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Title Page

The Nuclear Receptors CAR and PXR Cross Talk with HNF4α to Synergistically Activate the Human *CYP2C9* Promoter

Yuping Chen, Grace Kissling, Masahiko Negishi, Joyce A. Goldstein

Human Metabolism Section, Laboratory of Pharmacology and Chemistry (Y.C., J.A.G.) Biostatistics Branch (G.K.), and Pharmacogenetics Section (M.N.), Laboratory of Reproductive and Developmental Toxicology, Department of Health and Human Services, National Institutes of Health, National Institutes of Environmental Health Sciences, Research Triangle Park, NC 27709

Running Title Page

Running Title: HNF4α Cross-talks with CAR/PXR to Regulate *CYP2C9*

Address all correspondence to:

Dr. Joyce Goldstein

National Institute of Environmental Health Sciences

Research Triangle Park, NC 27709

Telephone 919-541-4495 Fax 919-541-4107

Email goldste1@niehs.nih.gov

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Nonstandard abbreviations: CYP and P450, Cytochrome P450; hRXR, human retinoid X receptor; hCAR, human constitutive androstane receptor; hPXR, human pregnane X receptor; hGR, human glucocorticoid receptor; EMSA, electrophoretic mobility shift assays; tk, thimidine kinase promoter; CAR-RE, constitutive androstane receptor-responsive element; GRE, glucocorticoid-responsive element; DR, direct repeat; TCPOBOP, (1,4-bis[2-(3,5-dichloropyridyloxy)]benzene); PEPCK, phosphoenolpyruvate carboxykinase; Glc6Pase, glucose-6-phophatase; L-CPT1, liver

carnitine palmitoyltransferase I; PGC-1α, PPARγ coactivator-1α; HNF4α, hepatic nuclear factors 4α, HPF-1, HepG2-specific P450 2C factor-1

Abstract

CYP2C9 is an important human drug-metabolizing enzyme which is expressed primarily in liver. Recent studies in our laboratory have shown that the nuclear receptor PXR is important in the transcriptional activation of the *CYP2C9* promoter by drugs such as rifampicin, and that the essential element is a CAR/PXR site -1839 bp upstream of the translation start site. Both CAR and PXR transcriptionally upregulate the *CYP2C9* promoter via these elements. In the present study, we ask whether additional sites in the proximal promoter also play a role in this induction. We identify two proximal HNF4α binding sites at -152 bp and -185 bp of the *CYP2C9* promoter, both of which bind HNF4α in gel-shift assays and transcriptionally upregulate this promoter in response to HNF4α in HepG2 cells. HNF4α synergizes with CAR and with PXR in HepG2 cells treated with rifampicin. The synergy only occurs when the CAR/PXR binding site at -1839 bp is present. Mutation of the two HNF4α binding sites differentially prevented upregulation of *CYP2C9* promoter by both CAR as well as HNF4α, synergy between the two receptors, and essentially abolished induction by rifampicin in HepG2 cells transfected with PXR. These studies strongly support the hypothesis that there is cross-talk between distal CAR/PXR sites and HNF4α binding sites in the *CYP2C9* promoter and that the HNF4α sites are required for maximal induction of the *CYP2C9* promoter.

Introduction

CYP2C9, the major member of the CYP2C subfamily in human liver, metabolizes more than 16% of clinically used drugs, including the hypoglycemic agents tolbutamide and glipizide, the anticonvulsant phenytoin, the anticoagulant warfarin, numerous non-steroidal anti-inflammatory drugs such as fluriprofen, diclofenac (Goldstein, 2001), as well as some newly developed drugs such as the antihypertensive losartan and the diuretic torsemide (Goldstein and de Morais, 1994; Goldstein, 2001). It also metabolizes endogenous compounds such as arachidonic acid. It is well known that the presence of genetic polymorphisms in the *CYP2C9* gene results in individual variability in the metabolism of CYP2C9 substrates in humans (Sullivan-Klose et al., 1996; Goldstein, 2001; Blaisdell et al., 2002).

Another potential source of variation in the metabolism of CYP2C9 substrates is induction by previous exposure to drugs, which may result in tolerance or therapeutic failure. Previous clinical reports have shown that the clearance of typical substrates of CYP2C9 are increased in humans after the administration of certain drugs, such as rifampicin, phenobarbital, and the herbal medicine St. John's Wort (Zilly et al., 1975; Kay et al., 1985; Williamson et al., 1998; Henderson et al., 2002). *In vitro* studies in human primary hepatocytes have also demonstrated that CYP2C9 is induced at the level of mRNA, protein, and catalytic activity by drugs such as rifampicin, hyperforin (the active constitute in St John's Wort), phenobarbital, and the glucocorticoid dexamethasone (Chang et al., 1997; Gerbal-Chaloin et al., 2001; Raucy et al., 2002; Madan et al., 2003; Komoroski et al., 2004). Promoter studies have revealed two constitutive androstane receptor binding elements (CAR-REs) within the *CYP2C9* promoter (at -2898 and -1839

bp from the translation start site) and one glucocorticoid responsive element (GRE) at -1697 bp (Ferguson et al., 2002; Gerbal-Chaloin et al., 2002; Chen et al., 2004). These sites bind CAR/and pregnane X receptors (PXR) or glucocorticoid receptors (GR) respectively to mediate the induction of *CYP2C9* by various drugs including rifampicin, phenobarbital, hyerforin and dexamethasone.

CYP2C9 is preferentially expressed in the liver and appears to be regulated by various hepatic transcriptional factors such as HNF4α and HNF3γ (Ibeanu and Goldstein, 1995; Jover et al., 2001; Bort et al., 2004). HNF4α, one nuclear receptor expressed mainly in the liver, intestine, kidney and pancreas, activates the transcription of target genes either through its recognition of a direct repeat DR1 motif or its recruitment of chromatin remodeling systems (Sladek and Darnell, 1992; Hu and Perlmutter, 1999). In liver, HNF4α sustains the constitutive expression of a large number of hepatic genes, including P450s such as *CYP2A6, 2B6, 2D6, 3A, 7A1*, as well as the glucuronyl transferase UGT1A1, certain hepatic transporters and even regulatory factors such as PXR and HNF1α (Watt et al., 2003). Importantly, HNF4α is involved in the transcriptional responses of hepatic genes to endogenous compounds or xenobiotics, such as induction of several major enzymes involved in gluconeogenesis (PEPCK, Glc6Pase, and L-CPT1) by glucagon or glucocorticoid (Stafford et al., 2001; Louet et al., 2002; Gautier-Stein et al., 2005), drug induction of the P450 gene *CYP3A* (Tirona et al., 2003) and inhibition of *CYP7A1* by rifampicin (Li and Chiang, 2004). Moreover, inactivation of HNF4α results in suppression of PXR and *CYP3A* expression in fetal hepatocytes (Hayhurst et al., 2001) and the hepatic fasting response mediated by PGC-1α in adult liver (Rhee et al., 2003).

HNF4α has been shown to increase endogenous CYP2C9 mRNA expression when overexpressed in HepG2 cells (Jover et al., 2001). One putative HNF4α binding site has been reported in the *CYP2C9* basal promoter region by our laboratory (Ibeanu and Goldstein, 1995). In the present study we ask whether HNF4α has a role in the transcriptional regulation of *CYP2C9*. We used reporter assays, mutagenesis, and EMSA to identify and functionally characterize two HNF4α sites in the *CYP2C9* promoter. We then examined whether these proximal HNF4α sites have a role in the regulation of *CYP2C9* by CAR and PXR. We show herein that these proximal HNF4α binding sites are required for the optimal activation of the *CYP2C9* promoter by both CAR and PXR probably through cross talk between HNF4α and CAR/PXR. Importantly, this study shows evidence for cross-talk between HNF4α and CAR/PXR involving both distal CAR/PXR sites and proximal HNF4α elements in the *CYP2C9* promoter.

Methods

Chemicals:

DMSO, rifampicin, dexamethasone, and other common reagents were purchased from Sigma Aldrich (St. Louis, MO). Rifampicin and dexamethasone were dissolved in DMSO. Cell culture media was purchased from Invitrogen (Carlsbad, CA). Desalted oligonucleotides were purchased from Genosys, Inc. (The Woodlands, TX). Restriction enzymes were purchased from New England Biolabs (Beverly, MA). All other reagents were of the highest grade available.

Transient transfection constructs

The wild type *CYP2C9*-3k/pGL3_Basic, and three mutants (*CYP2C9*-3k/-2898m, *CYP2C9*-3k/-1839m and *CYP2C*-3k/dmut) were as described previously (Chen, et al. 2004). All of these constructs start at -2920 to -1 upstream the translation start site. For the subsequent promoter deletion constructs, *CYP2C9*-1874/pGL3_Basic construct (previously named *CYP2C9*-1.9k/pGL3_Basic) (Chen et al., 2004) was first cleaved by EcoRI, incubated with Klenow Fragment (New England Biolabs, Beverly, TX) to blunt the two ends, and then further digested by EcoRV. Gel purified large fragments were self-ligated to produce one deletion construct *CYP2C9*-1874/Δ-1358/-362. Another deletion construct *CYP2C9*-1874/Δ-250/-114, was produced by digesting *CYP2C9*-1874/pGL3_Basic with AvrII, followed by a gel purification of the large fragment and religation. To produce the chimeric construct *CYP2C9*/SV40, 1416 bp of the *CYP2C9* promoter fragment from plasmid *CYP2C9*-1874 was digested through double digestion with EcoRV and SacI, then inserted into a SV40 promoter driven luciferase vector pGL3_Promoter linealized by SacI and SmaI.

pSG5-hPXR was kindly provided by Steve Kliewer (GlaxoSmithKline) (Kliewer et al., 1998). (XREM)-*3A4*-362/+53 was obtained from Brian Goodwin (Goodwin et al., 1999). pCR3-hGR was described previously (Chen, et al. 2003). The cDNAs of hHNF4α was amplified from total RNA of human primary hepatocytes with forward primer: 5'-CTCGTCGACATGGACATGGCCGACTAC3' and reverse primer: 5' GGCTTGCTAGATAACTTCCTGCTTGGT 3' (underlined are the start codon and stop codon, respectively). Gel purified PCR amplicons were cloned into TOPO-pCR2.1 (Invitrogen, Carlsbad, CA) and then sequenced. Mutations in PCR products were corrected through quick-change mutagenesis (QuickChange Site-directed mutagenesis, Stratagene, La Jolla, CA). The corrected cDNAs of hHNF4α were excised from pCR2.1 by HindIII and XbaI, and then inserted into the same restriction enzyme sites of expression vector pCR3.

Cell culture and transfection

HepG2 cells were maintained in the Eagle's minimal essential medium supplemented with 10% fetal bovine serum and penicillin-streptomycin at 37°C under 5% CO_2. Luciferase constructs and receptor constructs (or empty vectors, 100 ng of each) were combined with 2 ng of internal control pRL-TK, then mixed with Effectene transfection reagent (QIAGEN, Valencia, CA), and transfected into HepG2 cells 12-24 hours after seeding into 24-well plates (1-1.5 x 10^5 cells per well). Twenty-four hours later, medium was replaced, and drugs were added at the appropriate concentrations (0.1% of DMSO, 10 μM of rifampicin and 100 nM of dexamethasone). Drugs were incubated with the cells for 24 hr, followed by dual luciferase assays (Promega, Madison, WI). Firefly

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luciferase activities were normalized against Renilla luciferase readings of the internal control plasmids to calculate promoter activity.

Site-directed mutagenesis

The promoter construct *CYP2C9*-1874/pGL3_B was used as the template to mutate HPF1 sites in four *CYP2C9* promoter mutants: *CYP2C9*-1874/-152m1, *CYP2C9*-1874/-152m2, *CYP2C9*-1874/-185m and *CYP2C9*-1874/pdmut, respectively, through using QuickChange Site-directed mutagenesis kits (Stratagene, La Jolla, CA). The forward primers utilized for mutagenesis are as follows (hexamer half-sites are indicated by bold capital letters, mutated nucleotides are underlined and deletions are indicated by dots):

-152 mut1, 5' CTGTATCAGT**<u>CCC</u>TCA**A**AGTCCT**TTC 3';

-152 mut2, 5' GTATCAGT**GGGTC<u>T..</u>GTCCT**TTCAGAAG 3';

-185 mut, 5' GAACA**AGACC<u>T..G</u>GACA**TTTTATTTTTATC 3'.

CYP2C9 promoter DNA fragments containing expected mutations were verified by DNA sequencing, and then subcloned into the fresh pGL3_B vector.

Gel shift assays

Human hHNF4α was synthesized *in vitro* using the TNT Quick-Coupled In Vitro Transcription Translation System (Promega, Madison, WI), following the manufacturer's protocol. Empty vector pCR3 was also used as the template in parallel synthesis reactions to prepare the control. Nuclear extracts were attained from HepG2 cells following the standard approach in Current Protocols in Molecular Biology. Klenow Fragment (New England Biolabs, Beverly, MA) was employed to incorporate ^{32}P-dCTP at the 5' ends of the double-stranded oligonucleotides. Approximately 50,000 cpm of labeled probe was incubated with 2 μl of the synthesized nuclear receptors or approximately 1 μg of nuclear

extracts in a 10 µl binding reaction containing 10 mM Tris- HCl, pH 7.5, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 4% (v/v) glycerol, 50 mM NaCl and 1 µg of non-specific competitor poly (dI-dC) (Sigma, St. Louis, MO). In parallel reactions, specific cold competitors or specific hHNF4α antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were added to the mixture before the addition of proteins. After 20 min incubation at room temperature, 9.5 µl of the reaction mixture was loaded onto a 5% non-denaturing polyacrylamide gel for electrophoresis in 0.5X TBE buffer for 2 hours at 150V. The gels were dried and exposed to film. The following are the sequences of the oligonucleotides used as probes, wild type or mutated specific cold competitors (hexamer half-sites are indicated by bold capital letters and mutated nucleotides are underlined and deletions are indicated by dots):

-152 wt: 5'-ctagCTGTATCAGT**GGGTCA**A**AGTCCT**TTC -3'

-152 mut1: 5' CTGTATCAGT**CCCTCA**A**AGTCCT**TTC 3'

-152 mut2: 5' GTATCAGT**GGGTCT..GTCCT**TTCAGAAG 3'

-185 wt: 5' ctagAACA**AGACCA**A**AGGACA**TTTTAT 3'

-185 mut: 5' GAACA**AGACCT..GGACA**TTTTATTTTTATC 3'

APF1 wt: 5' ctagGCGC**TGGGCA**A**AGGTCA**CCTGC 3'

APF1 mut: 5' GCGC**TGGCGA**A**AGGAGA**CCTGC

Statistical analysis

One-way analysis of variance (ANOVA) was followed by bootstrapped multiple comparisons (Westfall and Young, 1993) to compare across constructs or receptors, with the following exceptions. Two-way ANOVA with interaction was utilized to test for synergism. For the first experiment, ANOVA was followed by the Bonferri test.

Supplemental two-sample t-tests were used for specific comparisons of two groups in a few cases as noted.

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Results

hHNF4α activates the human *CYP2C9* promoter in HepG2 cells and synergizes with the nuclear receptor hCAR

To determine whether HNF4α activates the *CYP2C9* promoter and whether it influences the activation by the nuclear receptor hCAR, expression plasmids containing hCAR and hHNF4α were cotransfected into HepG2 cells individually or in combination with a 1874bp *CYP2C9* luciferase promoter construct, the empty vector pGL3_B or a positive control *3A4*-XREM-362/+53, respectively. As shown in Fig. 1, both CAR and HNF4α significantly upregulated *CYP2C9*-1874 (3.5- and 5.4- fold, p of <0.05), while the empty vector was not upregulated by CAR or HNF4α. When both nuclear receptors were cotransfected simultaneously activation was synergistic rather than additive (24-fold rather than 9-fold). This synergy was statistically significant, p<0.001. The *(XREM)-3A4-362/+53* positive control was activated either by CAR or HNF4α as expected, but there was only a weak synergism (p=0.037) after cotransfection with both receptors.

We then investigated the location and contribution of possible HNF4α responsive elements to the synergistic upregulation of the promoter by CAR and HNF4α using various deletion constructs. A chimeric construct *CYP2C9*/SV40, in which the proximal 1356 bp of the *CYP2C9* promoter region was replaced by the SV40 promoter, was activated by CAR (p<0.001) but not by HNF4α, and there was no synergism between CAR and HNF4α (Fig. 2). The CAR activation and HNF4α activation were significantly decreased compared to that of the wild-type *CYP2C9*-1874 promoter. In contrast, when the promoter region from -1358bp to -362bp was deleted, the activation of the resulting *CYP2C9*Δ-1358 /-362 by CAR and HNF4α (p<0.001) was comparable to that of the full

CYP2C9-1874 construct, and the synergy between the two receptors was still observed ($p<0.001$). We finally deleted a very small region (-250 bp to -114 bp) within the *CYP2C9*-1874 construct surrounding containing the putative HPF1 site (Venepally et al., 1992). CAR activation was decreased from 4-fold to 2- fold ($P<0.001$), and the HNF4α activation and synergistic transactivation by HNF4α and CAR were abolished (Fig. 2). These data clearly suggest the presence of HNF4α binding site(s) localized within the basal promoter of *CYP2C9* (-250 bp to -114 bp), which are required for the synergistic activation by CAR and HNF4α.

Identification of two HNF4α binding sites which are required for full activation of *CYP2C9* by CAR and HNF4α

Within this region between -250 bp to -114 bp, one putative HPF1 site has been reported at –152 bp from the translation start site (Ibeanu and Goldstein, 1995). To confirm that this putative HPF1 site binds HNF4α, gel shift assays were first performed with nuclear extracts from HepG2 cells and a ^{32}P labeled oligonucleotide probe *2C9*-wt containing this sequence (as shown in Fig. 3B, left panel). A strong complex was formed, which was essentially eliminated by competition with 5X or 50X excess of wild type cold competitors *2C9*-wt, while 50X excess of two cold competitors containing a mutated HPF1 site (shown in Fig. 3A) competed only weakly for the formation of the complex. Antibody against HNF4α retarded the mobility of the complex and produced a supershifted band at the top, further suggesting the existence of HNF4α in this complex. Finally, we examined the binding of this probe to *in vitro* transcribed HNF4α. Transcribed products from the expression plasmid pCR3-hHNF4α formed a strong complex with the probe, while products from the empty pCR3 vector did not produce any

bands. All of the wild type cold competitors *2C9*-wt, *2C19*-wt and a positive control HNF4α binding oligonucleotide APF1-wt from the human APOCIII gene (Jiang and Sladek, 1997) strongly suppressed the formation of this complex. Mutated oligonucleotides competed less effectively. When antibodies against HNF4α were included in the binding reaction, there was marked supershifting of the band (Fig. 3B, right panel).

To verify whether the HPF1 site at –152 bp plays a functional role in the activation of *CYP2C9* by CAR and HNF4α, mutations were introduced into the *CYP2C9*-1874 construct, and constructs were examined with transient transfection assays in HepG2 cells (Fig. 4A). Two different mutations of the -152 HPF1 site significantly decreased CAR activation ($p<0.001$), but the HNF4α activation was only decreased slightly by the -152 mut1 mutant ($p>0.05$) and the -152 mut2 mutant ($p=0.03$). Synergistic activation by CAR and HNF4α was still observed with both mutants ($p<0.001$). These results suggest that while cross-talk may occur between the HPF1 site at –152 bp and the proximal CAR-RE for full CAR activation, other HNF4α binding sites may be involved in full activation by HNF4α and for the synergistic response between HNF4α and CAR.

Using a HPF1 consensus motif (RRRNCAAAGKNCAYY, see Venepally et al., 1992), we searched the *CYP2C9* basal promoter region for additional HNF4α binding sites and found another putative site -185 bp from the translation start codon. To determine whether HNF4α also binds this new site, new gel shift assays were performed. A series of complexes were produced by the incubation of nuclear extracts of HepG2 cells and radiolabeled oligonucleotides containing the new site (lane 2 in Fig. 5, left panel). The denser complex with lesser mobility indicated by the arrow was eliminated

by competition with excess of the wild type competitors but to a lesser extent by unlabeled mutated -185 wt oligo-nucleotides. Since the mutated cold competitors also competed for the complexes with greater mobility, these may be nonspecific products. Another wild type HNF4α binding oligonucleotide (APF1-wt) essentially eliminated complexes with lower mobility (indicated by arrow) by competition but had less effect on the complexes with greater mobility. Specific HNF4α antibodies decreased the intensity of the two complexes with lower mobility (and perhaps the complex with the greatest mobility), while a supershifted band appeared at the top (Lane 11 in Fig. 5, left panel), indicating that HNF4α is involved in these complexes. Importantly, when *in vitro* synthesized HNF4α was incubated with labeled probes (left panel), a single band was observed for HNF4α proteins but not for empty pCR3. All wild type cold competitors including an oligonucleotide from a known HNF4α binding site APF1 strongly inhibited the formation of this complex, while two mutated oligonucleotides did not. Antibodies against HNF4α effectively abolished this complex, providing further support that the -185 HPF1 site is a HNF4α binding site (Fig. 5, right panel).

Mutagenesis of both the new -185 HPF1 site and the -152 HPF1 site was performed singly or together in *CYP2C9*-1874 to functionally evaluate their roles in transactivation of *CYP2C9* promoter by CAR and HNF4α. (Fig. 6A). As shown in Fig. 6B, the -185 HPF1 mutation decreased CAR activation from 4.5-fold for wild type construct to 2.9-fold ($p<0.001$), but this change was smaller than that produced by the -152 HPF1 mutation (to 1.8-fold, $P<0.001$). However, the decrease in HNF4α activation produced by the -185 mutant (from 8.6-fold for wild-type to 1.8-fold, $p<0.001$) was greater than that of the -152 mutant (from 8.6-fold to 3.4-fold, $p<0.001$). The synergistic activation by

CAR and HNF4α of the -152 HPF1 mutant (P<0.001) was almost comparable to that of the wild type construct, but the synergism was dramatically decreased for the -185 HPF1 mutant. When both sites were mutated, activation by CAR and HNF4α and their synergistic effects were essentially abolished, clearly showing a cooperative contribution of both HPF1 sites to CAR activation of the *CYP2C9* promoter.

Two HPF1 sites are required for PXR-mediated rifampicin but not hGR-mediated dexamethasone induction of *CYP2C9* in HepG2

Earlier studies have shown that the CAR-REs of *CYP2C9* also interact with hPXR which mediates induction of *CYP2C9* by rifampicin (Gerbal-Chaloin et al., 2002; Chen et al., 2004). To examine whether the two HNF4α binding sites of the basal *CYP2C9* promoter region are also involved in the activation of the induction of the *CYP2C9* gene by PXR and rifampicin and activation by PXR, we performed cotransfection assays in HepG2 cells with *CYP2C9* promoter constructs and nuclear receptor expression plasmids for PXR and HNF4α. HNF4α appeared to be very important in the induction of *CYP2C9*-1874 construct by rifampicin and PXR (Fig. 7A). PXR and HNF4α activated this construct (1.6- and 3.8- fold respectively) when transfected into cells individually, and an additive 6-activation fold was seen when cells were cotransfected with both receptors. Rifampicin caused 3-fold induction in cells cotransfected with PXR (p<0.001). When HNF4α and PXR were coexpressed in rifampicin treated HepG2 cells, activation was synergistic (p<0.001) rather than additive (21-fold). Mutation of the -152 HNF4α site significantly decreased rifampicin induction of the *CYP2C9* promoter construct (p<0.001) in cells cotransfected with PXR (from 3-fold to 1.5-fold), but did not prevent the synergistic response with HNF4α. Mutation of the -185 HNF4α site did not decrease the

PXR-mediated induction by rifampicin, but essentially abolished HNF4α activation in DMSO treated cells as well as the synergistic response to HNF4α and PXR in cells treated with rifampicin. A double mutation of both HNF4α sites almost completely eliminated activation by PXR or HNF4α and almost abolished the induction by rifampicin. These data indicate that HNF4α and two proximate HNF4α sites are involved in the activation of the *CYP2C9* promoter by CAR, and the optimum induction of *CYP2C9* by rifampicin via PXR. HNF4α thus synergizes with both CAR and PXR.

Due to the location of both HNF4α binding sites in the very basal promoter region, it seemed possible that the mutations of the two HNF4α binding sites could exert an effect on basal promoter structure which affects CAR and PXR activation indirectly. In this case, these mutations should presumably affect other drug responses nonspecifically, such as the activation by dexamethasone which acts through interaction with a glucocorticoid receptor with a GRE at -1697 bp. To investigate this possibility, the effects of single and double mutations of the two HNF4α binding sites on dexamethasone induction were examined. Though *CYP2C9*-1874 was strongly activated by dexamethasone (60-fold); the -152 mutation did not affect this response. The construct with the -185 mutation and the double mutation exhibited comparable or even slightly higher induction (90-fold) compared to the DMSO vehicle (Fig. 7B). In summary, it appears that the HNF4α site mutants do not alter the *CYP2C9* basal promoter structure nonspecifically, and the cooperativity of HNF4α and its two binding sites appears to be specific for activation of the *CYP2C9* promoter by PXR and CAR.

The synergistic activation of the *CYP2C9* promoter by CAR and HNF4α requires an intact CAR/PXR-RE

CAR and PXR have been shown to activate the *CYP2C9* promoter acting through two CAR/PXR-REs located at -2898 bp and -1839 bp upstream of the translation start site respectively (Ferguson et al., 2002; Gerbal-Chaloin et al., 2002; Chen et al., 2004). The proximal site has been shown to be essential for CAR activation and PXR mediated induction. To determine whether these elements were required for the synergistic activation of *CYP2C9* promoter by CAR and HNF4α, CAR and HNF4α were transiently transfected into HepG2 cells along with the wild type *CYP2C9*-3k promoter construct, and mutants in which the two CAR/PXR-REs were mutated either individually or in combination (Fig. 8A). Results shown in Fig. 8B revealed that all constructs could be significantly activated by HNF4α ($p<0.001$) although mutation of the proximal CAR/PXR-RE decreased HNF4α activation by ~50% ($p<0.01$), again suggesting possible cross talk between the CAR/PXR-RE and the HNF4α responsive element(s). Moreover, mutation of the proximal CAR/PXR-RE at -1839 bp, either alone or together with the mutation of the distal CAR-RE at -2898 bp, prevented the synergy between CAR and HNF4α indicating that the proximal CAR site is necessary for the synergy between CAR and HNF4α.

Discussion

The present study identifies two proximal HNF4α binding sites which mediate transactivation of the *CYP2C9* promoter. These sites are located -185 and -152 bp from the translation start site respectively. HNF4α and CAR synergistically activated the *CYP2C9* promoter. A distal drug responsive element CAR/PXR-RE at -1839/-1824 bp and these two HNF4α binding sites were necessary for maximum activation by CAR as well as PXR-mediated drug induction by rifampicin. HNF4α was previously shown to transactivate the basal promoter of *CYP2C9* (Ibeanu and Goldstein, 1995; Jover et al., 2001) in HepG2 cells, and a putative HNF4α binding site was identified at -152 bp. However, our present studies showed that mutation of this site produced only a 50% decrease in HNF4α activation, which was considerably less than might be expected if this were the principle HNF4α binding site. Moreover this site did not appear important for the synergy between HNF4α and CAR.

In the present study, we identify and an additional DR1 site at -185 bp, which plays an essential role in HNF4α activation of the *CYP2C9* promoter. Mutation of the -185 site abolished most of the HNF4α activation of the *CYP2C9* promoter in HepG2 cells and was more important in the synergy between CAR and HNF4α. Mutation of both HPF1 sites was necessary to completely abolish activation of *CYP2C9* by HNF4α. These data show that the two HNF4α binding sites function differently but collaboratively to produce optimum transactivation of *CYP2C9* promoter by HNF4α as well as maximal activation by CAR.

Our studies also indicate that HNF4α appears to play a role in rifampicin induction of *CYP2C9*. These observations add *CYP2C9* to the list of hepatic genes, such as PEPCK,

CYP3A, and *CYP7A1* (Rhee et al., 2003; Tirona et al., 2003; Li and Chiang, 2004) which need HNF4α for maximum stimulatory responses by other nuclear regulatory factors. In these previously described studies, the crucial HNF4α sites that permit optimal response are often in the vicinity of the second responsive elements. The adjacent localization of HNF4α sites to other regulatory sites may facilitate the stability of DNA binding of transcriptional factors to their responsive elements and protein interactions involved in transactivation to produce maximum activation of certain genes (Stroup and Chiang, 2000; Stafford et al., 2001). Li and Chiang suggested an interaction between HNF4α bound to a bile acid responsive element II (BARE II) which positively activates *CYP7A1* promoter, and PXR bound to a second element approximately one hundred base pairs away which negatively regulates the promoter after treatment with the ligand rifampicin (Li and Chiang, 2004). However, in contrast to these previously reported studies, no HNF4α binding site was discovered adjacent to either of the two CAR/PXR binding sites in the *CYP2C9* promoter. The critical HNF4α binding sites of the *CYP2C9* promoter were >1500 base pairs downstream of the most proximal CAR/PXR binding site, suggesting a more complex mechanism. Recently, Negishi and coworkers (Swales et al., 2005) have found that maximal induction of *CYP2B6* by CAR involves a synergy between the distal CAR binding site (PBREM, at -1732/-1685bp) and a proximal okadaic acid responsive element (OARE, at -256/-233). This synergy involved association of CAR with the proximal OARE.

Further studies are underway to investigate the mechanism of the cross-talk between CAR/PXR and the HNF4α sites of *CYP2C9*. When CAR and RXR were added along with HNF4α in gel shift assays of the -152 or -185 HNF4α sites, we were unable to

demonstrate direct binding of CAR/PXR to either site (data not shown). Moreover mutation of the essential CAR/PXR site prevented the synergy between CAR and HNF4α, suggesting this site must be present for the synergy to occur. Possibly, other hepatic protein cofactors or corepressors must be present for an interaction between HNF4α and CAR or PXR. In our studies, coexpression of HNF4α and CAR with the *CYP2C9* promoter construct yielded synergistic effects in HepG2 cells but not in HeLa cells (data not shown) suggesting the possible involvement of liver-enriched factors, while the synergistic activation of *(XREM)-3A4-362/+53* by PXR and HNF4α was reported to be greater in HeLa cells than in HepG2 cells (Tirona et al., 2003).

In summary, two proximal HNF4α binding sites were identified which mediate transactivation of *CYP2C9* promoter and synergize with CAR/PXR. We provide evidence for a possible cooperative cross-talk between a distal CAR/PXR site and two proximal HNF4α binding sites. HNF4α and CAR synergistically activated the *CYP2C9* promoter. Both the distal CAR/PXR drug responsive element at -1839/1824 and the proximal HNF4α binding sites are necessary for the maximum transcriptional activation of the *CYP2C9* promoter by CAR and PXR. HNF4α sites in the proximal promoter appear to be important in the PXR-mediated induction of *CYP2C9* by drugs such as rifampicin as well as its upregulation by CAR.

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Footnotes

Address reprint requests to: Dr. Joyce Goldstein, NIEHS, PO Box 12233, Research Triangle Park, NC 27709

Legends for Figures

Fig 1. HNF4α synergizes with the nuclear receptor CAR in transactivation of the *CYP2C9* promoter in HepG2 Cells. 1874bp of a *CYP2C9* luciferase promoter was cotranfected into HepG2 cells with an internal control pRL-TK, parallel to empty luciferase vector constructs pGL3_B or a positive control *(XREM)-3A4-362/+53* containing PXR/CAR binding elements of *CYP3A4*. The nuclear receptors hCAR and/or hHNF4α were cotransfected into cells with promoter constructs either individually or in combination. Cells were refreshed 24 hours after transfection and grown for another 24 hours, then assayed for luciferase activity. Values represent the means ± standard deviation (S.D.) of three independent transfections. CAR,HNF4α or a combination of these two expression factors upregulate activity of the appropriate promoter construct compared to the empty vector transfected control with p values of <0.05, <0.01, <0.001 respectively (ANOVA with Bonferri. When given in combination, the transactivation by HNF4α and CAR was statistically synergistic, rather than additive, at †p<0.05, ††p<0.01, †††p<0.001 (two-way ANOVA with interaction).

Fig 2. The *CYP2C9* basal promoter region is required for the activation of the *CYP2C9* promoter by HNF4α and the synergetic activation by CAR and HNF4α. A, diagram of promoter constructs for transfection. EcoRI and EcoRV sites were used for a chimeric construct with the SV40 promoter and a 997bp fragment deletion in the *CYP2C9* promoter. AvrII was used to produce a short deletion in the basal promoter of *CYP2C9*-1874 construct. **B,** *CYP2C9*-1874 (wild type and two deleted) and one chimeric promoter construct were transfected into HepG2 cells along with an internal control pRL-

TK and nuclear receptor expression plasmids containing hCAR and hHNF4α. Medium was refreshed on the second day and luciferase activities were analyzed on the third day. Luciferase activities were normalized to the internal control pRL-TK and fold activation were relative to the value of empty vector cotransfection. Values represent the means of three independent transfections ± standard deviation (S.D.). CAR or HNF4α significantly upregulate promoter constructs when compared to the empty vector transfected control at *p<0.05, **p<0.01, or *** p<0. 001 (ANOVA followed by bootstrapped multiple comparisons). The ‡ indicates that the response of the mutated *CYP2C9* promoter construct to HNF4α or CAR is less than that of the wild-type construct at ‡p<0.05, ‡‡p<0.01, or ‡‡‡p<0.001. The ††† indicates synergistic rather than additive response to HNF4α and hCAR at p<0.001 (ANOVA with interaction).

Fig 3. Electrophoutic mobility shift assays (EMSA) demonstrate the binding of the putative HPF1 site of *CYP2C9* at -152 bp to HNF4 α. A, sequences of the oligos used for EMSA. Mutated nucleotides are underlined. **B**, ^{32}P labeled probe containing the putative HPF1 of *CYP2C9* was incubated at room temperature for 20 min with either nuclear extracts of HepG2 cells or hHNF4α synthesized *in vitro*. 5X or 50X excess of various cold competitors (CC) were added into binding reactions respectively for competition analysis. Antibody against hHNF4α was included in the last lane showing a supershifting. s, shifted complex; ss, supershifted band.

Fig 4. Mutation of the HPF1 site at -152 bp decreases but does not abolish transactivation of the *CYP2C9* promoter by CAR or HNF4α and does not effect the

synergistic activation by CAR and HNF4α. A, mutations of the -152 HPF1 site of *CYP2C9*. Mutated nucleotides are as underlined. **B**, HepG2 cells were transfected with wild type *CYP2C9*-1874 promoter constructs, two mutants, or the positive *(XREM)-3A4-362/+53* control, respectively. Expression plasmids for hCAR or hHNF4α were cotranfected either alone or in combination. Luciferase activity was measured on the third day and normalized to the internal control pRL-TK to calculate promoter activities. Fold activation was based on the value of empty vector cotransfection. Values represent the means ± SD of three independent transfections. An * indicates significantly greater than empty vector control at *p<0.05, **p<0.01, or ***p<0.001. The ‡ indicates that the response of the mutated *CYP2C9* promoter construct to HNF4α or CAR is less than that of the wild-type construct at ‡p<0.05, ‡‡p<0.01, or ‡‡‡p<0.001 (ANOVA followed by bootstrapped multiple comparisons). The ††† indicates that the response to HNF4α and hCAR is synergistic rather than additive at p<0.001 (two-way ANOVA with interaction)

Fig 5. EMSA demonstrates the binding of a new putative HPF1 site of *CYP2C9* at -185 bp to HNF4α. A, sequences of the oligos used for EMSA, are shown at the top. Mutated nucleotides were marked as underlined. **B**, ^{32}P labeled probe containing the new putative HPF1 site of *CYP2C9* was incubated with either nuclear extracts of HepG2 (left panel) or hHNF4α synthesized *in vitro* (right panel) at room temperature for 20 min. Excess (5X or 50X) of various cold competitors (CC) were added into binding reactions respectively for competition analysis. Antibody against hHNF4α was included in the last lane showing supershifting. s, shifted complex; ss, supershifted band.

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Fig 6. Comparative effects of mutation of both HPF1 sites at -152 and/or -185 bp on transactivation of *CYP2C9* promoter by CAR or hHNF4α and the synergetic activation from CAR and hHNF4α. A, diagram of constructs used in transfection assay. **B**, HepG2 cells were transfected by the wild type and deleted *CYP2C9*-1874 promoter constructs and three mutants respectively. Expression plasmids for hCAR or hHNF4α were cotranfected in parallel either alone or in combination. Luciferase activity was measured on third day and normalized to the internal control pRL-TK to calculate promoter activities. Fold activation was based on the value of empty vector cotransfections. Values represent the means ± SD of three independent transfections. An * indicates that significant upregulation of promoter constructs at *p<0.05, **p<0.01, or *** p<0.001 compared to the empty vector transfected control (ANOVA followed by bootstrapped multiple comparisons). The ‡ indicates that the response of the mutated *CYP2C9* promoter construct to HNF4α or CAR is less than that of the wild-type construct at ‡p<0.05, ‡‡p<0.01, or ‡‡‡p<0.001. The ††† indicates a synergistic rather than additive response to HNF4α and hCAR at p<0.001 (ANOVA with interaction).

Fig 7. Mutation of the two HPF1 sites at -152 and -185 bp abolishes A) hPXR-mediated rifampicin induction of *CYP2C9* promoter B) but not hGR mediated dexamethasone induction of *CYP2C9*. HepG2 cells were transfected with either the wild type or deleted *CYP2C9*-1874 promoter constructs, or one of three mutant constructs. Expression plasmids for various nuclear receptors were cotranfected into cells alone or in combination. Twenty-four hours after transfection, cells were refreshed with

new medium and treated with the appropriate drug for 24 hours. (**A**) Rifampicin (RIF) was added to hPXR transfected cells at a final concentration of 10 μM, Values for rifampicin induction are expressed as "Fold" relative to the value obtained with empty vector and the vehicle dimethylsulfoxide (DMSO) while values for dexamethasone (DEX) are expressed relative to dimethylsulfoxide alone. Values represent the means ± S.D. of three independent transfections. An * indicates that the effect of rifampicin treatment on a particular transfected construct is significantly greater than the vehicle control *p<0.05, **p<0.01, or *** p<0.001 (ANOVA and paired t-tests). The ‡‡‡ indicates that the induction response of the mutated *CYP2C9* promoter construct is less than that of the wild-type construct at p<0.001 (ANOVA followed by bootstrapped multiple comparisons). The ††† indicates a synergistic induction rather than additive response at p<0.001 (ANOVA with interaction).

(B) hGR transfected cells were treated with 100 nM dexamethasone . Luciferase activity was measured on the third day and normalized to internal control pRL-TK to calculate promoter activities. An * indicates that the effect of dexamethasone on a particular *CYP2C9* wild-type or mutated construct (containing a mutated HNF4α site) is significantly greater than the vehicle control at *p<0.05, **p<0.01, or *** p<0.001 (ANOVA and paired t-tests).

Fig 8. The proximal but not the distal *CYP2C9* CAR/PXR-RE is essential for the synergetic transactivation of the *CYP2C9* promoter by CAR and HNF4α as well as the full HNF4α activation of *CYP2C9* in HepG2 cells. A, diagram of the promoter constructs used in transfections. X represents a mutated CAR/PXR RE in the constructs.

B, mutations in the proximal CAR/PXR RE abolished the synergy of CAR and HNF4α and decreased the activation of the *CYP2C9* promoter by HNF4α. HepG2 cells were transfected with wild type *CYP2C9*-3k or the three mutants along with nuclear receptors (either with the empty vector, hHNF4α or hCAR alone, or in combination). After 24 hours, medium was refreshed and cells grown for another day. Promoter activities were determined by luciferase activity assays performed on the third day. Values represent the means ± SD of three independent transfections. An * indicates significantly greater than empty vector control at *p<0.05, **p<0.01, or ***p<0.001 (ANOVA followed by bootstrapped multiple comparisons). The ‡ indicates that the response of the mutated *CYP2C9* promoter construct to HNF4α or CAR is less than that of the wild-type construct at ‡p<0.05, ‡‡p<0.01, or ‡‡‡p<0.001. The ††† indicates that the response to HNF4α and hCAR is synergistic rather than additive at P<0.001 (two way ANOVA with interaction).

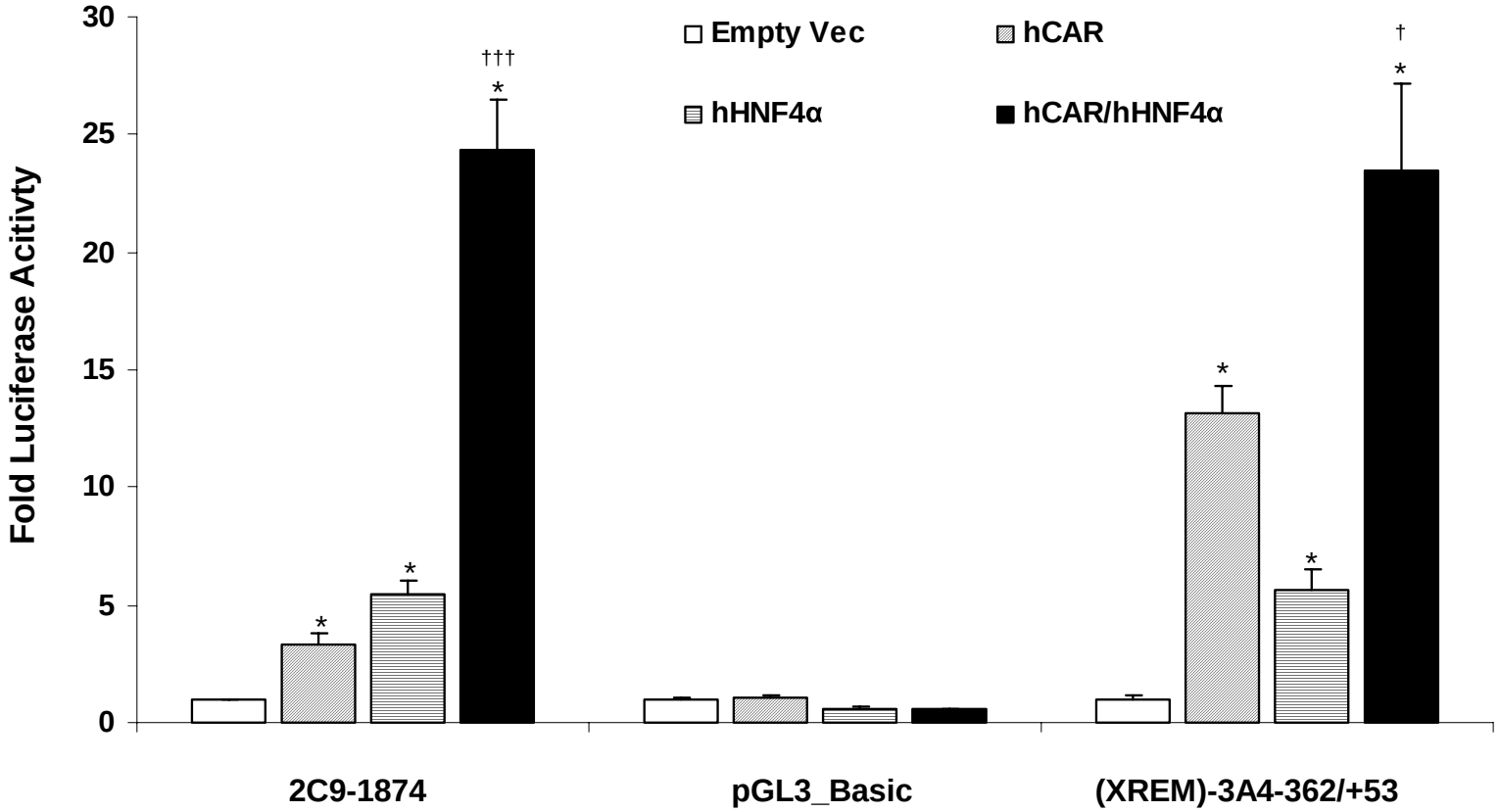

Fig 1

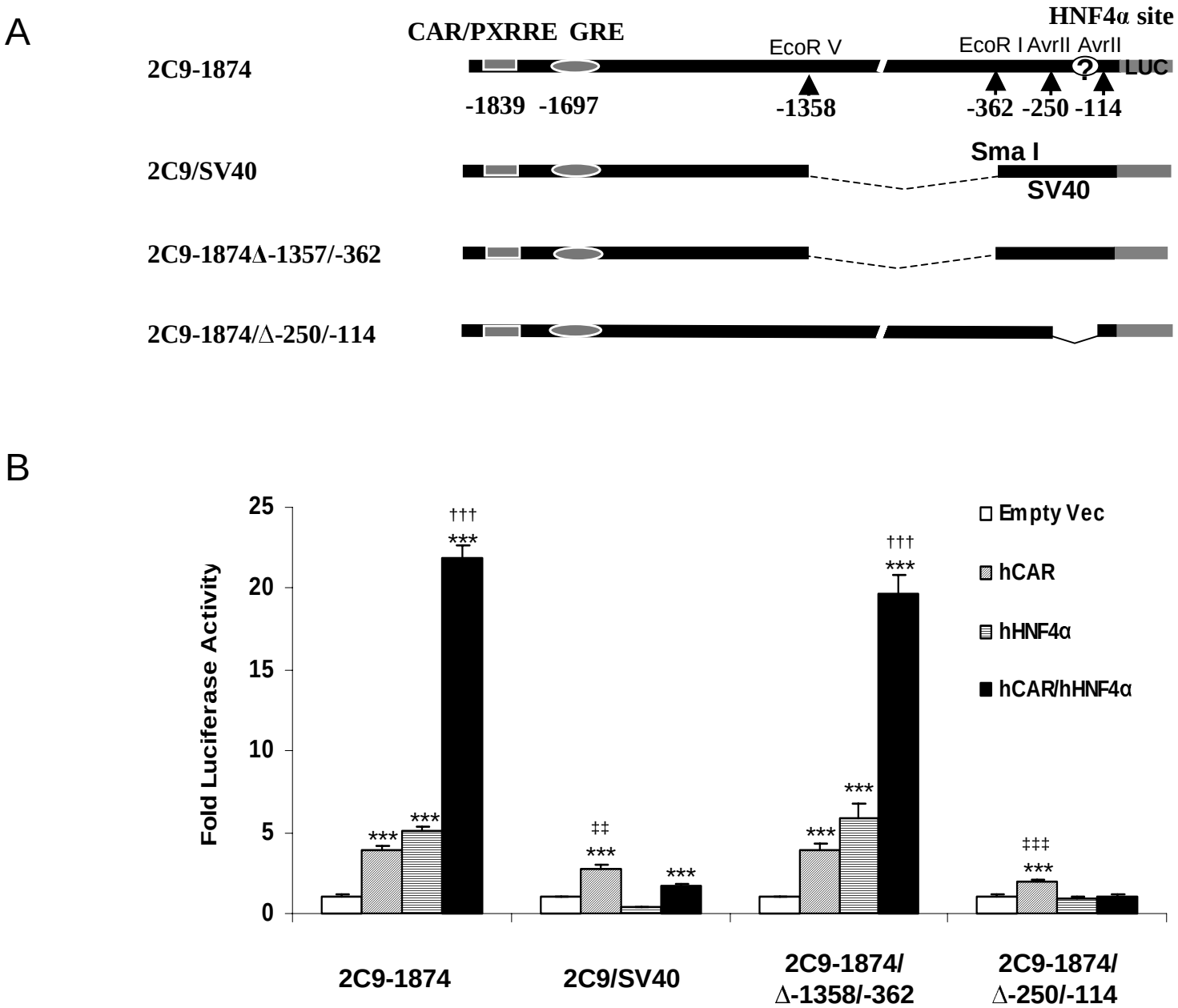

Fig 2

A

2C9-wt	-152	GTATCAGT**GGGTCA**A**AGTCCT**TTC
2C19-wt		CTATCAGT**GGGTCA**A**AGTCCT**TTC
-152 mut1		GTATCAGT**CCCTCA**A**AGTCCT**TTC
-152 mut2		GTATCAGT**GGGTC**T . .**GTCCT**TTC
APF1-wt		GCGC**TGGGCA**a**AGGTCA**CCTGC
APF1-mut		GCGC**TGGCG**A**AGGAGA**CCTGC

B

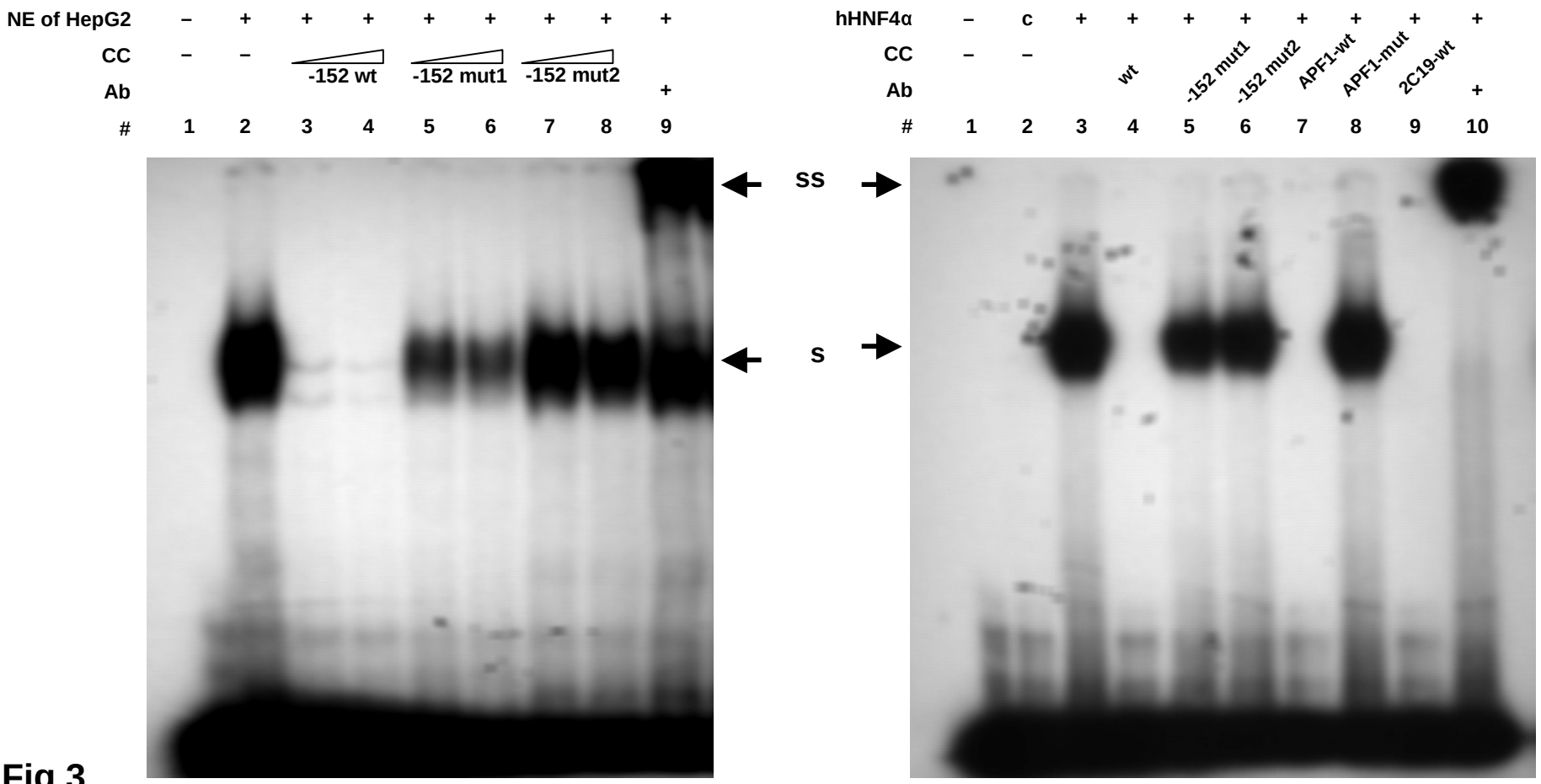

Fig 3

A

2C9-wt	GTATCAGTGGGTCAAAGTCCTTTC
-152 mut1	GTATCAGTCCCTCAAAGTCCTTTC
-152 mut2	GTATCAGTGGGTCT . .GTCCTTTC

B

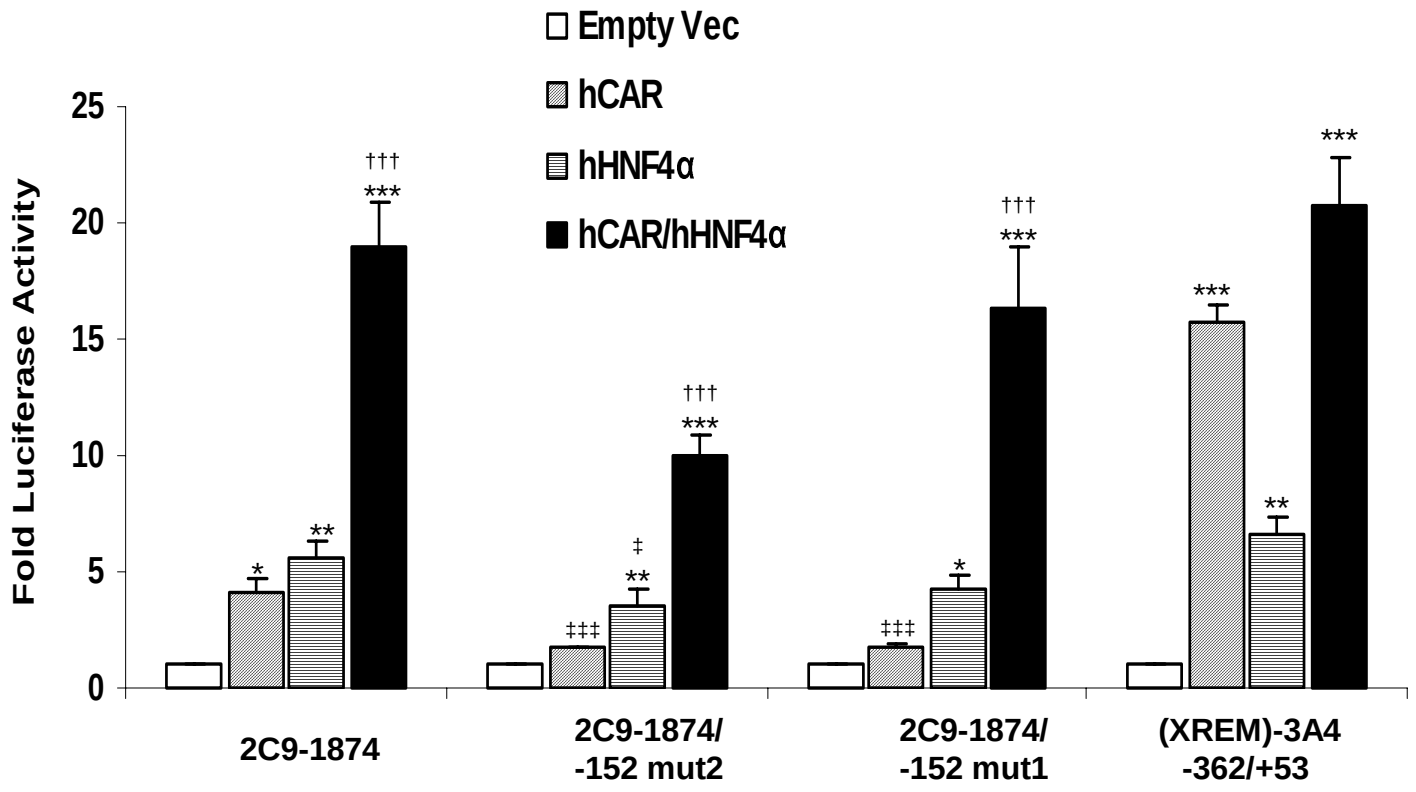

Fig 4

A

-185 wt	AACAAGACCAAAGGACATTTTAT
-185 mut	AACAAGACCT . .GGACATTTTATTTTTATC
-152 wt	GTATCAGTGGGTCAAAGTCCTTTC
APF1 wt	GCGCTGGGCAAAGGTCACCTGC
APF1 mut	GCGCTGGCGAAAGGAGACCTGC

B

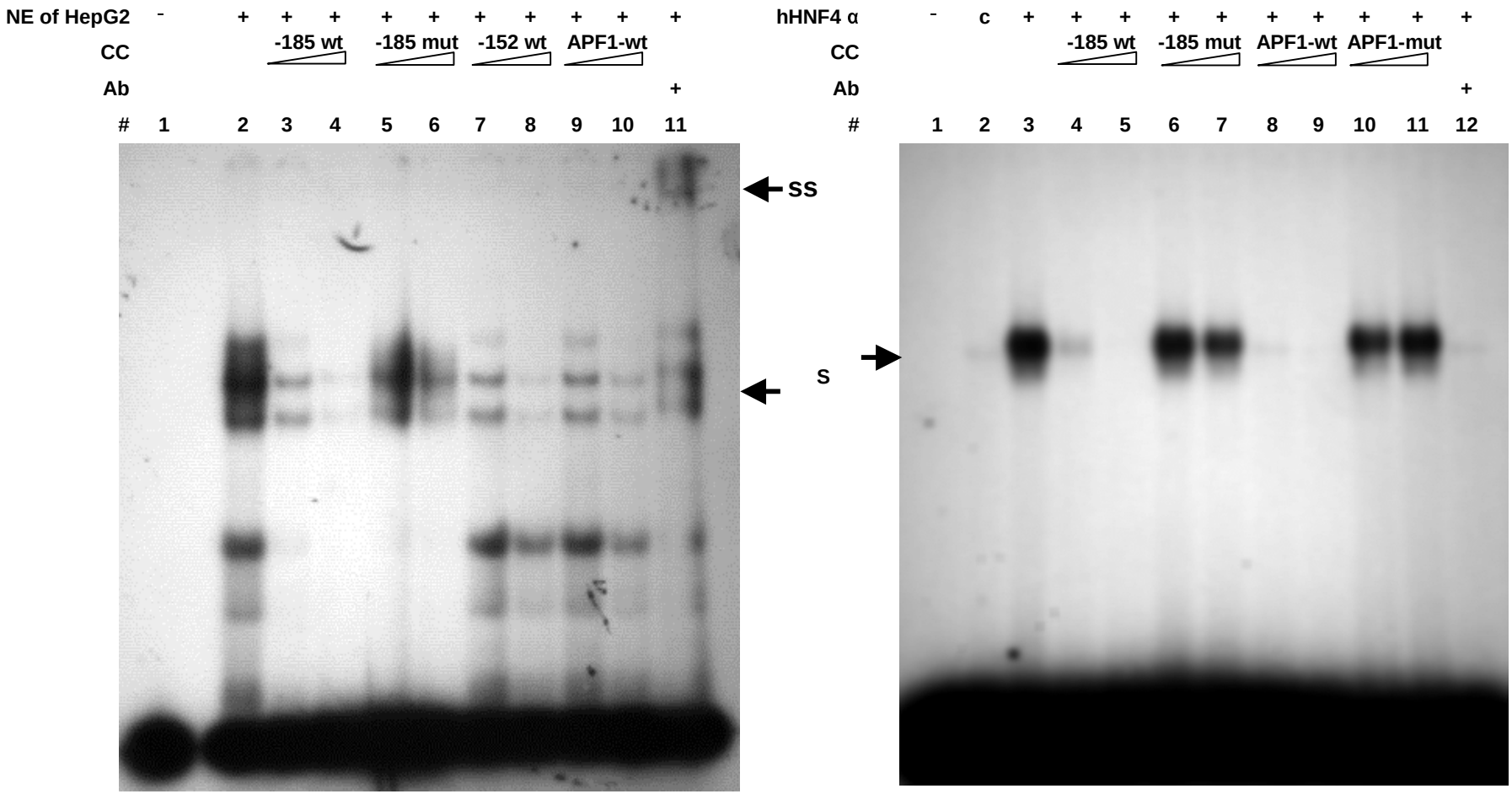

Fig 5

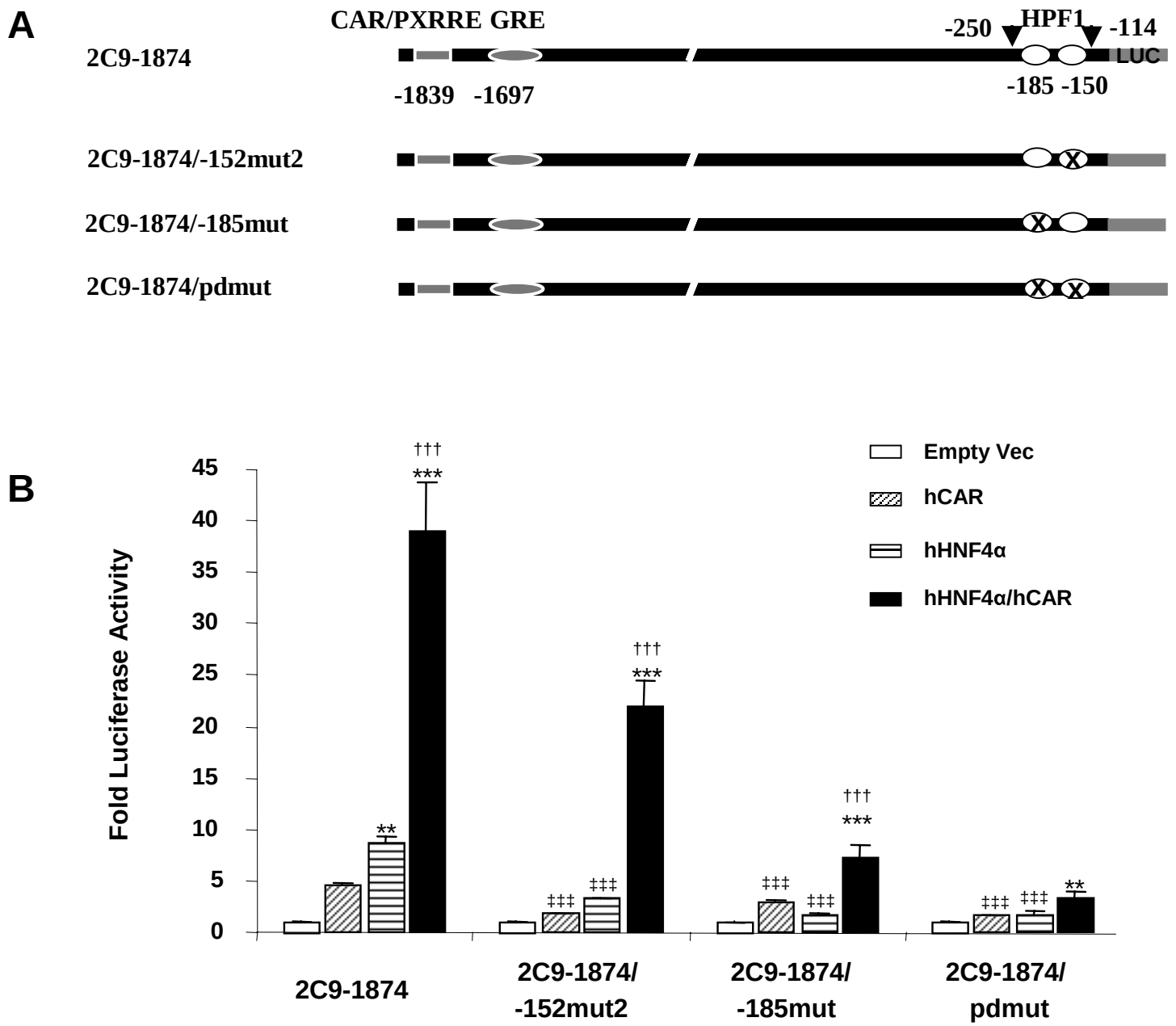

Fig 6

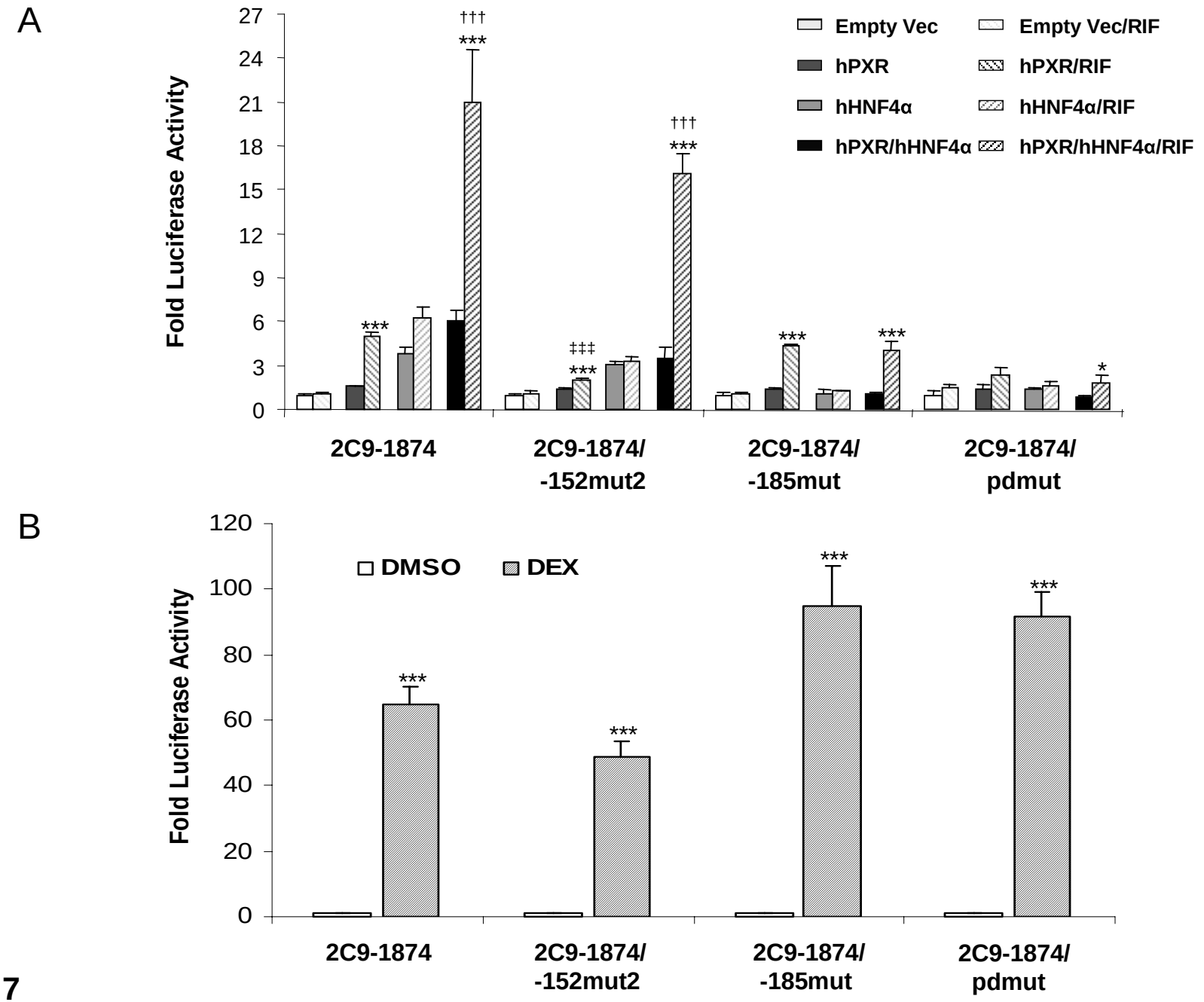

Fig 7

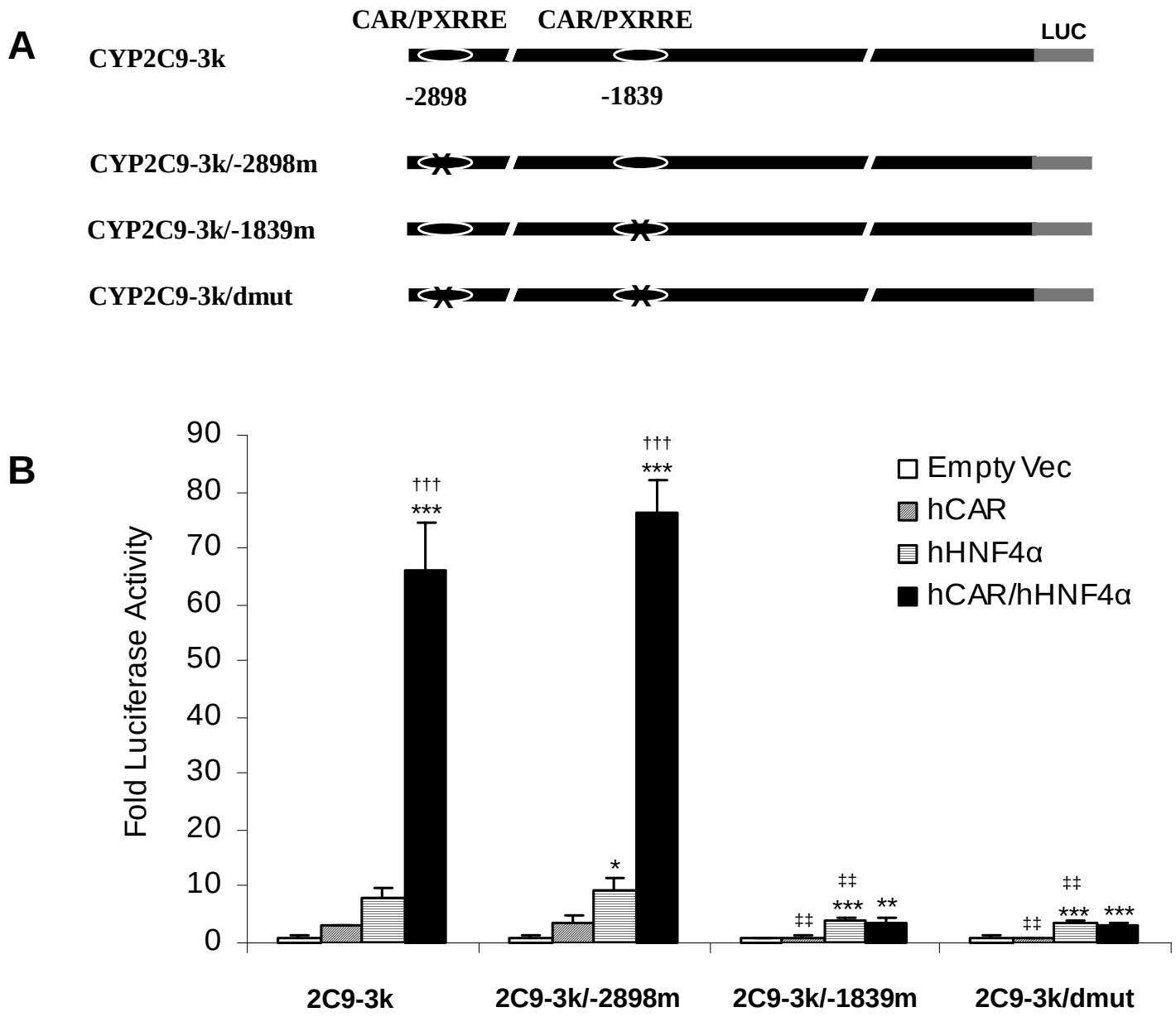

Fig 8