2C19* are directly regulated by CAR and PXR (Chen et al., 2003; Chen et al., 2004; Ferguson et al., 2002). The clinically prescribed synthetic glucocorticoid dexamethasone has also been shown to induce *CYP2C* expression (Gerbai-Chaloin et al., 2001). The glucocorticoid receptor (GR) binds/activates identical GRE half-sites within the 5'-flanking regions of the *CYP2C9* and *CYP2C19* genes, and is essential for

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dexamethasone induction (Chen et al., 2003; Gerbal-Chaloin et al., 2002). We have also previously identified a site in the 5'-flanking region of the *CYP2C9* that binds to the nuclear receptor hepatic nuclear factor 4 α (HNF-4 α) and enhances basal promoter activity (Ibeanu and Goldstein, 1995).

Although *CYP2C8* is reported to be the most inducible member of the human CYP2C subfamily, there is a paucity of information concerning the transcription factors and binding sites in the *CYP2C8* promoter which control these responses. To explore these regulatory mechanisms, we treated primary human hepatocytes with drugs and isolated RNA for RT-PCR, cloned upstream regions into luciferase reporter vectors and transfected them into cells, and performed gel shift assays to identify nuclear receptor binding sites. Through these studies, we have identified a distal site for PXR and CAR that is essential for the induction of *CYP2C8* reporters in primary human hepatocytes, a putative glucocorticoid receptor (GR) binding site essential for dexamethasone induction, and a hepatic nuclear factor 4 (HNF4 α) binding site that is responsive to HNF4 α cotransfection.

Materials and Methods

Rifampicin, dimethyl sulfoxide, dexamethasone, paclitaxel, CITCO, phenytoin, and lithocholic acid were purchased from Sigma (St. Louis, MO). Hyperforin was purchased from Chromadex (Laguna Hills, CA). Cell culture media, fetal bovine serum, and trypsin/EDTA were purchased from Invitrogen/Gibco (Carlsbad, CA). Oligonucleotides were purchased from Sigma-Genosys (The Woodlands, TX) and desalted. All other reagents if not specified were obtained from standard sources. All restriction endonucleases and modifying enzymes were purchased from New England Biolabs (Beverly, MA).

Bioinformatics

Regulatory regions of the *CYP2C8* gene were obtained via the Celera Discovery System and NCBI databases. CAR/PXR binding sites were identified via customized search motifs and mined using the GCG (Wisconsin Package) Findpatterns tool as described previously (Chen et al., 2003; Ferguson et al., 2002; Jackson et al., 2004; Wang et al., 2003). The tfsites.dat database within the Wisconsin package, which contains the Transfac database, was utilized to identify putative transcription factor binding sites within the proximal *CYP2C8* promoter. All DNA sequencing analyses, vector mapping, and construction, and sequence alignments were performed with Vector NTI 9. (Informax).

Isolation of total RNA and quantitative RT-PCR analysis

Total RNA was extracted using RNeasy mini prep system (QIAGEN, Valencia, CA) following the manufacturer's procedure. RT-PCR analysis was performed in two steps by initial reaction with Superscript II (Life Technologies, Rockville, MD) reverse transcriptase.

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Subsequently, PCR with SYBR green PCR Master Mix (Applied Biosystems, MD) was performed with gene specific primers using standard curves and relative quantitation methods. For the RT reaction, 200 ng of total RNA was combined with 2 μ l (40 units) RNase Inhibitor (Perkin Elmer, Boston, MA), 1X First Strand Buffer (final), 10 mM DTT (final concentration), 0.5 mM dATP, dTTP, dGTP, and dCTP (each, final), and 1 μ l (200 units) of Superscript II to total volume of 20 μ l, and incubated at 42°C for 50 min, then inactivated at 70°C for 15 min, and stored at -20°C or immediately used in PCR analysis. PCR was performed on an ABI 7900HT using the standard curves method of relative quantitation. Briefly, pooled RT reaction products from 3 μ l, 0.3 μ l, 0.03 μ l, and 0.003 μ l were run for each target gene and endogenous control gene primer set, and threshold temperatures were determined. Samples were amplified and quantitated via standard curves for each gene. Relative concentrations were normalized for endogenous control gene content and each sample was calibrated to the vehicle control (DMSO). Specific primers for *CYP2C8* (T570F 5'-AGATCAGAATTTTCTCACCC-3' and T730R 5'-AACTTCGTGTAAGAGCAACA-3'), *CYP2C9* (65.815F 5'-AGGAAAAGCACAACCAACCA-3' and 65.903R 5'-TCTCAGGGTTGTGCTTGTC-3'), and β -actin (actinF 5'-GAGCTATGAGCTGCCTGACG-3' and actinR 5'-CACTTGCGGTGCACGATG) for 45 cycles at annealing temperatures of 50°C, 53°C, and 60°C, respectively. Standard error calculations are shown in figures, and *p*-values are indicated via asterisks calculated via ANOVA analysis and student's *t*-test.

Cloning of *CYP2C8* promoter sequences and nuclear receptors

CYP2C8 5' flanking regions were cloned by PCR amplification of BAC clone DNA RP11-63F3 (Children's Hospital Oakland Research Institute, CA). 2C8-2.5kb was amplified

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with 2C8-2527F-NheI_F (5'-GCTAGCAATGCTAATACTCTTGATC-3'), and 2C8-1bpHindIII_R (5'-AAGCTTTGAAGCCTTCTTCTTATTAAG-3'), subcloned into pCR2.1 vector, sequenced via Big Dye Terminator Cycle Sequencing method (Applied Biosystems, MD), and cloned into pGL3-Basic luciferase reporter vector (Promega, Madison WI) via NheI and HindIII restriction sites. 2C8-3kb luciferase reporter construct was prepared by amplification of BAC clone DNA with primers 2C8-2966bpMluI_F (5'-ACGCGTAACTAAAGTGAAGTGTGG-3') and 2C8-2461bp_R (5'-AATCCCAGTTTTCTGCTTGGTG-3'), subcloning into pCR2.1, sequencing, and final cloning via MluI and an endogenous EcoRI site within the 2C8-2.5kb luciferase reporter described above. 2C9-3kb#1 was constructed and described previously (Chen et al., 2004). 2C8-(-8.9to-8.5)-tk-Luc was constructed by amplification of a 417 bp region flanking the putative CAR/PXR binding site located at -8,806 bp from the translation start site with 2C8-8888SacI-F (5'-GAGCTCTAGCCATACTAATATCAGAC-3') and 2C8-8471PacIR (5'-TTAATTAATAGAAACATTGTCTCAGTATGT-3') primers, subcloning, and subsequent digestion with SacI to insert upstream of the pGL3-tk reporter construct (Promega, Madison WI). This region was subsequently inserted upstream of the 2C8-2.5kb and 2C8-3kb constructs to make 2C8-(-8.9to-8.5)-2.5kb and 2C8-3kb (2C8-(-8.9to-8.5)-3kb), respectively at the SacI sites of each vector. Construction of 2C8-300bp was analogous to 2C8-2.5kb but using 2C8-301bpNheI_F (5'-GCTAGCAGTGTTTCTCCATCATC-3') primer. Mutation of the putative CAR/PXR binding site at -8806 was performed via the QuikChange method (Stratagene, La Jolla, CA) using primers Qchange2C8-8806F (5'-GAAGAAACAAATAAGTCAAGGTTGAGGACCCCATTTAATGATAAT-3') and Qchange2C8-8806R (5'-

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ATTATCATTAATGGGGTCCTCAACCTTGACTTATTTGTTTCTTC-3') primers.

Analogous mutations were performed to make 2C8-(-8.9to-8.5)-3kbmut8806 which were verified by DNA sequencing. Mutation of the putative CAR/PXR binding site at -2796bp was generated with Qchange2C8-2796_F (5'-

CAAACAGTAAACCCTATGGACACTTCGAACCTTGGTTG-3') and Qchange2C8-2796_R

(5'-CAACCAAAGTTCGAAGTGTCCATAGGGTTTACTGTTTG-3') primers to make 2C8-(-

8.9to-8.5)-3kbmut2796 and 2C8-(8.9to-8.5)-3kbboublemut which was verified by DNA

sequencing. Mutation of the putative GRE at -1927bp of the *CYP2C8* promoter was performed via the Quikchange method using primers: Qchange2C8-GREmut_F

(CAATCTTGGTGGCCCGGTTTCCCTGGATGTTTTGCTTAAAAGG-3') and Qchange2C8-

GREmut_R (5'-CCTTTTAAGCAAAACATCCAGGGAAACCGGGCCACCAAGATTG-3') to

make 2C8-2.5kb-GREmut. Mutation of the putative HNF4 α binding site at -154bp was

accomplished by amplifying with mutant PCR primers 2C8-mutPCR-HNF4 α -NcoI-154bp_F

(5'-CCATGGGCGTTTCACCACTCAGAAAAAAGTAT-3') and 2C8-1bpHindIII_R, and

subcloning and cloning this insert to make 2C8-300bpmutHNF4#5 and 2C8-2.5kbmutHNF4 α .

Mammalian expression vectors pCR3.0-hCAR, pCR3.1-GR, and pSG5-hPXR(atg) were

described previously (Ferguson et al., 2002), (Chen et al., 2003). pCR3.0-HNF4 α was

constructed by amplification of cDNA with gene specific primers (forward primer: 5'-

CTCGTCGACATGGACATGGCCGACTAC3' and reverse primer: 5'-

GGCTTGCTAGATAACTTCCTGCTTGGT 3' and subcloned into pCR2.1 for sequence

verification. This insert was then cloned into pCR3.0 via HindIII and XbaI enzymes and DNA

sequencing confirmed the final mammalian HNF4 α expression vector.

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Cell Culture and Transfections

HepG2 and HeLa (obtained from ATCC) cells were cultured in Eagle's Minimal Essential Medium with 31mg/L penicillin and 50 mg/L streptomycin and supplemented with 10% fetal bovine serum. Primary human hepatocytes were purchased from CellzDirect (Pittsboro, NC). Primary hepatocytes were maintained in William's E Medium with 31mg/L penicillin and 50 mg/L streptomycin without phenol red, and supplemented with ITS (Collaborative Biomedical Products, Bedford, MA) on collagen coated plates (Biocoat, Fort Washington, PA). Transfections were performed with Effectene transfection reagent (QIAGEN, Valencia, CA) using manufacturers' procedures and conditions were optimized for either HepG2/HeLa cells or primary human hepatocytes. All cells for transfection experiments were plated in 24-well format, transfected for 12-18 hours, and fresh media was applied prior to drug treatments. All transfected plasmids were prepared via QIAGEN Maxi-prep methods (Valencia, CA), quantitated, and diluted to 100 ng/ μ l prior to transfection. Cells were subsequently lysed with 100 μ L passive lysis buffer (Promega, Madison WI) for 0.5 hours at room temperature with gentle rocking. Dual Luciferase Assays (Promega, Madison WI) were then performed on cell lysates per manufacture's procedure.

Gel Mobility Shift Assays

Electrophoretic mobility shift assays (EMSA) were performed as described previously (Ferguson et al., 2002). Briefly, human HNF4 α , human RXR, human CAR, and human PXR were synthesized in vitro using the TNT Quick Coupled In Vitro Transcription/Translation system (Promega, Madison WI) following the manufacturer's protocol. Probes were labeled with γ -³²P-dCTP purified by Microspin G-25 columns (Amersham, Piscataway, NJ). Labeled

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probe (150,000 cpm) was applied to each binding reaction in 10 mM HEPES (pH=7.6), 0.5 mM DTT, 15% glycerol, 0.05% Nonidet P-40, 50 mM NaCl, 1 μ g poly(dI-dC), and 1-2 μ L of *in vitro* transcribed/translated proteins to a final reaction volume of 10 μ L. The reactions were incubated at room temperature for 20 minutes, then loaded on 5% acrylamide gels in TBE buffer, dried, and exposed to film for 6-18 hours at -70°C . Wild type and mutant cold competitors were generated by denaturing and annealing unlabeled primers and applied to binding reactions at the indicated concentrations. Antibodies to RXR α and HNF4 α were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA).

Statistical Analyses

All error bars indicate standard error measurements. P values for each experimental comparison were determined using the Analysis of Variance (ANOVA) and student's t-test. For comparison, all *p*-values were determined relative to the vehicle or untreated controls.

Results

***CYP2C8* induction in primary human hepatocytes**

To confirm that *CYP2C8* mRNA was induced by drugs in our hands, we first treated primary human hepatocytes with 10 μ M rifampicin and 500 μ M phenobarbital for 48 hours, isolated RNA, and performed quantitative RT-PCR with gene specific primers (Fig. 1A). We observed an 8-fold induction of *CYP2C8* mRNA by rifampicin, and a more modest 2-fold activation ($p=0.15$) by phenobarbital in hepatocytes from this donor. *CYP2C9* was also induced 3-fold by rifampicin ($p<0.001$); however, a modest induction by phenobarbital was not statistically significant. In Fig. 1B, an analogous experiment was performed using rifampicin and 10 μ M paclitaxel, which is a potent anticancer drug and substrate for *CYP2C8* and a ligand for PXR. In this donor, both rifampicin and paclitaxel induced *CYP2C8* expression, consistent with previous reports in primary human hepatocytes (Gerbai-Chaloin et al., 2001; Raucy et al., 2002).

Fig. 1C contains quantitative RT-PCR data from *CYP2C8* mRNA in a third set of primary human hepatocytes treated with various inducers for 24 hours. Here, PXR-type ligands such as rifampicin produced a 4-fold induction of *CYP2C8* expression. Hyperforin (active component of the antidepressant herbal remedy St. John's Wort) has been shown to mediate induction of *CYP3A4* and *CYP2C9* via the nuclear receptor PXR (Moore et al., 2000), (Chen et al., 2004). Here, we show that treatment of primary human hepatocytes with 400 nM hyperforin for 24 hours induces *CYP2C8* mRNA accumulation 5-fold relative to DMSO control samples, and comparable to the 4.4-fold induction observed with rifampicin. Another reported PXR ligand, lithocholic acid, produced a marginal but statistically significant induction of *CYP2C8* mRNA at a concentration of 50 μ M. In addition to prototypical PXR ligands, we also examined

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induction by two reported CAR agonists, phenytoin and the recently reported human CAR specific ligand CITCO (Maglich et al., 2003). Both phenytoin and CITCO induced *CYP2C8* expression in hepatocytes from this patient, indicating that CAR may also regulate *CYP2C8*. Treatment with 10 μ M dexamethasone weakly (< 2-fold) increased *CYP2C8* expression in primary human hepatocytes under the experimental conditions used. From these data, we confirm the reports of others that rifampicin, phenobarbital, and paclitaxel can induce *CYP2C8* expression in primary human hepatocytes. In addition, we show for the first time that human CAR-type agonists phenytoin and CITCO induce *CYP2C8* expression, while the herbal ingredient hyperforin strongly induces *CYP2C8* mRNA accumulation similar to rifampicin.

What factors may regulate *CYP2C8* expression?

To explore the mechanisms of induction, we examined the 5'-flanking region of the *CYP2C8* gene. We identified a number of putative transcription factor binding sites within the *CYP2C8* proximal 2,000 bp 5' flanking sequence. *CYP2C8* contains several TATA boxes in this segment. Beaune and coworkers published 2,477 bp of 5' flanking sequence, suggested several putative glucocorticoid receptor response elements, and reported that the transcription start site for *CYP2C8* resides at -23bp relative to the translation start site (Ged and Beaune, 1991). This observation is consistent with initiation at the most proximal TATA box at -56 to -51 bp. However, a *CYP2C8* cDNA sequence from human liver deposited in Genbank (Accession Number: BC020596) contains 95 bp of 5'-flanking sequence that is consistent with transcription initiation from the more distal TATA box at -126 to -121 bp (Strausberg et al., 2002). Thus, it is possible that both TATA boxes are functional in liver. We also observed a putative HNF4 α binding site at -154 bp that has previously been observed, (Cairns et al., 1996);

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however, this site has not been characterized to date. Putative sites for Hepatic Nuclear Factor-5 (-1130 bp, -1352 bp), HNF-6 (-1508 bp, -1945 bp), and HNF-3 (-1042 bp, -1132 bp, 1341 bp) were also found along with more general transcription factor sites like the GATA-binding protein (multiple sites), and two CCAAT Enhancer binding protein (C/EBP) sites (-1007 bp, previously noted by Klose et al. (Klose et al., 1999) and an additional site at -185 bp). All of these sites are upstream of the transcription start site and may function as enhancers or in the basal transcription machinery. HNF3 has recently been shown to activate transcription of all of the CYP2C promoters (Bort et al., 2004). Interestingly, we observed that a site at -2056 bp in *CYP2C8* was homologous to a CAR-RE site identified for *CYP2C9* at -1836 bp (Chen et al., 2004; Gerbal-Chaloin et al., 2002). However, the site in *CYP2C8* did not match our previously described consensus CAR/PXR binding site search motif (Ferguson et al., 2002). We also found that *CYP2C8* contained a putative glucocorticoid receptor response element at -1927 bp that is homologous to the GRE identified for *CYP2C9*.

CYP2C8 induction mechanism distinct from CYP2C9/19

Due to the inducibility of *CYP2C8* by rifampicin and phenobarbital, and the clear evidence for PXR and CAR in the regulation of *CYP2C9* via a proximal CAR-RE, we first tested whether the homologous element within the *CYP2C8* 5'-flanking region may be responsible for the rifampicin induction through PXR. Thus, we examined the ability of CAR/PXR to bind to these homologous elements in gel shift assays. Fig. 2 shows that *in vitro* transcribed/translated CAR and PXR, in the presence of the retinoid X receptor (RXR), binds strongly to the CAR-RE at -1836 bp of the *CYP2C9* positive control, while the homologous *CYP2C8* element at -2056 bp does not significantly bind to either CAR or PXR proteins in the

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same experiment. These data indicate that this site is not capable of binding CAR/PXR, and is likely not responsible for CAR/PXR mediated drug induction of *CYP2C8*.

We further tested whether elements located in the proximal 3kb of the *CYP2C8* 5'-flanking region could be activated by CAR or mediate rifampicin induction via PXR in gene reporter assays. Luciferase reporter constructs for *CYP2C9* and *CYP2C8* were transfected into HepG2 cells and cotransfected with mammalian expression vectors for human CAR or PXR. Fig. 3 shows that while *CYP2C9*-3kb reporter constructs were significantly activated by human CAR, and induced by rifampicin in the presence of human PXR in HepG2 cells, the *CYP2C8*-3kb reporter construct was not activated by either receptor. These data are consistent with the lack of CAR/PXR binding to the *CYP2C8* element at -2056 bp.

Identification of a putative far-upstream CAR/PXR binding site within the *CYP2C8* promoter

Due to the absence of CAR/PXR activation within the proximal 3kb of the *CYP2C8* promoter, and the clear induction of *CYP2C8* by CAR/PXR agonists, we obtained genomic DNA sequence from the Celera Discovery database and searched for CAR/PXR binding sites further upstream using the Findpatterns tool in the GCG software suite. In the case of *CYP3A4*, a DR-3 element at -7,733 bp was shown to play a major role in PXR mediated drug induction. We discovered that *CYP2C8* contains a perfect match to our consensus DR-4 CAR/PXR binding sequence in the far upstream of the *CYP2C8* promoter at -8,806 bp (Fig. 4). This element is nearly identical to the *CYP3A4* element at -7,733 bp, and similar to the *CYP2C9* CAR-RE element at -1,839 bp. Moreover, this element bound CAR and PXR in gel shift assays (Fig. 4). From this representative assay, it is clear that both CAR and PXR, in the presence of RXR, are

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able to bind both oligonucleotides from the *CYP2C9* element (positive control) and the newly discovered *CYP2C8* element located at –8,806 bp. These binding interactions were specifically decreased by competition with 20X cold competitor double stranded oligonucleotides.

In addition to the CAR-binding site at –8,806 bp, we also identified a putative DR-5 element at –2,796 bp (Fig. 5). This site is homologous with the DR-5 identified in the *CYP2C9* promoter, but differs by two nucleotides from *CYP2C9* making the *CYP2C8* sequence a better match to our consensus CAR/PXR binding sites. Consistent with this observation, CAR/RXR efficiently bound this *CYP2C8* element in gel shift assays, 10X cold competitor (Fig. 5) decreased the intensity of this complex, while 100X cold competitor essentially eliminated the band (data not shown). Treatments with anti-RXR antibodies demonstrated a clear supershifted band. Although the apparent expression/binding of PXR with RXR to the positive control was much lower in this particular experiment than that of CAR/RXR, PXR/RXR bound equally well to our positive control element and the –2,796 bp element, demonstrating that this element can bind both CAR and PXR. However, because of the absence of activation/induction of the 2C8-3kb luciferase reporter as shown in Fig. 3, the possible role of this binding site in *CYP2C8* regulation by CAR and PXR remains unclear.

Rifampicin induction for *CYP2C8* is observed in primary human hepatocytes, but not HepG2 cells

Subsequent to the identification of the CAR/PXR binding site at –8,806, and positive gel shift data for both CAR and PXR, we cloned a 400bp region flanking this sequence and inserted it upstream of the *CYP2C8*-2.5kb reporter construct. The proximal *CYP2C8*-2.5kb reporter is not responsive to CAR/PXR in either HepG2 cells (data not shown) or primary human

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hepatocytes (Fig. 6B). This new construct (2C8-(-8.9to-8.5)-2.5kb) was transfected into HepG2 cells along with CAR and PXR expression plasmids. Surprisingly, while the *CYP2C9* control reporters were activated by both CAR and PXR-rifampicin, no rifampicin induction was observed with the 2C8-(-8.9to-8.5)-2.5kb construct in HepG2 cells (Fig. 6A). A modest 2.5-fold activation was observed with this reporter by the addition of CAR expression plasmids, but this was much lower than that observed for the *CYP2C9* positive control.

Since *CYP2C8* is induced in primary human hepatocytes by phenobarbital, rifampicin, and other CAR/PXR activators, we then transfected fresh primary human hepatocytes with this *CYP2C8* reporter construct and control reporters in the presence of either 0.2% DMSO vehicle, or 10 μ M rifampicin (Fig. 6B). These data show that although the *CYP2C8*-2.5kb construct remains non-responsive to rifampicin treatment, insertion of the 400 bp distal region within 2C8-(-8.9to-8.5)-2.5kb confers rifampicin inducibility to the *CYP2C8* reporter in primary hepatocytes. These data indicate that HepG2 cells may be insufficient to support *CYP2C8* induction via CAR/PXR. However, in primary human hepatocytes, which are the best current model system for human liver, we observe both induction of endogenous mRNA by rifampicin and increased transcription with *CYP2C8* reporter constructs containing the distal element.

Induction within a heterologous thymidine kinase reporter shows the element at -8,806bp is essential for rifampicin induction

To further test the inducibility of this distal region of the *CYP2C8* promoter, it was cloned upstream of the heterologous thymidine kinase promoter to make 2C8-8.9to-8.5-tk. We transfected this construct into both HepG2 cells (no response to CAR/PXR analogous to the lack of response observed upstream of the 2C8-2.5kb reporter, data not shown) and primary human

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hepatocytes (Fig. 7). Rifampicin induced transcription of this construct in primary human hepatocytes in a manner similar to the induction seen when the distal region was present upstream of the natural *CYP2C8* promoter (Fig. 6B). Furthermore, mutation of the CAR/PXR binding site at –8,806 bp abolished this induction, indicating that this DR-4 element is essential for the rifampicin induction response within this heterologous promoter reporter construct.

Both CAR and PXR agonists induce *CYP2C8* reporter constructs in primary hepatocytes

To further address the *CYP2C8* induction mechanism in primary human hepatocytes, we transfected various *CYP2C8* promoter reporter constructs into primary cells and treated with either 0.2% DMSO, 10 μ M rifampicin, or 400 nM CITCO (Fig. 8). We show for the first time that a human CAR agonist (CITCO) is effective in activating a *CYP2C9* reporter constructs (3.7-fold). A 5-fold induction response was seen with the PXR agonist rifampicin. No induction was observed for *CYP2C8* reporter constructs containing the proximal –3kb alone, or mutant of a putative CAR/PXR binding site at –2,796 bp. Consistent with previous experiments, 2C8-8.9to-8.5-3kb was activated by rifampicin (3.5-fold), while the hCAR specific agonist CITCO induced *CYP2C8* reporter constructs to an even greater extent (5.3-fold). These data demonstrate that both CAR and PXR specific agonists regulate *CYP2C8* transcription in primary human hepatocytes. Mutation of the CAR/PXR binding site at –8,806 bp (2C8-8.9to-8.5-3kbmut8806) completely abolished the induction response from both rifampicin and CITCO. Thus, this single element appears to be essential for the PXR mediated, and the CAR mediated induction of *CYP2C8* in primary hepatocytes. Mutation of the putative proximal element at –2,796 alone had no effect on rifampicin or CITCO activation of the 2C8-8.9-to-8.5-3kb reporter construct, while mutation of both the –8,806 distal and –2,796 proximal elements

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(doublemut) abolished the drug induction responses. In total, these data demonstrate that the CAR/PXR binding site at $-8,806$ bp is required for both rifampicin and CITCO induction, and that no additional responsive elements in the proximal -3 kb of the *CYP2C8* promoter appear to contribute to the CAR or PXR-mediated induction of *CYP2C8*.

Dexamethasone induction of *CYP2C8* is regulated via a glucocorticoid responsive element

Previously, Maurel and coworkers demonstrated that dexamethasone induced *CYP2C8* mRNA accumulation in primary human hepatocytes (Gerbal-Chaloin et al., 2001). In the present study, we identified a putative GRE at -1927 bp in the *CYP2C8* promoter that is homologous with the GREs identified in the *CYP2C9* (Gerbal-Chaloin et al., 2002) and *CYP2C19* (Chen et al., 2003) genes (Fig. 9). In both HepG2 and HeLa cells transfected with glucocorticoid receptor (GR) mammalian expression plasmid, the synthetic glucocorticoid dexamethasone induced *CYP2C8* reporter constructs. Mutation of the putative GRE-binding site (GRE-mut) resulted in a complete loss of dexamethasone induction indicating that this is the essential site for induction of *CYP2C8* by glucocorticoids. In total, these data indicate that unlike CAR and PXR agonists, dexamethasone, a GR specific ligand, is capable of inducing *CYP2C8* gene reporter constructs in HepG2 cells, and the response in HeLa cells may suggest that glucocorticoids may also regulate *CYP2C8* expression in extrahepatic tissues.

HNF4 α regulation of *CYP2C8* via proximal binding site

CYP2Cs have previously been shown to be regulated by hepatic nuclear factor 4 alpha (HNF4 α), however no studies have been reported regarding demonstrating its involvement in the regulation of *CYP2C8*. We cloned the coding region of human HNF4 α into a mammalian

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expression vector to study its role in CYP2C regulation. Fig. 10A shows a gel shift assay with oligonucleotides from a putative HNF4-binding site at –154 bp from the translation start site that is homologous with an HNF-4 site identified previously for *CYP2C9* (Ibeanu and Goldstein, 1995). These data show that this site is capable of strongly binding to *in vitro* transcribed/translated HNF4 α . This binding interaction is specifically inhibited by wild type cold competitor but is not inhibited by mutant cold competitor. In addition, anti-HNF4 α antibodies were capable of supershifting the HNF4 α complex.

Next we transfected HeLa cells and HepG2 cells with a *CYP2C8* luciferase reporter construct in the presence/absence of HNF4 α . Although exogenous HNF4 α did not appear to activate the *CYP2C8* promoter in HepG2 cells, this promoter was strongly activated by HNF4 α (29-fold) in HeLa cells (Fig. 10B). HNF4 α activation was greatly attenuated (85% decrease) by mutation of the element at –154 bp of the *CYP2C8* promoter. Since the empty pGL3-Basic control vector was activated slightly by HNF4 α in these assays, the observed 85% attenuation can be adjusted to ~93% by accounting for this non-specific activation of the luciferase vector backbone. Thus, the element at –154 bp of the *CYP2C8* promoter appears to account for the majority of HNF4 α mediated activation within this 300 bp region.

Discussion

In the present study, we have demonstrated functional roles for the nuclear receptors CAR, PXR, GR, and HNF4 α in the transcriptional regulation of *CYP2C8* expression, and identified specific regulatory elements within the 5'-flanking region that are essential for these cell-signaling pathways. A CAR/PXR binding site was identified at -8,806 bp (DR-4) that is essential for the activation of the *CYP2C8* promoter by both the PXR ligand rifampicin and the human CAR ligand CITCO (Maglich et al., 2003) in primary human hepatocytes. In addition, along with rifampicin and CITCO, other CAR/PXR agonists such as phenobarbital, phenytoin, hyperforin, and paclitaxel were also shown to induce *CYP2C8* mRNA, further supporting a role for these receptors in the regulation of *CYP2C8* expression by drugs. The glucocorticoid receptor was shown to mediate dexamethasone induction of *CYP2C8* reporter expression in both HepG2 and HeLa cells, and mutation of a single putative GR binding site (DR-4) at -1,927 bp resulted in a complete loss of dexamethasone induction. Finally, HNF4 α was shown to bind to the *CYP2C8* promoter at a DR-1 element in the proximal promoter region (-154 bp), and mutation of this element resulted in a marked decrease in the constitutive activation by HNF α of *CYP2C8* reporter activity in HeLa cells.

Examination of the *CYP2C8* basal promoter region revealed several putative binding sites that may be involved in *CYP2C8* transcription: TATA binding protein (TATA) (two sites as previously reported), hepatic nuclear factor-3 (HNF-3), CCAAT enhancer binding protein (c/EBP), hepatic nuclear factor 4 (HNF4 α), and GATA-binding protein. HNF3, a member of the FOXO family of transcription factors, has recently been shown to regulate *CYP2C8* expression in cell lines (Bort et al., 2004). Thus, many potential nuclear factors may be involved in controlling *CYP2C8* transcription in liver and extrahepatic tissues, and influence endogenous

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CYP2C8-mediated cellular metabolism. In the present study, we have shown that *CYP2C8* transcription can be regulated by several nuclear receptors, and future studies with some of these other factors may elucidate a more complete understanding of the signal transduction pathways that control *CYP2C8* expression.

Preliminary examination revealed that *CYP2C8* contains putative sites in the proximal 3kb promoter that are homologous to those shown to be essential for the regulation of *CYP2C9* by CAR and PXR agonists (Fig. 11). Surprisingly, although CAR and PXR agonists induced *CYP2C8* mRNA, the proximal 3kb region of *CYP2C8* did not appear to be transcriptionally regulated by drugs in either HepG2 cells transfected with PXR or in primary human hepatocytes. Close inspection of the proximal elements from *CYP2C8* and *CYP2C9*, and gel shift assays of these elements with CAR and PXR proteins revealed that two base differences between the *CYP2C9* and *CYP2C8* elements prevented CAR/PXR from binding to the proximal element from *CYP2C8* at -2,056 bp. The location and identity of the more distal half-site within this element appears to be critical for CAR/PXR binding. A more distal DR-5 element at -2,796 bp does bind CAR and PXR; however, it does not appear to participate in the transcriptional activation of the *CYP2C8* promoter in our experiments. Thus, no CAR/PXR responsive elements could be identified within the proximal 3,000 bp of the *CYP2C8* promoter, which is unique for the *CYP2C* promoters. It is possible that other sites are present between our distal region and the proximal 3kb of the *CYP2C8* promoter; however our software search techniques failed to identify any strong candidates.

Thus, unlike *CYP2C9*, which is primarily regulated by PXR through a proximal element at -1,839 bp, *CYP2C8* is regulated instead by a distal responsive element at -8.8 kb. This far upstream region from -8.9 to -8.5 kb was able to confer increased transcription after treatment

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of primary hepatocytes with both PXR and CAR agonists. This region contains several imperfect AGGTCA half-sites similar to the far upstream modules found for CYP3A4, MDR1, and OATP2 (Geick et al., 2001; Goodwin et al., 1999; Guo et al., 2002). This type of configuration is not unprecedented in CAR/PXR inducible genes. The rat OATP2 transporter is induced by the PXR ligand pregnenolone-16 α -carbonitrile via an element in the far-upstream (~8.7kb, and a small contribution from an element at -5.5 kb) yet does not appear to contain a proximal CAR/PXR binding site (Guo et al., 2002). Thus, the mechanism of induction of *CYP2C8* by CAR and PXR agonists is unique to this subfamily.

In humans, *CYP2C8* has also been shown to be the most profoundly induced isoform in response to inducers such as rifampicin and phenobarbital, and is also induced by the anticancer therapeutic and *CYP2C8* substrate paclitaxel, and the synthetic glucocorticoid dexamethasone (Raucy et al., 2002; Synold et al., 2001). Our data from primary human hepatocytes confirm these reports with rifampicin, paclitaxel, and phenobarbital, and expands the list of inducers to include hyperforin, phenytoin, and the CAR agonist CITCO (Maglich et al., 2003). These drugs are known to induce transcription of other P450s such as CYP3A and CYP2B, as well as drug transporters such as P-glycoprotein through activation of the nuclear receptors CAR and PXR. This is the first study to show that *CYP2C8* and *CYP2C9* are inducible by both CAR and PXR agonists. Moreover, the distal element of *CYP2C8* confers inducibility with response to both types of agonists.

CYP2C8, like other human CYP2Cs is also inducible by the synthetic glucocorticoid dexamethasone. Although we did not observe dexamethasone induction in our culture system with primary human hepatocytes, this probably is due to the 100 nM dexamethasone media supplement used to support maximal induction responsiveness to rifampicin/phenobarbital

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masking the response. We did, however, observe dexamethasone induction when *CYP2C8* reporter constructs were cotransfected with human glucocorticoid receptor expression plasmids. This induction was mediated solely by a single site at -1,927 bp indicating that several other putative GR sites (Ged and Beaune, 1991) are probably not functional. The mechanism of GR induction of *CYP2C8* appears to be identical to that observed for *CYP2C9* and *CYP2C19*, and this conservation throughout the subfamily suggests that a selective pressure may maintain glucocorticoid inducibility in humans. *In vivo*, glucocorticoids are primarily known to decrease inflammatory response and stimulate apoptosis, and knockout mice have proven that GR is essential for life (Cole et al., 1995). Interestingly, the *CYP2C* substrate arachidonic acid is also known as a pro-inflammatory endogenous precursor chemical when metabolized by the cyclooxygenase enzymes into prostaglandins (Turini and DuBois, 2002). Thus, it is reasonable to suggest that the glucocorticoid induction of the *CYP2Cs* may also play a role in inflammation and/or apoptosis through perturbation of arachidonic acid metabolism in certain tissues. It is also noteworthy that the observed GR/dexamethasone induction response of *CYP2C8* is also observed within the proximal 2.5kb of the promoter, indicating that the lack of induction response with CAR/PXR in this region in HepG2 cells is not likely due to a general lack of promoter function or enhancibility within this region.

HNF4 α was also identified as a regulator of *CYP2C8* transcription in these studies. We show that HNF4 α activation occurs in the basal promoter region primarily through an element at -154 bp that is homologous with the site identified in the *CYP2C9* promoter. Although HNF4 α is well known as a hepatic transcription factor, it is also expressed in kidney, intestine, stomach, skin, as well as cell lines from the pancreas in mammals. HNF4 α has also been shown to play an important role in the basal expression of many genes, including *CYP2C9* (Ibeanu and

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Goldstein, 1995). In addition to its role in constitutive expression in these tissues, a recent report by Kim and coworkers showed that HNF4 α plays an essential role in the drug induction of *CYP3A4* (Tirona et al., 2003), and an HNF4 α binding site was identified within the far-upstream XREM sequence. One interesting observation in this study is that while HepG2 cells support both induction of *CYP2C9* by rifampicin and activation by HNF4 α , neither of these responses for *CYP2C8* could be elicited in HepG2 cells. Only primary human hepatocytes were capable of supporting induction by CAR/PXR agonists. This coupled with the lack of HNF4 α activation of the *CYP2C8* promoter in HepG2 cells suggests that factors specifically necessary for *CYP2C8* mediated induction may be poorly expressed in the HepG2 cell model. However, we observe a robust activation of *CYP2C8* reporters HNF4 α in HeLa cells that is comparable to the response with *CYP2C9* gene reporters, yet HeLa cells do not support CAR/PXR induction. Thus, the HepG2 cell system is not a good surrogate for studying CAR/PXR or HNF4 α activation of *CYP2C8*.

In conclusion, we have identified CAR, PXR, GR, and HNF4 α as key regulators of *CYP2C8* transcription. Through these studies we have identified specific elements that are essential for transcriptional activation by these receptors, and unraveling these cellular signaling pathways will help better understand the physiological role of the CYP2Cs and the factors that control their inducibility and contribute to the variability observed in humans. In addition, this is the first clear evidence that the human CYP2Cs are transcriptionally activated by CAR agonists as well as PXR agonists.

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Acknowledgments

We would like to thank Dr. Hongbing Wang (University of North Carolina, Chapel Hill, NC) for his assistance with primary human hepatocytes, and Joyce Blaisdell and Dr. Tatsu Sueyoshi (Laboratory of Reproductive and Developmental Toxicology, NIEHS, RTP, NC) for helpful discussions in the preparation of this manuscript.

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Footnotes

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Legends for Figures

Fig. 1. Quantitative RT-PCR of CYP2C8 and CYP2C9 mRNA in primary human hepatocytes. Primary human hepatocytes were cultured in William's E media and treated with chemicals for 24 hours as described in Materials and Methods at the following concentrations: Dimethyl Sulfoxide (DMSO, 0.2%), rifampicin (10 μ M), phenobarbital (500 μ M), paclitaxel (10 μ M), CITCO (400 nM), phenytoin (50 μ M), hyperforin (400 nM), dexamethasone (Dex, 10 μ M), and lithocholic acid (Lith. Acid 50 μ M). Quantitative RT-PCR was performed with gene specific primers on an ABI 7900 instrument. Relative concentrations were calculated using standard curves method. Figures A, B, and C represent human hepatocytes isolated from three different donors. Each treatment group was analyzed in triplicate, and error bars indicate standard error for each treatment. * Indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$ as compared to vehicle (DMSO) control samples.

Fig. 2. Gel shift assay with 2C9 proximal CAR-RE and homologous 2C8 sequence. Electrophoretic mobility shift assays were performed with oligonucleotide probes labeled with 32 P and binding reactions were performed with in vitro transcribed/translated CAR/RXR or PXR/RXR proteins. The top panel shows a comparison of homologous elements from CYP2C8 and CYP2C9 with two mismatches in their half-sites. The left panel show the positive control CYP2C9 sequence known to bind and be activated by CAR and PXR/rifampicin, while the right panel shows the homologous sequence for CYP2C8 indicating that neither CAR and PXR nor their heterodimers with RXR are able to bind the putative CAR-RE CYP2C8 element at -2,056 bp. Competition for binding was done with 20X cold competitor oligonucleotides to show probe specificity. These data indicate that the element homologous to the principle element

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responsible for *CYP2C9* induction is not capable of efficiently binding CAR or PXR in the *CYP2C8* promoter.

Fig. 3. *CYP2C8* reporter construct is not activated by CAR or PXR/rifampicin in HepG2 cells. Luciferase constructs containing the proximal 3,000 bp of *CYP2C8* and *CYP2C9* 5'-flanking sequences were cloned upstream of the luciferase gene within the promoterless pGL3-Basic vector. 100 ng of the empty mammalian expression vector (pCR3.1), pCR3.0-human CAR or pSG5-human PXR were cotransfected where indicated with 100 ng of luciferase reporter and 10 ng of the pRL-TK internal control plasmid. Error bars indicate standard error calculations of three independent samples. * Indicates $p < 0.05$, ** indicates $p < 0.01$ relative to the pCR3.1 empty vector control.

Fig. 4. Gel Shift Assay with 2C8 DR-4 (-8,806 bp) CAR/PXR binding site. Electrophoretic mobility shift assays were performed with oligonucleotide probes labeled with ^{32}P and bound with *in vitro* transcribed/translated CAR/RXR or PXR/RXR proteins. The top panel shows a comparison of validated CAR/PXR binding sites from *CYP2C9*, *CYP3A4* far upstream, and our newly identified element at -8,806 bp with mismatches indicated by boxes. The gel shown below contains our newly identified *CYP2C8* element in the far upstream of the *CYP2C8* promoter showing strong binding to both CAR/RXR and PXR/RXR heterodimers. Competition for binding was done with 20 X cold competitor oligonucleotides to verify probe specificity.

Fig. 5. Gel Shift Assay with *CYP2C8* proximal DR-5 element at -2796 that does bind CAR and PXR heterodimers with RXR α . Electrophoretic mobility shift assays were performed with

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oligonucleotide probes labeled with ^{32}P and bound with *in vitro* transcribed/translated CAR/RXR or PXR/RXR proteins and supershifted with anti-RXR α antibodies. The top panel shows a comparison of these homologous elements from *CYP2C8*, *CYP2C9*, and *CYP2C19* (the homologous element from *CYP2C19* does not bind CAR/RXR or PXR/RXR), with boxes indicating sequence differences. This representative gel image shown below contains our positive control *CYP2C8* element at $-8,806$ (left) that typically binds both CAR/RXR and PXR/RXR with equal affinity (in this gel the PXR/RXR binding to this control was significantly weaker than CAR/RXR, but clearly observable). 10X cold competitor (cc) was applied in this experiment, however higher concentrations have been used in other experiments that completely abolish this interaction (data not shown). The DR-5 element at -2796 appears to bind both CAR/RXR and to a PXR/RXR, but slightly weaker than the positive control. Anti-RXR α antibodies (Santa Cruz) were used to supershift complexes and show the RXR α is present in the shifted complexes for both CAR and PXR heterodimers.

Fig. 6. Primary human hepatocytes support *CYP2C8* induction via the far upstream region. The proximal 2,500 bp of *CYP2C8* and 3,000 bp of *CYP2C9* 5'-flanking sequences were cloned upstream of the luciferase gene within the promoterless pGL3-Basic vector. The far-upstream region of *CYP2C8* from -8.9 to 8.5kb was inserted upstream of *CYP2C8*- 2.5kb to make *2C8*($-8.9\text{to}-8.5$)- 2.5kb . These reporters were transfected into HepG2 (A) or primary human hepatocytes (B). In HepG2 cells 100 ng of luciferase reporter and hPXR, empty pCR3.1, and hCAR mammalian expression plasmids were cotransfected at 100 ng each with 10 ng of the pRL-TK internal transfection control plasmid. 200 ng of luciferase reporters were transfected into primary human hepatocytes. Rifampicin concentrations were $10\ \mu\text{M}$, while all samples

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contained 0.2% DMSO. Error bars indicate standard error calculations of a minimum of three independent samples. * Indicates $p < 0.05$, ** indicates $p < 0.01$ relative to the pCR3.1 empty vector control.

Fig. 7. Primary human hepatocytes support *CYP2C8* induction via the far upstream region with a heterologous thymidine kinase promoter. Luciferase constructs containing the 400 bp region including the CAR/PXR binding site at $-8,806$ bp, and mutant construct at the $-8,806$ bp element (mutant generated indicated in top panel) upstream of the thymidine kinase promoter, along with control plasmids were transfected into primary human hepatocytes. 200 ng of each construct with 20 ng of the pRL-TK internal transfection control plasmid were. Rifampicin concentrations were $10 \mu\text{M}$, while all samples contained 0.2% DMSO for 24 hours after transfection. Error bars indicate standard error calculations of a minimum of three independent samples. * Indicates $p < 0.05$, relative to the DMSO vehicle controls.

Fig. 8. The far-upstream CAR/PXR binding site is essential for rifampicin and CITCO induction for *CYP2C8* reporter constructs in human hepatocytes. Luciferase constructs containing the proximal 3,000bp of the *CYP2C8* promoter, or both proximal element and far-upstream 400 bp region containing the CAR/PXR binding site at $-8,806$ bp, along with control plasmids were transfected into primary human hepatocytes. 200 ng of each construct with 20 ng of the pRL-TK internal transfection control plasmid were transfected using the Effectene method (QIAGEN). Drug treatments with rifampicin ($10 \mu\text{M}$) and CITCO (400 nM) were performed for 24 hours and compared with vehicle (0.2% DMSO) controls. Error bars indicate standard error

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calculations of a minimum of 3 independent samples. * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$ relative to respective DMSO vehicle controls.

Fig. 9. The glucocorticoid receptor mediates dexamethasone induction of *CYP2C8* via an essential DR-3 element in the proximal promoter region. Luciferase reporter constructs for *CYP2C8* containing 2.5 kb of the proximal promoter, and *CYP2C9* control reporters were transfected into HepG2 (A) and HeLa (B) cells along with our mutant GRE reporter in the presence of GR mammalian expression vectors or empty vector control (pCR3.1). The top left panel shows the GRE sites from *CYP2C8* and *CYP2C9* along with our mutated *CYP2C8* element (GRE-mut). 100 ng of luciferase reporters and human GR were transfected along with 10 ng of pRL-TK internal control plasmid. Cells were transfected for 16 hours, then treated with either 10 μ M dexamethasone or 0.2% DMSO vehicle and incubated another 36 hours before cell lysis. Error bars indicate standard error calculations of a minimum of 3 independent samples. ** Indicates $p < 0.01$, and *** indicates $p < 0.001$, relative to respective DMSO vehicle controls.

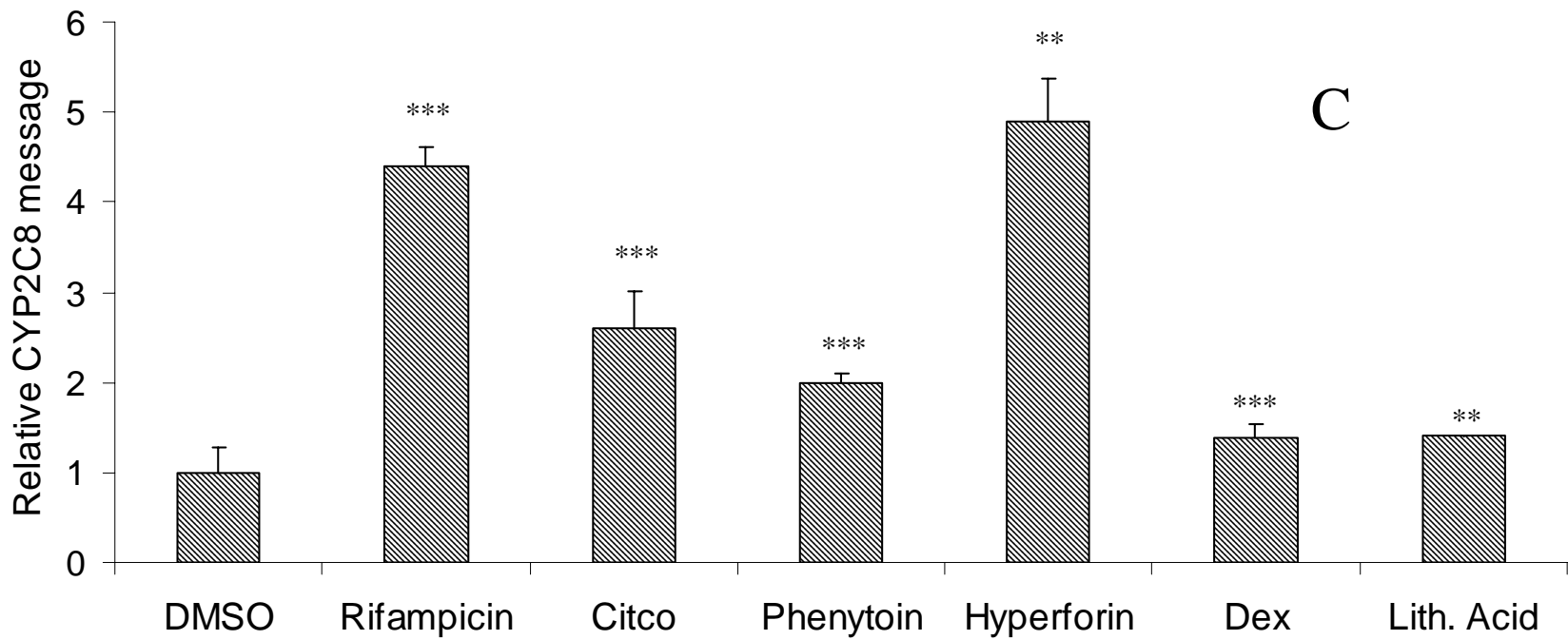
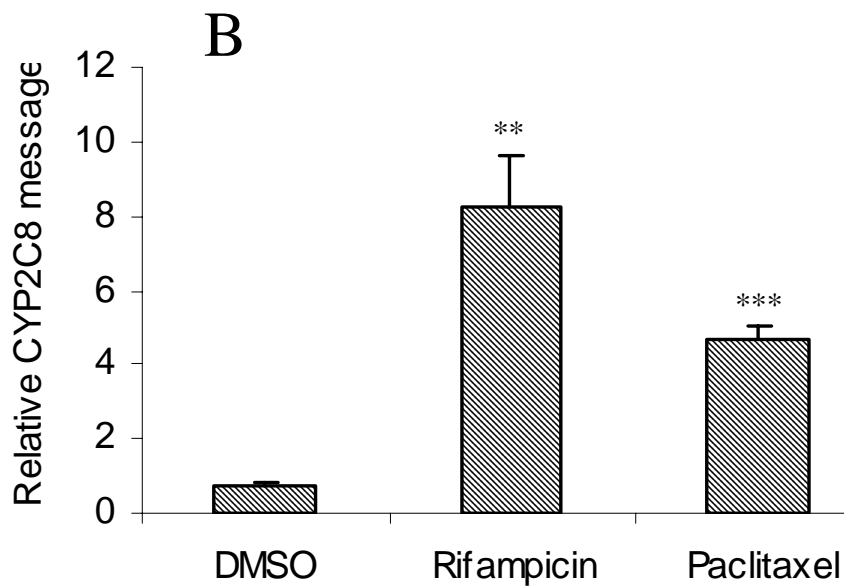
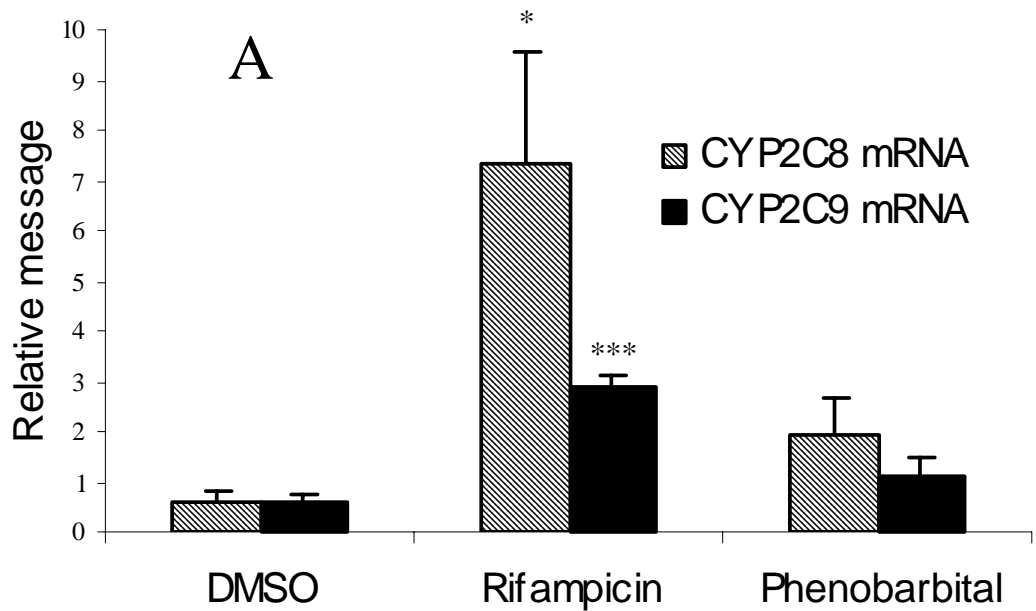
Fig. 10. Identification of a functional HNF4 α binding site in the basal promoter region of the *CYP2C8* promoter. (A) Gel shift assays were performed on a putative HNF4 α /HPF-1 binding site at -154 bp of the *CYP2C8* promoter with *in vitro* transcribed/translated HNF4 α . Wildtype and mutant cold competitors were used to show specificity of probe/protein binding, and anti-HNF4 α antibody (Santa Cruz) was used to verify HNF4 α presence in the complex. (B) Sequence comparison of the *CYP2C8* and *CYP2C9* promoters shows two differences between the HNF4 α binding site of *CYP2C9* and the putative site identified in *CYP2C8*. We mutated this element and transfected HeLa cells with 100 ng of luciferase reporter constructs containing

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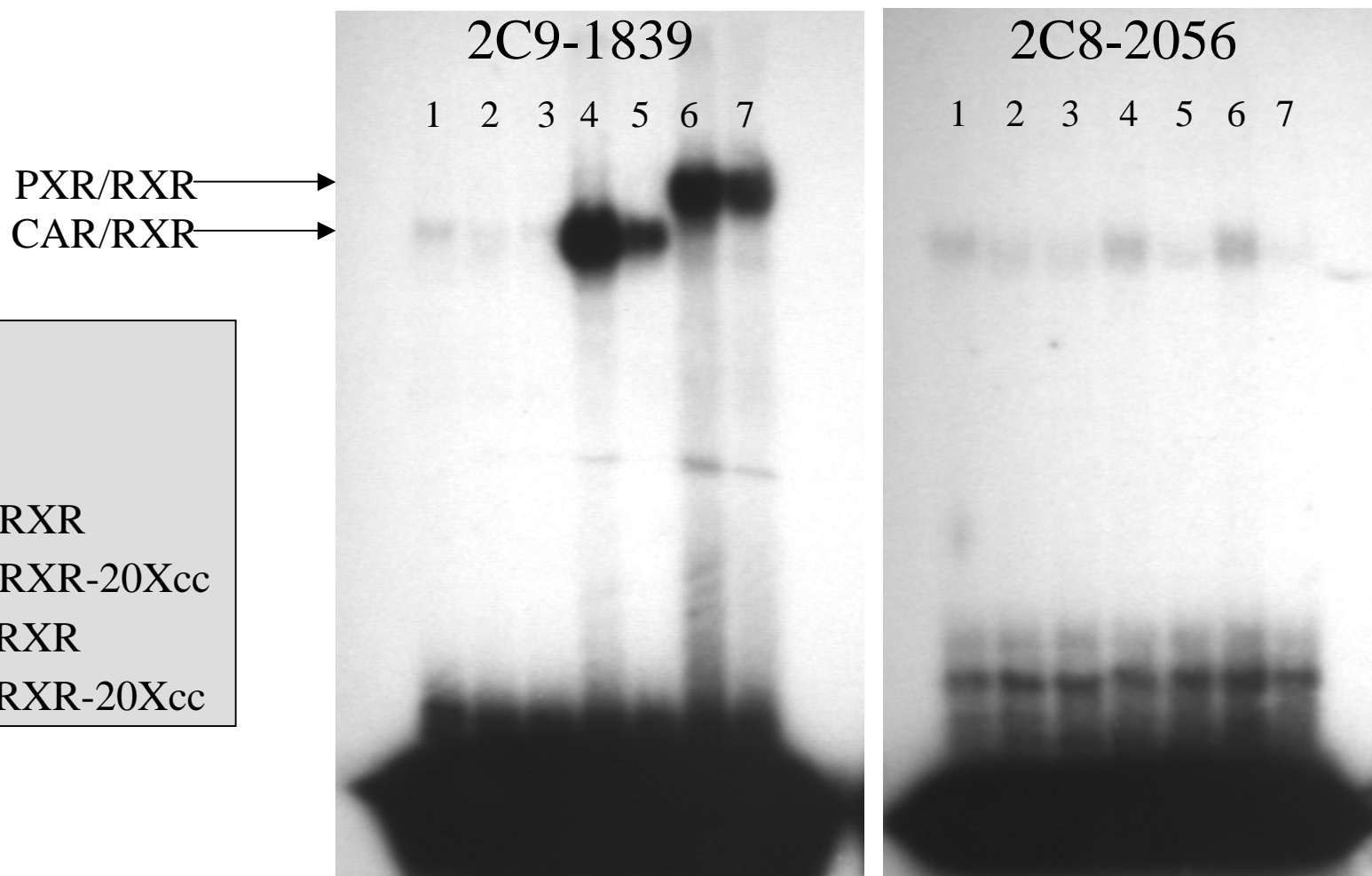
300 bp of *CYP2C8* 5'-flanking sequence along with control plasmids using 10 ng of the pRL-TK internal control plasmid. Error bars indicate standard error calculations of a minimum of three independent samples. ** Indicates $p < 0.01$, and *** indicates $p < 0.001$, relative to respective DMSO vehicle controls.

Fig. 11. Summary figure comparing known response elements for CAR, PXR, GR, and HNF4 α for the *CYP2C8* and *CYP2C9* genes. This figure summarizes our current view of the regulatory elements within the *CYP2C8* and *CYP2C9* promoters. The X indicates that although the element is homologous between these genes, this site in *CYP2C8* does not bind to CAR/PXR, while the ? indicates that although a second element in *CYP2C8* appears to bind CAR/PXR, it is not essential for the induction response to CAR and PXR agonists. All other elements shown on the promoter appear to be essential to the efficacy of the receptor's activation of gene reporters.

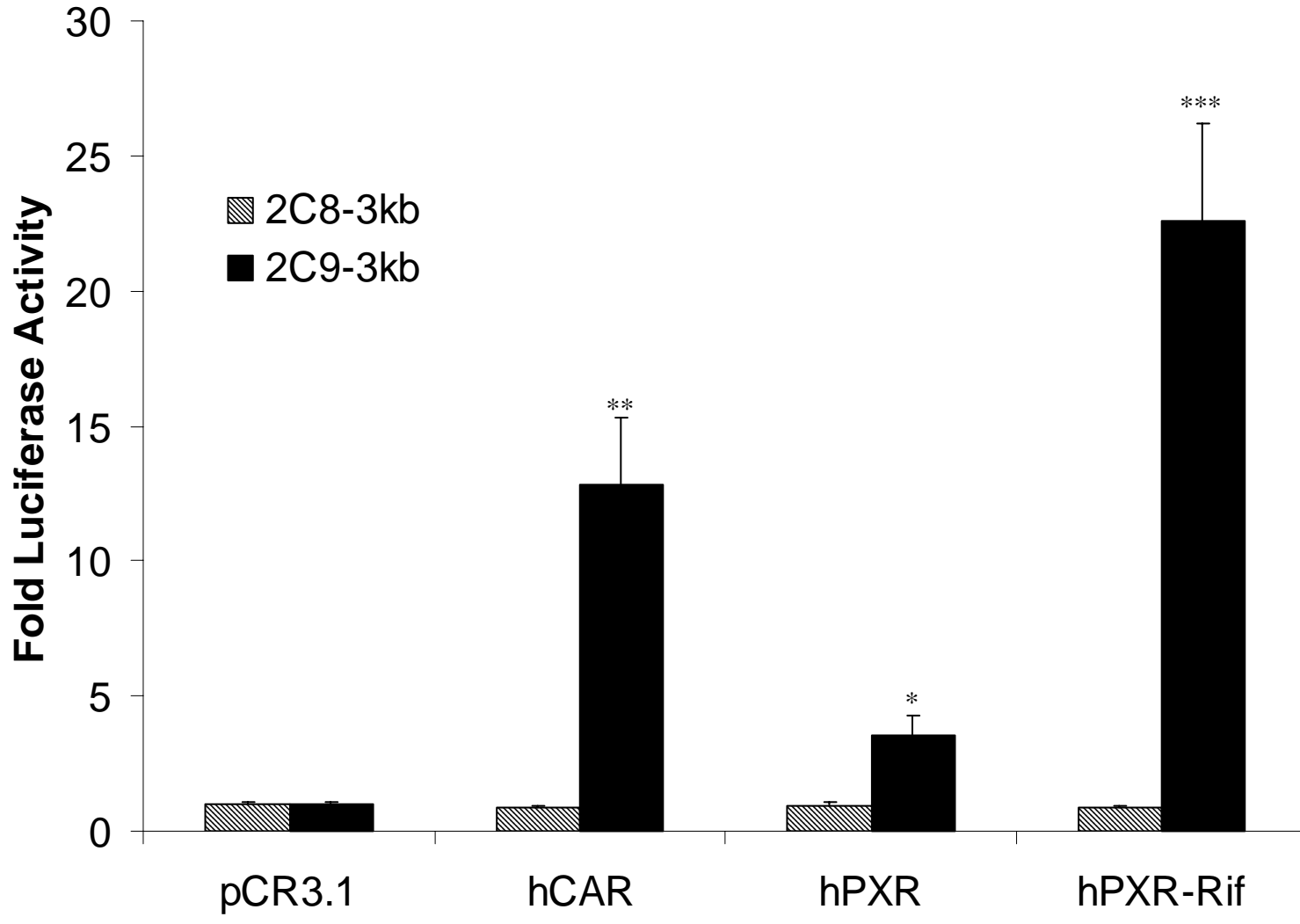
1



2C9-1839	aacCAAAC T cttcTGAC T ctc
2C8-2056	aacCAAAC A cgtcTGAC C aca



- | | |
|----|---------------|
| 1. | RXR |
| 2. | CAR |
| 3. | PXR |
| 4. | CAR/RXR |
| 5. | CAR/RXR-20Xcc |
| 6. | PXR/RXR |
| 7. | PXR/RXR-20Xcc |

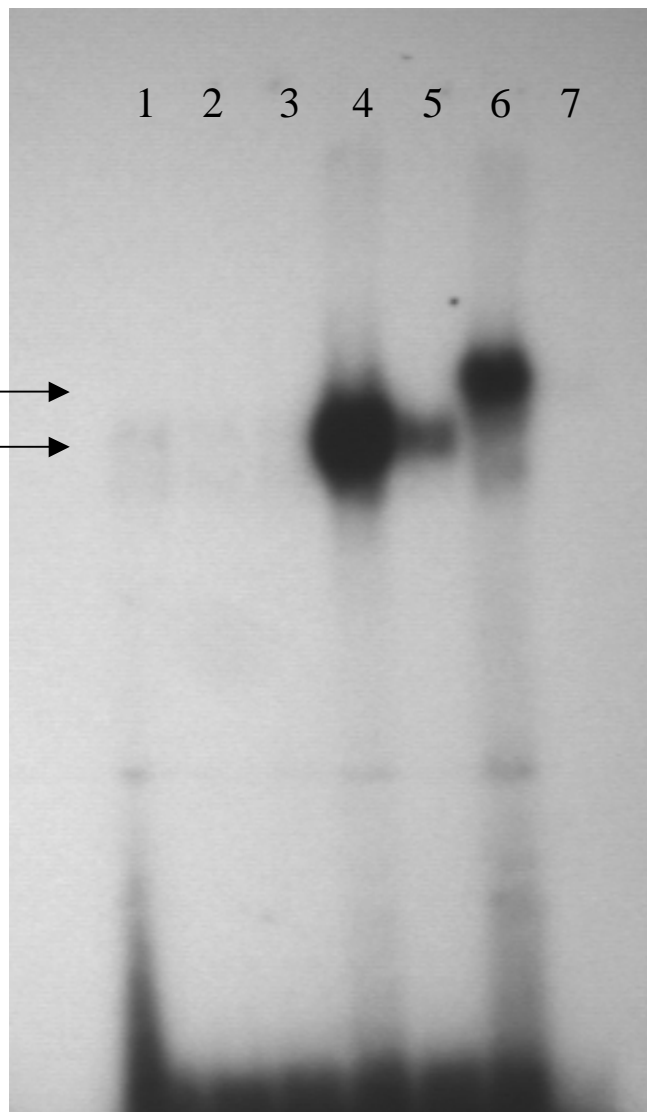


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2C8-8806	ag TC AACTttgaTGACC C ca
3A4-7733	aaTGAACTtgc.TGACC C tc

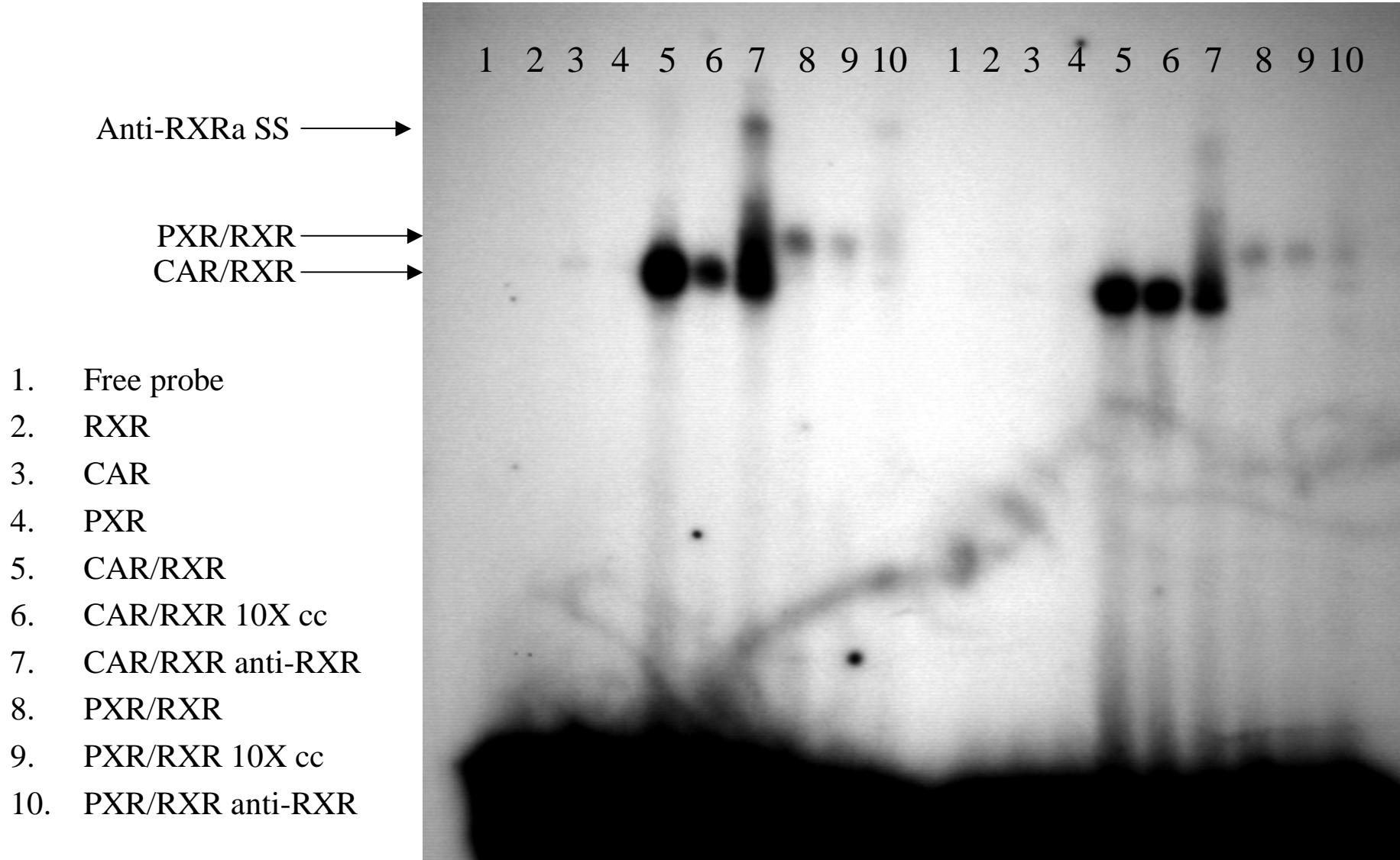
PXR/RXR →
CAR/RXR →

- | | |
|----|---------------|
| 1. | RXR |
| 2. | CAR |
| 3. | PXR |
| 4. | CAR/RXR |
| 5. | CAR/RXR-20Xcc |
| 6. | PXR/RXR |
| 7. | PXR/RXR-20Xcc |

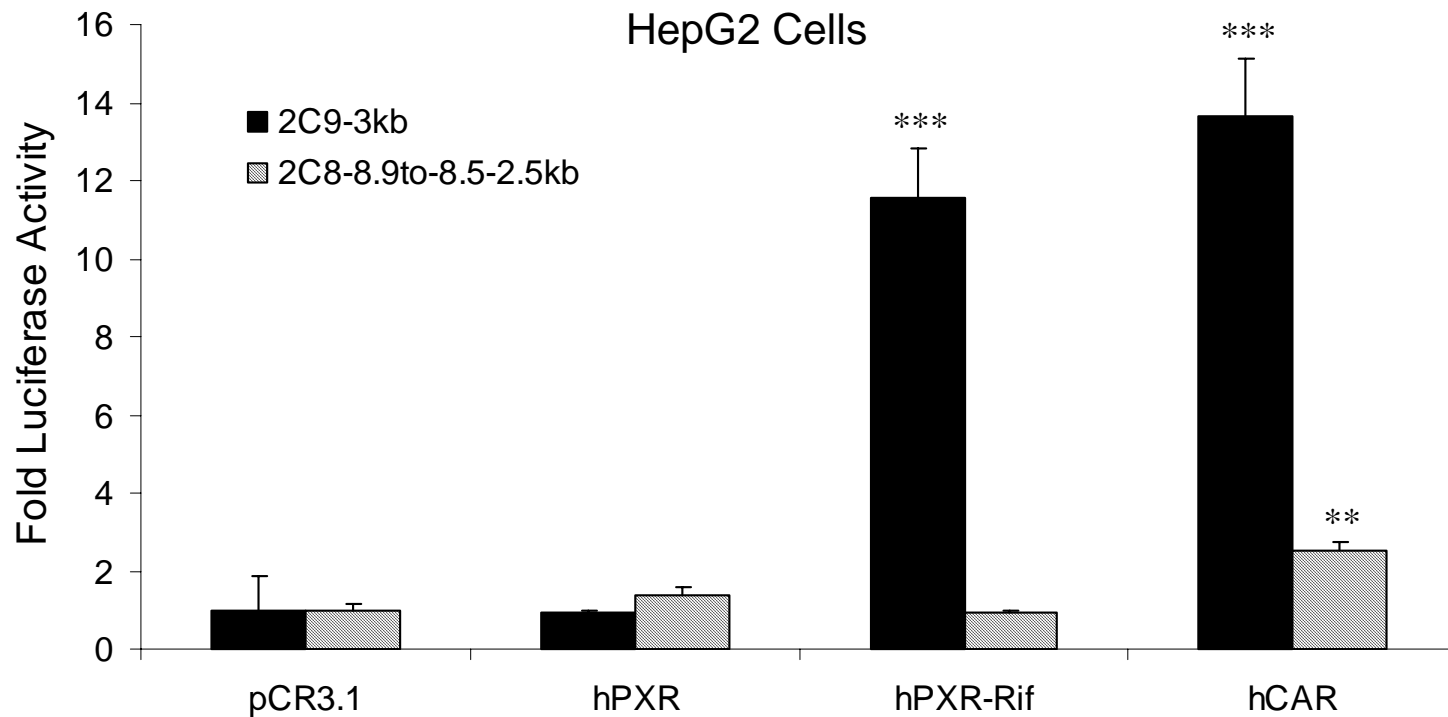
2C8-8806



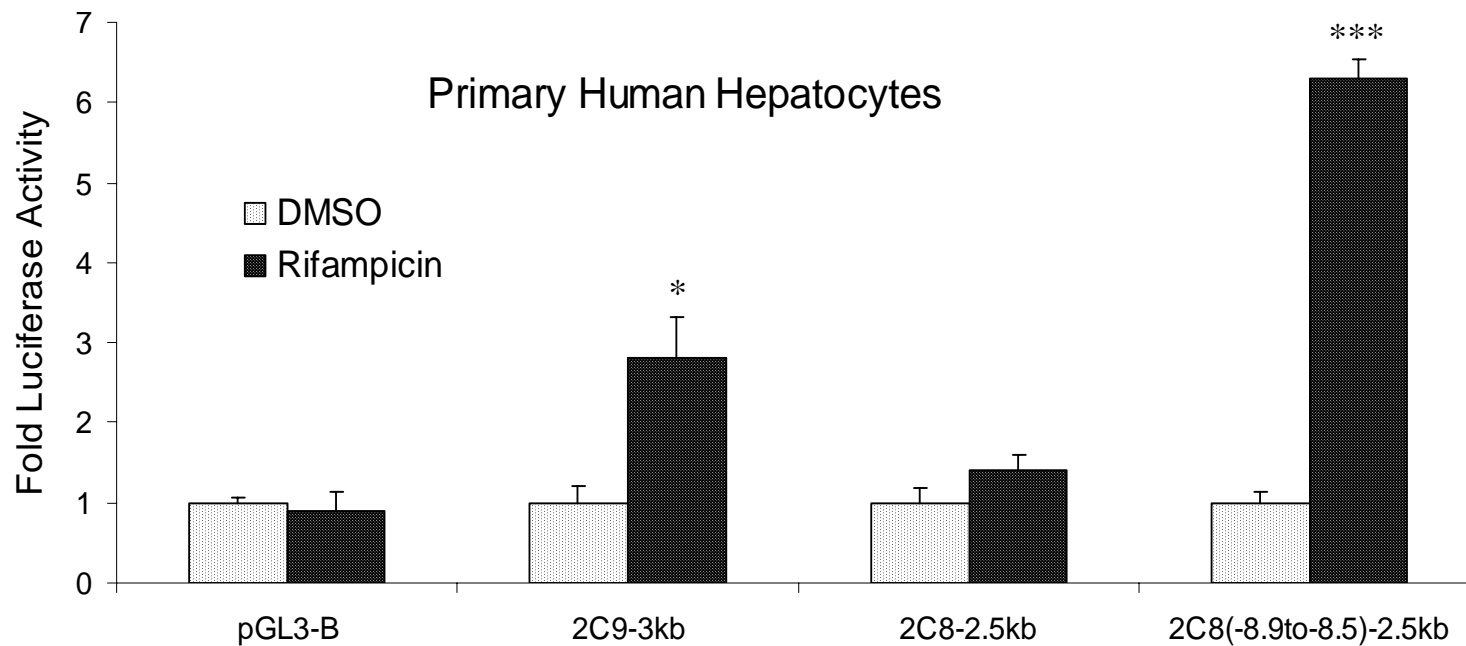
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2C9-2898	aaTGAACCctacaTAAACTat
2C19-2647	aaTGAACCccacaGAAACTat

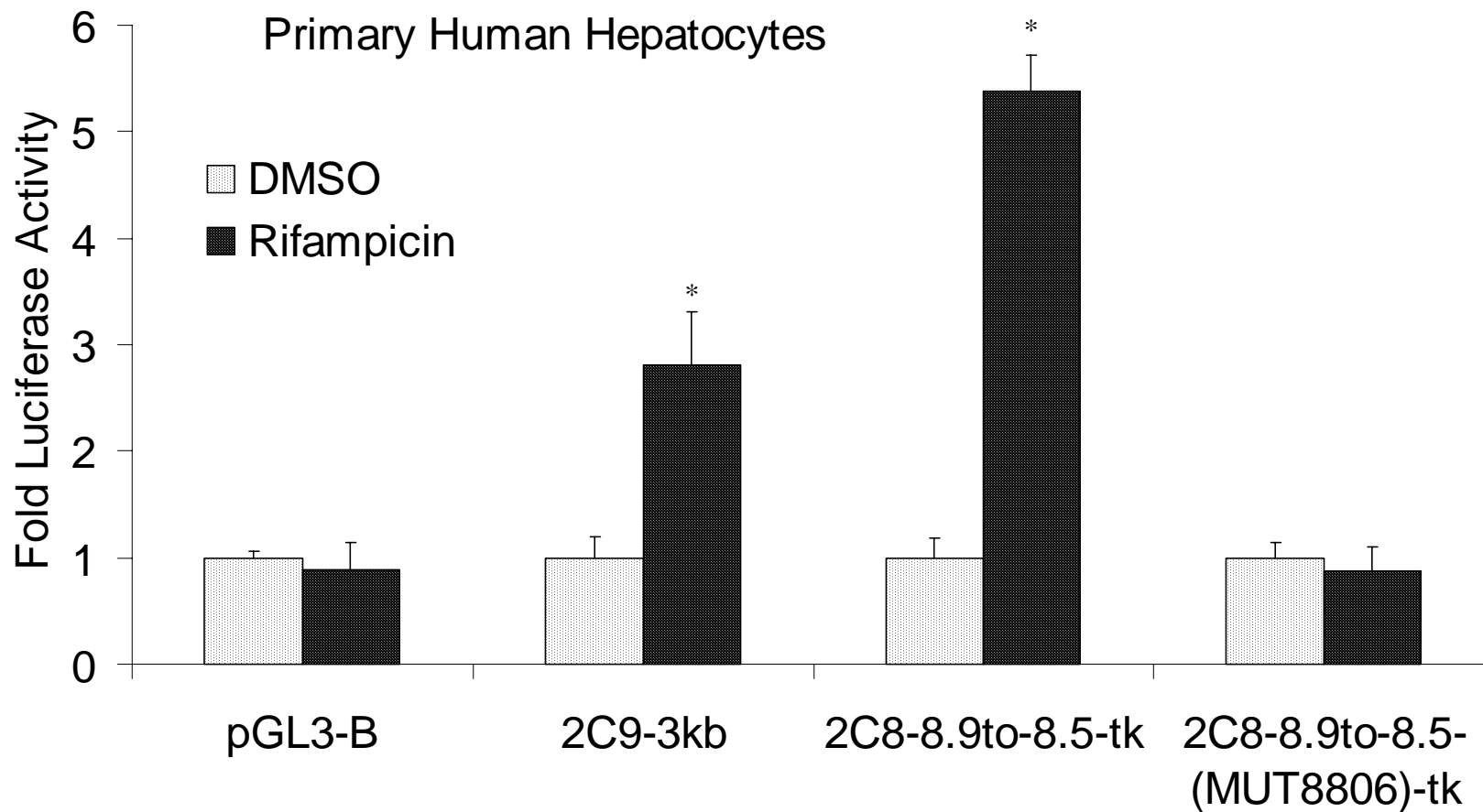
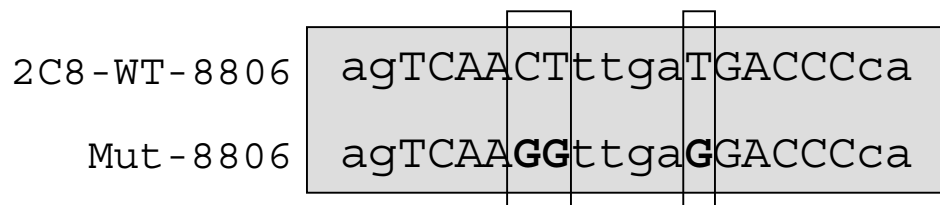


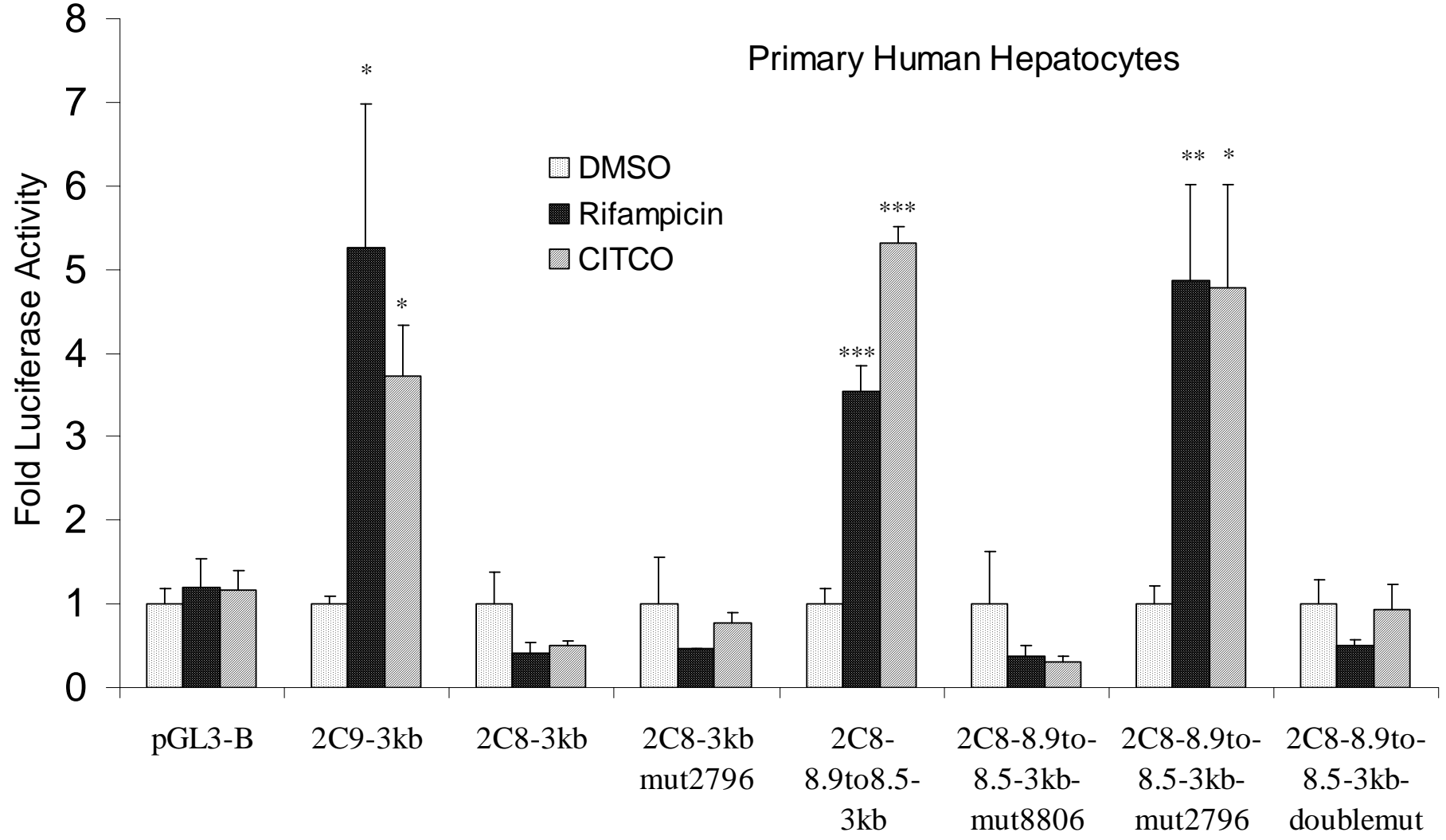
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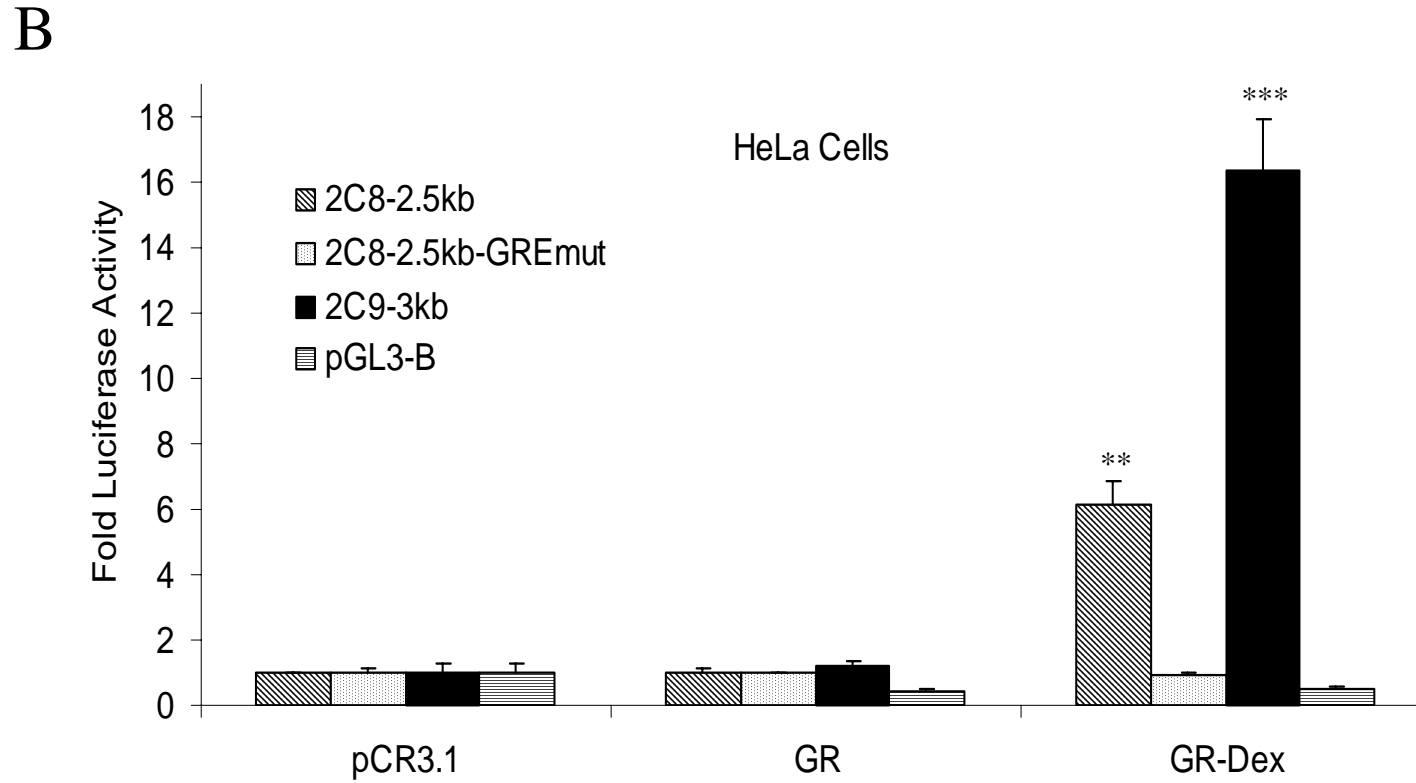
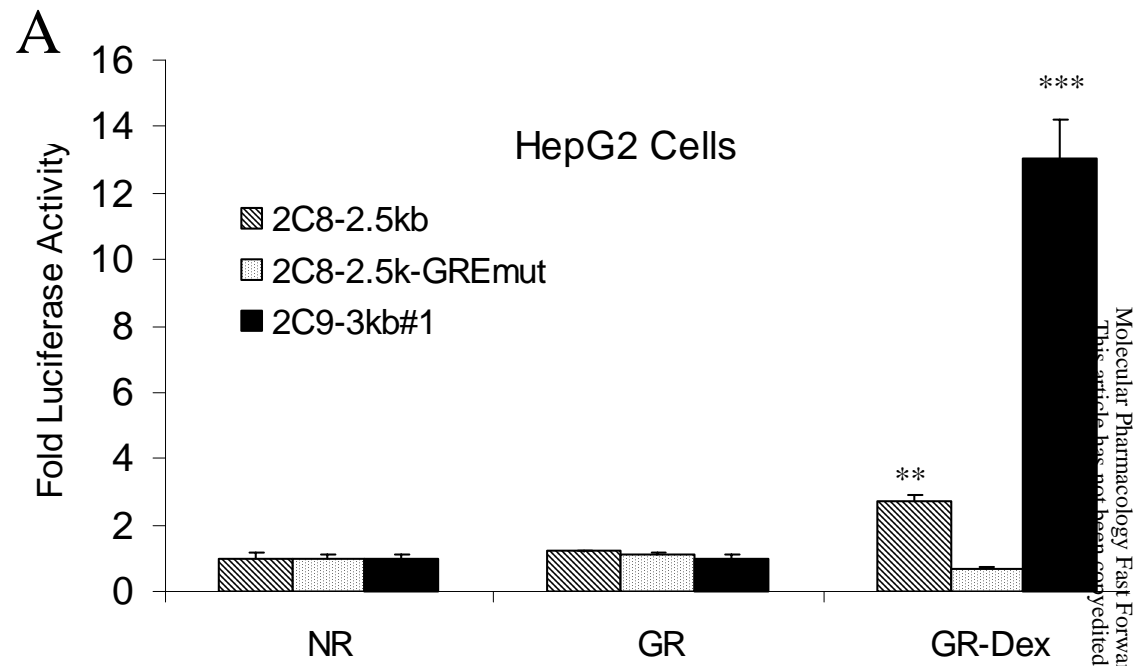


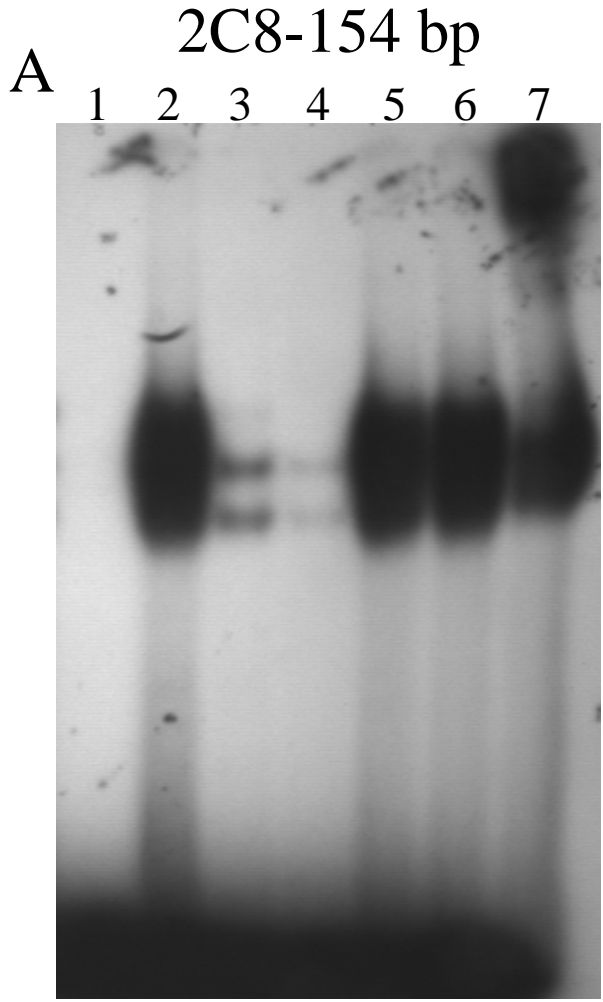
B











1. Free Probe
2. HNF-4
3. HNF-4 10X cc
4. HNF-4 100X cc
5. HNF-4 10X mut cc
6. HNF-4 100X mut cc
7. HNF-4 Anti-HNF-4alpha

HNF-4 Binding Site

2C9-150	agtGGGTCAaAGTCCTttc
2C8-154	catGGGCCAaAGTCCActc
HNF4-mut	catGGGCGTtTCACCActc

