

Unveiling a new essential cis-element for the transactivation of the *CYP3A4* gene by xenobiotics

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Running title

a) Running title: *CYP3A4* transactivation via multiple PXR response elements

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c) The number of text pages	36	
The number of tables	1	
The number of figures	8	
The number of references	33	
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d) Abbreviations used in this paper: AdCont, control adenovirus; AdhPXR, adenovirus expressing hPXR; ANOVA, analysis of variance; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; dNR, distal nuclear receptor-binding element; DR4, direct repeat separated by four nucleotides; EMSA, electrophoretic mobility shift assay; eNR3A4, essential distal nuclear receptor-binding element for *CYP3A4* induction; ER6, everted repeat separated by six nucleotides; hPXR, human PXR; hRXR α , human RXR α ; MEM, minimum essential medium; P450, cytochrome P450; prER6, ER6 in the *CYP3A4* proximal promoter; PXR, pregnane X receptor; RXR α , retinoid X receptor α ; TCID₅₀, 50% titer culture infectious dose; XREM, xenobiotic-responsive enhancer module.

Abstract

Pregnane X receptor (PXR) has been shown to form a heterodimer with retinoid X receptor α (RXR α), and to bind to the distal nuclear receptor-binding element 1 (dNR1) and an everted repeat separated by six nucleotides in the proximal promoter (prER6) of the *CYP3A4* gene. In the present study, a new rifampicin-responsive region, located at -7.6 kb upstream from the transcription initiation site, has been identified using reporter assays in HepG2 cells. This region contains a cluster of possible nuclear receptor-binding half-sites, AG(G/T)TCA-like sequence. Of these putative half-sites, we focused six half-sites and termed α - η half-sites. Introduction of a mutation into either α or β half-site of *CYP3A4* reporter genes almost completely diminished the rifampicin-induced transcription. In electrophoretic mobility shift assays, PXR/RXR α heterodimer bound to the direct repeat separated by four nucleotides (DR4), formed with α and β half-sites. HepG2-based transactivation assays with the reporter gene constructs with or without mutations in the PXR binding element(s) demonstrated that this DR4 motif is essential for the transcriptional activation not only by rifampicin but also by various human PXR activators. In addition, reporter assays performed in human hepatocytes and mice with adenoviruses expressing luciferase derived from various *CYP3A4* reporter genes and that expressing human PXR, supported the results of experiments in HepG2 cells. These results suggest the obligatory role of the newly identified DR4-type PXR binding element of the *CYP3A4* gene for xenobiotic induction of *CYP3A4*.

Introduction

Hepatic and intestinal cytochrome P450s (P450s) play key roles in the oxidative biotransformation of exogenous chemicals such as therapeutic drugs and environmental pollutants (Gonzalez, 1992; Nebert and Russell, 2002). CYP3A4 is predominantly expressed in human liver and intestine, where this form comprises approximately 30 to 70% of the total P450 contents (Kolars et al., 1994; Paine et al., 2006; Shimada et al., 1994). In addition, significant interindividual variations in hepatic and intestinal levels of CYP3A4 have been reported (Wolbold et al., 2003). The hepatic and intestinal expression levels of CYP3A4 are increased *in vivo* after the exposure to variety of drugs (Kocarek et al., 1995; Michalets, 1998; Schuetz et al., 1993), which leads to the accelerated metabolism of the drugs themselves and concomitantly used drugs. Thus, CYP3A4 induction is a physiological adaptation to the exposure, but also a risk factor associated with adverse drug-drug interactions in patients under the combination drug therapy.

A number of independent studies have established that chemicals so called “CYP3A4 inducers” activate the transcription of the *CYP3A4* gene through pregnane X receptor (PXR) (Bertilsson et al., 1998; Blumberg et al., 1998; Lehmann et al., 1998). PXR has been identified as a xenobiotic-responsive transcription factor that regulates multiple drug-metabolizing enzymes and transporters (Geick et al., 2001; Synold et al., 2001; Xie et al., 2000). PXR is abundantly and selectively expressed in the liver and intestine, where induction of CYP3A4 expression occurs (Moore and Kliewer, 2000). Human PXR (hPXR) is activated after the exposure of

structurally diverse xenobiotics, including a macrolide antibiotic rifampicin, an antiepileptic phenobarbital, and a pesticide pyributicarb (Luo et al., 2002; Matsubara et al., 2007). Ligand-activated hPXR was reported to stimulate the transcriptional activation of the *CYP3A4* gene through the binding to its cognate response element(s) within the regulatory region of this gene following dimerization with retinoid X receptor α (RXR α) (Honkakoski et al., 2003).

Studies on the *CYP3A4* gene promoter have uncovered the complex molecular mechanisms underlying the transcriptional regulation of this gene. At first, two copies of an AG(G/T)TCA hexamer were identified as recognition sequences for the nuclear receptor family of transcriptional factors in the proximal promoter of the *CYP3A4* gene (Barwick et al., 1996). Barwick et al. demonstrated that these half-sites, composing an ER6 (everted repeat separated by six nucleotides), conferred a rifampicin-responsiveness on heterologous reporter gene constructs transfected into rabbit, but not rat, hepatocytes. The isolation of hPXR gave a definitive evidence that the receptor binds to the ER6 in the *CYP3A4* proximal promoter (prER6) following the dimerization with RXR α to enhance the transcription of heterologous reporter gene containing multiple copies of the prER6 (Bertilsson et al., 1998; Blumberg et al., 1998; Lehmann et al., 1998). Goodwin et al. demonstrated the existence of another xenobiotic-responsive enhancer module (XREM) located at -8 kb upstream from the transcription start point, and identified distal nuclear receptor-binding element 1 (dNR1) (Goodwin et al., 1999). Both dNR1 and prER6 enhanced the transcription of the *CYP3A4* gene through ligand-activated hPXR. In the report, the

simultaneous mutation of both elements, however, did not completely abolish the rifampicin-mediated reporter gene activation. In addition, our previous study has shown that these two PXR response elements are not enough for the maximal rifampicin-induced transcriptional activation of the *CYP3A4* gene (Takada et al., 2004). These data have prompted us to investigate the possibility that an unidentified cis-element(s) exists in the *CYP3A4* promoter, especially for the rifampicin-induced transactivation of the *CYP3A4* gene.

In the present study, we have sought an additional cis-element(s) involved in the transcriptional activation of the *CYP3A4* gene in response to rifampicin. Using transient transfection assays with several deletion and mutated constructs, we have identified a distinct PXR response element as an essential distal nuclear receptor-binding element for *CYP3A4* induction. Electrophoretic mobility shift assays (EMSAs) detected hPXR binding toward this element. Our results indicate that hPXR binds to three elements in the *CYP3A4* gene promoter, consequently mediating *CYP3A4* induction in response to xenobiotics.

Materials and Methods

Materials

Restriction enzymes and DNA modification enzymes, unless otherwise stated, were purchased from Takara Shuzo (Ohtsu, Japan). T4 polynucleotide kinase and S1 nuclease were from New England BioLabs (Beverly, MA). [γ - 32 P]ATP was purchased from GE Healthcare Bio-Sciences Inc. (Piscataway, NJ). Dulbecco's modified Eagle's medium (DMEM) was from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). William's E medium and media supplements were from Invitrogen (Carlsbad, CA). Matrigel and ITS-premix were from Becton Dickinson (Heidelberg, Germany). KHEM5310 medium was from KAC Co., Ltd (Kyoto, Japan). Fetal bovine serum, rifampicin, RU486 [11 β -(4-dimethylamino)phenyl-17 β -hydroxy-17-(1-propynyl)estra-4,9-dien-3-one], SR12813 [tetraethyl 2-(3,5-di-*tert*-butyl-4-hydroxyphenyl)ethenyl-1,1-bisphosphonate] and nifedipine were purchased from Sigma-Aldrich (St. Louis, MO). T0901317 [*N*-(2,2,2-trifluoroethyl)-*N*-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl]-benzenesulfonamide] was purchased from Cayman Chemical (Ann Arbor, MI). Hyperforin was purchased from Alexis Biochemicals (San Diego, CA). All other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Oligonucleotides were synthesized by Nissinbo (Tokyo, Japan).

Reporter gene constructs and expression vectors

The luciferase reporter plasmid, pGL3-Basic, was purchased from Promega (Madison, WI). Preparation of the chimeric *CYP3A4*-luciferase reporter gene constructs, including pCYP3A4-362-7.7k, pCYP3A4-362-7.7kΔB, pCYP3A4-362-7.7kΔ*Spe*I, pCYP3A4-362-7.7km, pCYP3A4-362m-7.7k, pCYP3A4-362m-7.7km, pCYP3A4-362, was described previously (Takada et al., 2004).

Deletion constructs of pCYP3A4-362-7.7k were prepared as follows; pCYP3A4-362-7.7k was digested with *Pvu* II and *Bgl* II, and then the fragment was inserted into *Sma* I and *Bgl* II sites of pCYP3A4-362 (pCYP3A4-362-7.7kΔ*Pvu*II). pCYP3A4-362-7.7k was digested with *Mlu* I and *Spe* I, treated with S1 nuclease and then self-ligated [pCYP3A4-362-7.7k(dNR3)]. Polymerase chain reaction (PCR) was carried out using primers 5'-AGATGCTTTCATCAGATTAAG-3' and 5'-CTTGTTCTTGTCAGAAGTTCAG-3' with pCYP3A4-362-7.7k as a template. The PCR product was phosphorylated by using T4 polynucleotide kinase and self-ligated [pCYP3A4-362-7.7kΔ(7564-7386)]. pBlue3A4-362-7.7k, which was made by inserting the pCYP3A4-362-7.7k DNA fragment into *Kpn* I and *Hind* III sites of pBluescriptII-SK+ (Stratagene, La Jolla, CA), was digested with *Hinc* II, and self-ligated. This construct was further digested with *Kpn* I and *Hind* III, and then the fragment was inserted into the same restriction sites of pGL3-Basic (pCYP3A4-362-7.7kΔ*Hinc*II).

Mutated constructs targeted on each putative half-site at about 7.6 kb were prepared from the pCYP3A4-362-7.7k by PCR using the targeting primers shown in Table 1. The underlined letters of the primer sequences indicate the nucleotides substituted.

pCYP3A4-362-7.7kΔeNR3A4 was also prepared from the pCYP3A4-362-7.7k by PCR using the sense primer targeted on β half-site and the antisense primer targeted on α half-site shown in Table 1. In this construct, eNR3A4 (DR4 motif) was changed to CTCGAG (*Xho* I motif). The sequences of all the *CYP3A4* reporter gene constructs were verified by direct DNA sequencing.

The hPXR cDNA, in which CTG initiation codon was modified to ATG, was isolated by PCR with primers 5'-GGA₂CTCGAGATGGAGGTGAGACCCAAAGAAAGC-3' and 5'-GGATCTAGACTTTCAGCTACCTGTGATGCCGAA-3', and inserted into pGEM-T vector (Promega). The plasmid was digested with *Xho* I and *Not* I, and then the cDNA was inserted into the same restriction sites of pT_NTTM vector (Promega) for *in vitro* translation. The human RXR α (hRXR α) cDNA was isolated by PCR with primers 5'-GAACTCGAGGACATGGACACCAAACATTTGGTG-3' and 5'-CGCTCTAGACTAAGTCATTTGGTGCGGCGCCTC-3', and inserted into pT_NT as for hPXR. These products were named as pT_NT-hPXR Δ ATG and pT_NT-hRXR α , respectively.

Cell culture and transfections

HepG2 cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, MEM Non-Essential Amino Acids and Antibiotic-Antimycotic (Invitrogen). Cells were seeded onto 12-well plates (Becton Dickinson, Franklin lakes, NJ) at a density of 1×10^5 cells/well, and 24 hr later the cells were

transfected with 1 μ g of luciferase reporter gene construct and 1 μ g of pSV- β -Galactosidase Control Vector (Promega) for each well by using CellPfect Transfection Kit (GE Healthcare Bio-Sciences Inc.). After the transfection, cells were cultured in serum-free DMEM in the presence of various chemicals for 48 hr. Control cells were cultured with vehicle [0.1% dimethyl sulfoxide (DMSO)] alone. Subsequently, the cells were harvested, and suspended in 1x reporter lysis buffer (Promega). Luciferase activities were determined with Luciferase reporter assay system (Promega). To normalize transfection efficiency, β -galactosidase assays were performed as described elsewhere (Herbomel et al., 1984).

Electrophoretic mobility shift assay

hPXR and hRXR α were synthesized *in vitro* from pT_NT-hPXR Δ ATG and pT_NT-hRXR α respectively, using the T_NT[®] SP6 Coupled Reticulocyte Lysate Systems (Promega) following the manufacturer's protocol. Control lysate was prepared using the empty pT_NT vector. The sequences of oligonucleotide probes and competitors are shown in Fig. 4A. Double-stranded oligonucleotides were labeled with [γ -³²P]ATP using T4 polynucleotide kinase and purified with NAP5 columns (GE Healthcare Bio-Sciences Inc.). The binding reaction was carried out with a reaction mixture (10 μ l) containing 10 mM Tris-HCl (pH 8.0), 5% glycerol, 100 mM KCl, 2 mM dithiothreitol, 1 μ g of poly(dI-dC) (GE Healthcare Bio-Sciences Inc.), and 1 μ l each of synthesized hPXR- or hRXR α -containing lysate, or control lysate. Reactions were preincubated on ice for 1 hr before the addition of

³²P-labeled probe (35 fmol). Competitor oligonucleotides were included at various concentrations as indicated in each figure. Samples were kept on ice for additional 30 min and then separated on 4% polyacrylamide gel in 0.5 x Tris-boric acid-EDTA buffer at 40 mA. The gel was dried and exposed to an imaging plate to detect DNA-protein complexes with Fuji Bio-Imaging Analyzer FLA-3000 (Fuji Film, Tokyo, Japan). Antibody to hPXR was prepared in a previous study (Matsubara et al., 2007) and added to the reaction mixture before preincubation.

Construction of recombinant adenovirus

Construction of the *CYP3A4* reporter adenovirus, AdCYP3A4-362-7.7k, was performed previously (Matsubara et al., 2007). pCYP3A4-362m-7.7km and pCYP3A4-362-7.7kΔβγ were digested with *Kpn* I and *Xba* I, and the fragments were inserted into the same restriction sites of the pShuttle vector (Quantum Biotechnologies, Laval, QC, Canada). Then mutated *CYP3A4* reporter adenoviruses were obtained as described previously (Matsubara et al., 2007). The recombinant adenoviruses were isolated and propagated in HEK293 cells according to the manual from Quantum Biotechnologies.

Adenovirus that expresses hPXR (AdhPXR) was reported previously (Noracharttiyapot et al., 2006). Control adenovirus that expresses β-galactosidase (AdCont; AxCALacZ) was provided by Dr. Izumi Saito (The University of Tokyo, Tokyo, Japan). The titer of adenoviruses, 50% titer culture infectious dose (TCID₅₀), was

determined as reported previously (Matsubara et al., 2007).

Human hepatocyte culture and infection of recombinant adenovirus

Cryopreserved human hepatocytes (lot. H704; Caucasian, female, 49 years old) were purchased from XenoTech LLC (Lenexa, KS) and thawed using Hepatocytes isolation Kit (XenoTech LLC) according to the manufacture's protocol. The cells were plated onto collagen-coated 24-well plate (Becton Dickinson) at a density of 1×10^5 cells/well and maintained in KHEM5310 medium supplemented with 10% fetal bovine serum and Antibiotic-Antimycotic for 4 hr in an atmosphere of 5% CO₂/95% air at 37°C. The medium was then changed to serum-free Williams' E medium with 0.1 μM dexamethasone, ITS-premix, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL Matrigel, and the cells were cultured for 24 hr. After removal of the medium, the hepatocytes were incubated with 0.1 mL of the recombinant adenoviruse-containing medium for 1 hr followed by the addition of 0.4 mL of the medium containing a chemical. After 24 hr, the medium was changed to the fresh medium containing the chemical and the cells were cultured for an additional 24 hr. Subsequently, luciferase and β-galactosidase activities were measured with hepatocyte lysates using the methods as described above. Multiplicity of infection (MOI) was calculated by dividing the TCID₅₀ with the number of cells.

***In vivo* reporter assay in mice**

Male ICR mice purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan) were used at 6 weeks old weighing 30-33 g. After 20 hr fasting, the mice were injected intraperitoneally with adenoviruses (1×10^{10} TCID₅₀/mouse). Seven days after the infection, the mice were treated intraperitoneally with vehicle (25% polyethylene glycol 300, 15% DMSO) alone or rifampicin (100 mg/kg/day) for three consecutive days and sacrificed 24 hr after the last dose. S-9 fractions were prepared and used for the determination of luciferase activities and protein concentrations as reported previously (Furukawa et al., 2002).

Statistical analysis

GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA) was used for all the statistical analyses.

Results

Identification of a cis-element in the distal promoter region of the *CYP3A4* gene.

Using *CYP3A4*-luciferase reporter plasmids lacking portions of the distal promoter region (-7836 to -7200) of pCYP3A4-362-7.7k, transient transfection assays were performed in HepG2 cells (Fig. 1). Treatment of HepG2 cells with 10 μ M rifampicin resulted in the increase of luciferase activity 14-fold after transfection of pCYP3A4-362-7.7k, in which both -7836 to -7200 of the distal promoter region and -362 to +11 of the proximal promoter region of the *CYP3A4* gene are contained. Removal of nucleotides from -7836 to -7738 in the distal promoter region partially diminished but did not completely lose the reporter activity (8.2-fold). Further deletion of nucleotides from -7836 to -7493 resulted in the complete loss of the rifampicin response (0.9-fold).

The constructs lacking nucleotides from -7495 to -7200 or from -7565 to -7384 showed slightly reduced rifampicin responses compared to pCYP3A4-362-7.7k (9-fold and 6.7-fold, respectively vs 16-fold for the wild-type). The deletion of nucleotides from -7608 to -7215, however, completely abolished the response (1.1-fold), regardless of the presence of both dNR1 and prER6. These results suggest the existence of an unidentified region essential for the transcriptional activation of the *CYP3A4* gene in response to rifampicin, between -7608 and -7565.

This region contains a cluster of possible nuclear receptor-binding half-sites, AG(G/T)TCA and related sequences, as shown in Fig. 2. Of these putative half-sites, we focused six half-sites and termed them α - η

half-sites in a direction from the 5' to 3'. Only the δ half-site preserves a consensus AG(G/T)TCA sequence, and the others have one or two dissimilarities. To assess roles of these half-sites in the transcriptional activation of the *CYP3A4* gene in response to rifampicin, a series of mutated reporter gene constructs was used. As shown in Fig. 3, rifampicin-induced activations were substantially lost with the mutated construct of either α or β half-site (1.5-fold and 1.5-fold, respectively). On the other hand, the introduction of a mutation into other half-sites had no drastic effects on the rifampicin response. These results raised a possibility that hPXR/hRXR α heterodimer binds to the direct repeat separated by four nucleotides (DR4) formed with α and β half-sites. Thus, we termed the DR4 as eNR3A4 (the essential distal nuclear receptor-binding element for *CYP3A4* induction).

Binding of hPXR/hRXR α heterodimer to eNR3A4.

To examine the direct binding of hPXR/hRXR α heterodimer to eNR3A4, electrophoretic mobility shift assays (EMSAs) were carried out with *in vitro* synthesized hPXR and hRXR α and the double-stranded oligonucleotides corresponding to unmutated or mutated putative PXR binding motifs (Fig. 4A). Consistent with the previous reports (Goodwin et al., 1999), hPXR/hRXR α heterodimer bound to both dNR1 and prER6 with high affinities (Fig. 4B). hPXR/hRXR α heterodimer also bound to eNR3A4 with a similar affinity, but not to a DR4 motif that consisted of β and γ half-sites ($\beta\gamma$).

The binding specificity of eNR3A4 for hPXR/hRXR α was investigated with competition assays. The specific

complex including eNR3A4 was completely competed out by either a 50-fold excess of unlabeled eNR3A4 or dNR1 but not by mut_dNR1 (Fig. 4C). In agreement with these results, the binding of hPXR/hRXR α to dNR1 was effectively blocked with wild-type (dNR1 and eNR3A4) but not mutated eNR3A4 competitors (mut5'_eNR3A4 and mut3'_eNR3A4) (Fig. 4D). Furthermore, quantitative competition assays were performed with labeled eNR3A4 and dNR1, and respective cold probes at various concentrations (x1, x2, x4, x10 or x20 of the labeled probe). The results of these assays demonstrated that the affinity of hPXR/hRXR α for eNR3A4 is comparable with or slightly lower than those for dNR1 (Supplement data). When a specific antibody against hPXR was included in binding reactions, supershifted complexes were observed with both elements (Figs. 4C and 4D).

Functional evaluation of eNR3A4.

To elucidate the role of eNR3A4 in the rifampicin-induced transcriptional activation of the *CYP3A4* gene, we first performed transient transfection assays with the reporter gene constructs with mutations in putative hPXR binding element(s) (Fig. 5). As reported previously, the mutation of either dNR1 or prER6, and the mutation of both elements did not abolish the rifampicin response completely (4.8-fold, 7.4-fold, and 3.5-fold respectively vs 16-fold for the wild-type). On the other hand, the response was completely abolished by the introduction of a mutation into either α or β half-site of eNR3A4. This unresponsiveness was also observed with the construct

containing the proximal promoter alone.

To investigate whether eNR3A4 functions in the transcription of the *CYP3A4* gene in human hepatocytes, we performed reporter assays in human hepatocytes with adenoviruses expressing each of *CYP3A4*-luciferase reporters (AdCYP3A4-362-7.7k, AdCYP3A4-362m-7.7km and AdCYP3A4-362-7.7k $\Delta\beta\gamma$). As shown in Fig. 6, the treatment of cells with 10 μ M rifampicin for 48 hr increased the luciferase activity 34- and 10-fold in cells infected with AdCYP3A4-362-7.7k and AdCYP3A4-362m-7.7km, respectively. In contrast, the treatment had no effect on the reporter activities in cells infected with AdCYP3A4-362-7.7k $\Delta\beta\gamma$, lacking the β half-site.

To further confirm whether eNR3A4 plays an essential role in the hPXR-dependent activation of the *CYP3A4* gene transcription *in vivo*, *in vivo* reporter assays were performed in mouse livers using the adenoviruses expressing each of *CYP3A4*-luciferase reporters (AdCYP3A4-362-7.7k, AdCYP3A4-362m-7.7km and AdCYP3A4-362-7.7k $\Delta\beta\gamma$) and hPXR (AdhPXR) (Fig. 7). Treatment with rifampicin clearly increased luciferase activities in mouse livers infected with AdCYP3A4-362-7.7k and AdhPXR (7742-fold), whereas the rifampicin treatment insignificantly affected the reporter activities in mouse livers infected with AdCYP3A4-362-7.7k and AdCont. Consistent with the results of *in vitro* reporter assays in HepG2 cells and human hepatocytes, the introduction of mutations into both dNR1 and prER6 did not abolish the rifampicin response completely (339-fold). Not surprisingly, the construct with partial deletion of eNR3A4 showed no response to rifampicin even in mice infected with hPXR-expressing adenovirus.

eNR3A4 for the transcriptional activation of the *CYP3A4* gene by various CYP3A inducers.

To verify whether eNR3A4 is necessary for the chemical-induced transcriptional activation of the *CYP3A4* gene other than rifampicin, HepG2 cells were transfected with either pCYP3A4-362-7.7k or pCYP3A4-362-7.7kΔeNR3A4, in which eNR3A4 was deleted. As shown in Fig. 8, pCYP3A4-362-7.7k was potently transactivated by treatment with rifampicin (8.0-fold), modestly with phenobarbital (4.2-fold), RU486 (3.9-fold), SR12813 (3.8-fold), pyrbuticarb (3.6-fold), and T0901317 (3.1-fold) and weakly with hyperforin (2.0-fold) and nifedipine (1.9-fold). As expected, the deletion of eNR3A4 drastically diminished the chemical-mediated activations of the reporter gene.

Discussion

hPXR response elements such as dNR1 and prER6 have been identified in the *CYP3A4* gene promoter (Barwick et al., 1996; Goodwin et al., 1999). These dNR1 and prER6 are necessary for the maximal PXR-mediated activation of the *CYP3A4* gene transcription (Goodwin et al., 1999). Our previous results, however, have raised a possibility that an unidentified cis-element(s), which probably exists in the distal promoter sequence, is involved in the PXR-mediated transcriptional activation of the *CYP3A4* gene (Takada et al., 2004). In the present study, we have indicated the localization of a novel rifampicin-response region in the distal promoter of the *CYP3A4* gene and identified a distinct PXR response element, termed eNR3A4, to which hPXR binds as a heterodimer with hRXR α .

Functional evaluation of the role of eNR3A4 was performed with the *CYP3A4* reporter gene constructs that have a mutation(s) in three PXR response elements (dNR1, prER6 and eNR3A4) in HepG2 cells. Interestingly, the introduction of a mutation into eNR3A4 completely abolished the rifampicin-induced transcriptional activation, while those into dNR1 and/or prER6 did not (Fig. 5). In the human hepatocytes, the most reliable *in vitro* model for evaluating the xenobiotic-induced induction of P450s (Li et al., 1997), partial deletion of eNR3A4 completely eliminated the rifampicin response (Fig. 6). In contrast, the introduction of mutations into both dNR1 and prER6 only partially reduced the response (Fig. 6). In addition, *in vivo* reporter assays performed in mouse livers with adenoviruses (Fig. 7), supported the results of *in vitro* experiments in HepG2 cells and

human hepatocytes. These data are consistent with the idea that eNR3A4 is a key element for CYP3A4 induction to function as an on/off switch in the transcriptional activation of the *CYP3A4* gene mediated through hPXR. Further experiments showed that deletion of eNR3A4 caused clear disappearance of reporter gene activations in response to phenobarbital, RU486, SR12813, pyrbuticarb, T0901317, hyperforin, nifedipine as well as rifampicin (Fig. 8). These compounds were previously reported to activate the transcription of the *CYP3A4* gene through hPXR (Bertilsson et al., 1998; Drocourt et al., 2001; Duniec-Dmuchowski et al., 2007; Jones et al., 2000; Lehmann et al., 1998; Luo et al., 2002; Matsubara et al., 2007; Moore et al., 2000). These findings further support the general function of eNR3A4 in hPXR-mediated CYP3A4 induction.

Present results propose the hPXR-mediated transcriptional regulation of the *CYP3A4* gene through eNR3A4 as well as dNR1 and prER6. hPXR-mediated transcriptional regulation via multiple response elements is also observed in other genes such as *CYP2B6* and *CYP2C9* and the localization of plural PXR-interacting cis-elements have been defined with these P450 genes (Chen et al., 2004; Wang et al., 2003). These data may suggest that PXR-dependent induction of hepatic P450 forms is under the influence of the number of cis-elements interacting with PXR.

Until now, numbers of researchers have investigated the transcriptional regulation of the *CYP3A4* gene using many reporter gene constructs. Goodwin et al. (Goodwin et al., 1999) using a reporter gene construct containing the distal promoter (-7836 to -7208) and proximal promoter (-362 to +53) of the *CYP3A4* gene have

demonstrated that both sequences are required for the maximal transactivation through hPXR. Actually, eNR3A4 was included in all of the reporter gene constructs displaying rifampicin responsiveness in their works, although the role of eNR3A4 had not been recognized. This is also the case with several previous reports on established cell lines, stably expressing the luciferase reporter gene through the distal and proximal promoter of the *CYP3A4* gene, for assessing CYP3A4 induction (Lemaire et al., 2004; Noracharttiyapot et al., 2006).

In conclusion, we have identified a novel PXR response element, termed eNR3A4, located at around 7.6 kb upstream from the transcription initiation site of the *CYP3A4* gene, to which hPXR/hRXR α heterodimer binds. The results obtained from reporter assays in HepG2, human hepatocytes and mouse livers demonstrate that eNR3A4 is a key regulatory element for the xenobiotic induction of CYP3A4 through hPXR. Moreover, the present results suggest the distinct role of hPXR-interacting cis-elements for the transcriptional activation of the *CYP3A4* gene.

Acknowledgments

We thank Dr. Izumi Saito (The University of Tokyo, Tokyo, Japan) for providing the AxCALacZ and Tsubasa

Chikada (Tohoku University, Sendai, Japan) for his excellent technical assistance.

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Footnotes

This work was supported in part by Grant-in Aid from Ministry of Education, Culture, Sports, Sciences and Technology of Japan and Ministry of Health, Labor and Welfare of Japan.

This work was presented in part at 8th international meeting of the International Society for the Study of Xenobiotics (Oct 9-12, 2007, Sendai, Japan).

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Legends to Figures

Fig. 1: Influence of partial deletion of the distal promoter on rifampicin-induced transcription of the *CYP3A4* gene.

A series of *CYP3A4*-luciferase reporter gene constructs was prepared as described in *Materials and Methods* and shown on the left. Numbers indicate the positions relative to the transcriptional start site. These reporter gene constructs were transiently transfected into HepG2 cells. The cells were treated with rifampicin (10 μ M) or vehicle (0.1% DMSO) for 48 hr before harvest, and luciferase and β -galactosidase activities were determined. Luciferase activities were normalized with β -galactosidase activities. Fold induction represents the ratio of the activities in rifampicin-treated cells to those in vehicle-treated cells for each construct. Data are mean \pm S.D. of four determinations from a single representative experiment. * P < 0.05; ** P < 0.01; significantly different from the vehicle-treated cells based on one-way ANOVA followed by Tukey's post hoc test.

Fig. 2: Sequence of the novel rifampicin-responsive region of the *CYP3A4* gene.

The shaded box indicates the newly-identified rifampicin-responsive region, which includes a cluster of AG(G/T)TCA-like sequences. Of these putative half-sites, six half-sites underlined and indicated by arrows were named α - η half-sites from the 5' to 3' direction. Numbers indicate the positions relative to the transcriptional start site. Closed boxes represent sequences used for the construction of reporter genes. dNR1 and prER6 are

shown as open boxes.

Fig. 3: Influence of mutation of putative nuclear receptor-binding half-site on the response to rifampicin.

A schematic representation of the mutated reporter gene constructs is shown on the left. Using the *CYP3A4*-luciferase reporter gene construct (pCYP3A4-362-7.7k), each putative half-site contained in the cluster was mutated to CTCGAG (indicated by X) as described in *Materials and Methods*. Numbers indicate the positions relative to the transcriptional start site. These reporter gene constructs were transiently transfected into HepG2 cells. The cells were treated with rifampicin (10 μ M) or vehicle (0.1% DMSO) for 48 hr before harvest, and luciferase and β -galactosidase activities were determined. Luciferase activities were normalized with β -galactosidase activities. Fold induction represents the ratio of the activities in rifampicin-treated cells to those in vehicle-treated cells for each construct. Data are mean \pm S.D. of four transfections from a single representative experiment. ****** $P < 0.01$; significantly different from the vehicle-treated cells based on one-way ANOVA followed by Tukey's post hoc test.

Fig. 4: Binding of hPXR/hRXR α to eNR3A4 in electrophoretic mobility shift assays.

A. Sequences of the oligonucleotides used for EMSA are shown. Putative nuclear receptor-binding half-sites are shown in uppercase and bold letters. Mutated nucleotides are underlined. B. EMSA were performed with

radiolabeled prER6, dNR1, eNR3A4 and $\beta\gamma$. Incubations were carried out with *in vitro* synthesized hPXR, and/or hRXR α as indicated. An asterisk indicates nonspecific bindings. C and D. Competition assays were performed with radiolabeled eNR3A4 (C) or dNR1 (D) as probes. Ten- or fifty-fold molar excess of unlabeled competitors were added to the reaction, as indicated in figure. Supershift analyses were carried out with 1 μ g of polyclonal antibody against hPXR protein.

Fig. 5: Evaluation of the role of three PXR binding elements in the rifampicin-induced transactivation of the CYP3A4 gene in HepG2 cells.

PXR binding elements in the pCYP3A4-362-7.7k were mutated as described in *Materials and Methods*, and their rifampicin-responsiveness was analyzed in transient transfection assays. Open and closed boxes represent wild-type and mutated PXR binding elements, respectively. Numbers indicate the positions relative to the transcriptional start site. Each reporter gene construct was transiently transfected into HepG2 cells. The cells were treated with rifampicin (10 μ M) or vehicle (0.1% DMSO) for 48 hr before harvest, and luciferase and β -galactosidase activities were determined. Luciferase activities were normalized with β -galactosidase activities. Fold induction represents the ratio of the activities in rifampicin-treated cells to those in vehicle-treated cells for each construct. Data are mean \pm S.D. of six transfections from a single representative experiment. **** $P < 0.01$** ; significantly different from the vehicle-treated cells based on one-way ANOVA followed by Tukey's post hoc

test.

Fig. 6: Reporter assays with adenoviruses expressing *CYP3A4*-luciferase reporter genes in human hepatocytes.

The schematic structures of the *CYP3A4* reporter adenoviruses are shown on the left. Open and closed boxes represent wild-type and mutated PXR binding elements, respectively. Human hepatocytes were infected with each reporter adenovirus (MOI of 60) and β -galactosidase-expressing AdCont (MOI of 20). The cells were treated with rifampicin (10 μ M) or vehicle (0.1% DMSO) for 48 hr before harvest, and luciferase and β -galactosidase activities were determined. Luciferase activities were normalized with β -galactosidase activities. The normalized activities in the vehicle-treated cells are set at 1 for each construct. Data are mean \pm S.D. of four transfections from a single representative experiment. ** $P < 0.01$; significantly different from the vehicle-treated cells based on one-way ANOVA followed by Tukey's post hoc test.

Fig. 7: *In vivo* reporter assays with adenoviruses expressing *CYP3A4*-luciferase reporter genes in mice.

AdCYP3A4-362-7.7k, AdCYP3A4-362m-7.7km or AdCYP3A4-362-7.7k $\Delta\beta\gamma$ (5.0×10^9 TCID₅₀/mouse) in combination with AdCont or AdhPXR (5.0×10^9 TCID₅₀/mouse) was intraperitoneally injected into mice. These mice were orally administered with rifampicin (100 mg/kg/day) or vehicle for 3 days before sacrifice. The

animals were sacrificed on the tenth day and livers were collected. Luciferase activities were measured as described in *Materials and Methods*, and normalized with protein concentrations. Data are expressed as a ratio of the luciferase activities to those in the AdCont-infected and vehicle-treated mice for each construct. Columns and bars represent the mean \pm S.D., respectively (n = 3 - 5). ***P* < 0.01; significantly different from the corresponding vehicle-treated mice based on one-way ANOVA followed by Tukey's post hoc test.

Fig. 8: Role of eNR3A4 in the xenobiotic-induced transcriptional activation of the *CYP3A4* gene.

The ability of various CYP3A inducers to transactivate pCYP3A4-362-7.7k through eNR3A4 was tested. HepG2 cells were transiently transfected with pCYP3A4-362-7.7k or pCYP3A4-362-7.7k Δ eNR3A4, in which eNR3A4 was deleted. The cells were treated with rifampicin (10 μ M), phenobarbital (1 mM), RU486 (10 μ M), SR12813 (10 μ M), pyributicarb (3 μ M), T0901317 (100 nM), hyperforin (100 nM), nifedipine (10 μ M) or vehicle (0.1% DMSO) for 48 hr before harvest, and luciferase and β -galactosidase activities were determined. Luciferase activities were normalized with β -galactosidase activities. Fold induction represents the ratio of the activities in rifampicin-treated cells to those in vehicle-treated cells for each construct. Data are mean \pm S.D. of four transfections from a single representative experiment. **P* < 0.05; ***P* < 0.01; significantly different from the vehicle-treated cells based on one-way ANOVA followed by Dunnett's post hoc test. † *P* < 0.05; †† *P* < 0.01; significantly different from the cells transfected with pCYP3A4-362-7.7k based on unpaired Student's t test.

Table 1: Oligonucleotides used for the preparation of mutated and deletion constructs of the *CYP3A4*

reporter gene.

The underlined letters indicate the nucleotides substituted.

Half-site	Strand	Sequence (5' → 3')
α	Sense	GCG <u>CTCGAG</u> GTGTTGACCCAGGTGAATC
	Antisense	GCG <u>CTCGAG</u> AGGTTTAATAATCACTG
β	Sense	CGC <u>CTGGAG</u> CAGGTGAATCACAAGCTGAAC
	Antisense	CGC <u>CTCGAG</u> CAACACAGGACAAGG
γ	Sense	GCG <u>CTCGAG</u> ACAAGCTGAACTTCTGACAAGAACAAGC
	Antisense	GCG <u>CTCGAG</u> CTTGTGATTCACCTGGGGTCAACACAGG
δ	Sense	CGC <u>CTCGAG</u> TCTGACAAGAACAAGC
	Antisense	CGC <u>CTCGAG</u> GCTTGTGATTCACCTGG

ε	Sense	GCG <u>CTCGAG</u> TGTCAGAAAGTTCAGCTTGTG
	Antisense	CGC <u>CTCGAG</u> GAAGTTCAGCTTGTGATTAC
η	Sense	CGC <u>CTCGAG</u> GCTTGTGATTACCTGG
	Antisense	GCG <u>CTCGAG</u> TGTCAGAAAGTTCAGCTTGTG

Fig. 1.

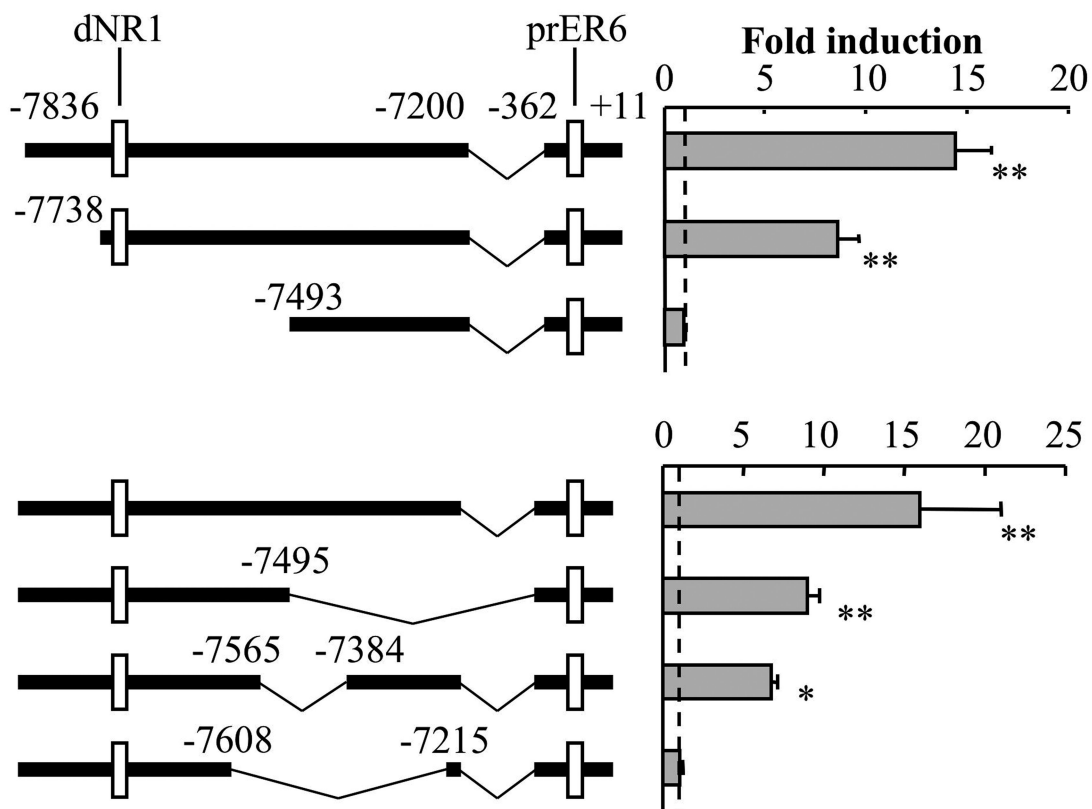


Fig. 2.

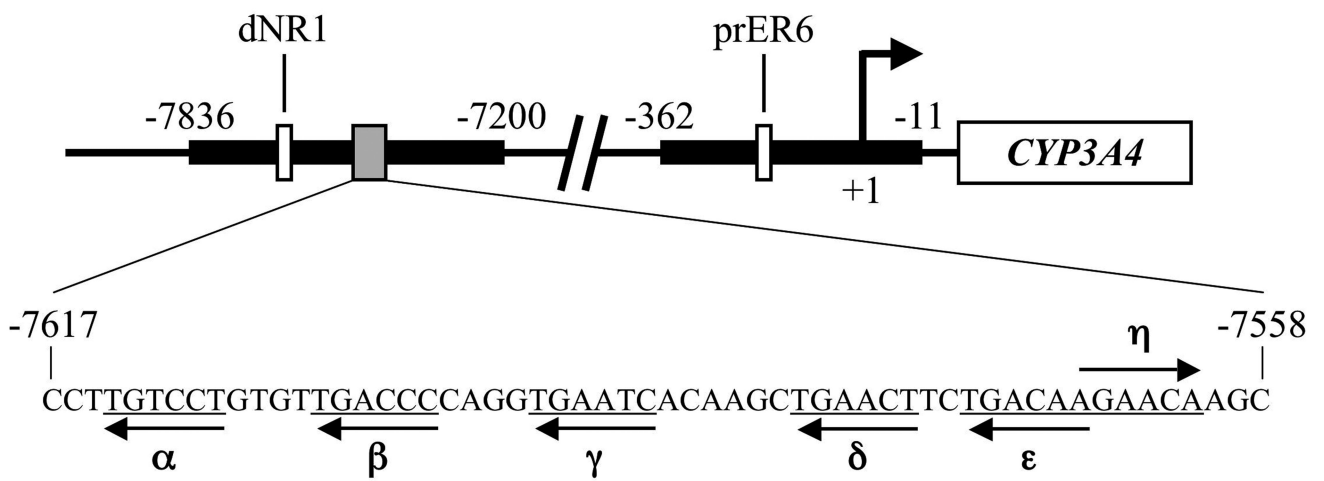


Fig. 3.

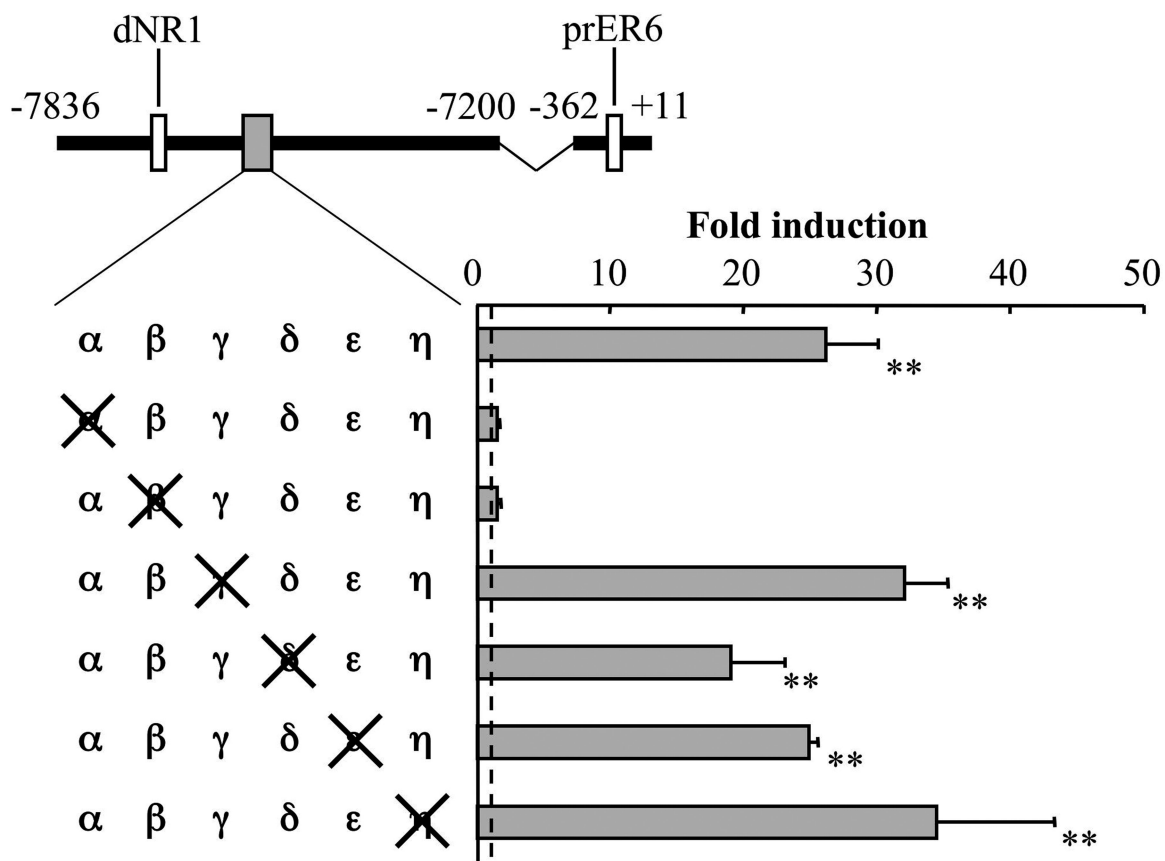


Fig. 4.

A

<u>Probe</u>	<u>Sequence</u>
prER6	ata TGAACT caaagg AGGTC Agtg
dNR1	gaa TGAACT tgc TGACCC tct
eNR3A4	cct TGTCCT gtgt TGACCC cag
$\beta\gamma$	tgt TGACCC cagg TGAATC aca
mut_dNR1	gaa T <u>T</u> AAT <u>T</u> tgc TGACCC tct
mut5'_eNR3A4	cct T <u>T</u> T <u>C</u> <u>T</u> gtgt TGACCC cag
mut3'_eNR3A4	cct TGTCCT gtgt T <u>A</u> <u>C</u> <u>T</u> Ccag

Fig. 4.

B

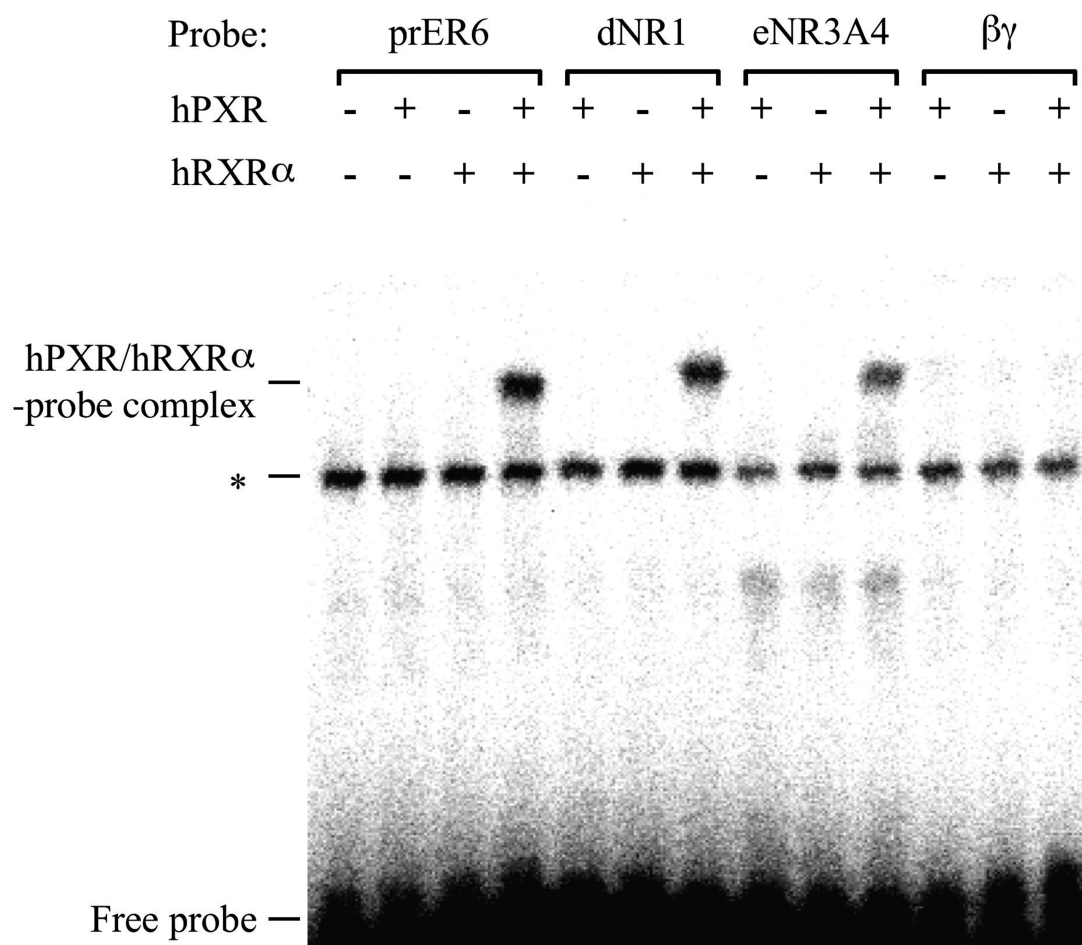


Fig. 4.

C

Competitor:			eNR3A4		dNR1		mut_		dNR1	
			x10 x50		x10 x50		x10 x50			
hPXR	+	-	+	+	+	+	+	+	+	+
hRXR α	-	+	+	+	+	+	+	+	+	+
Antibody	-	-	-	-	-	-	-	-	-	+

Supershifted complex —

hPXR/hRXR α
-probe complex —

*

Free probe —

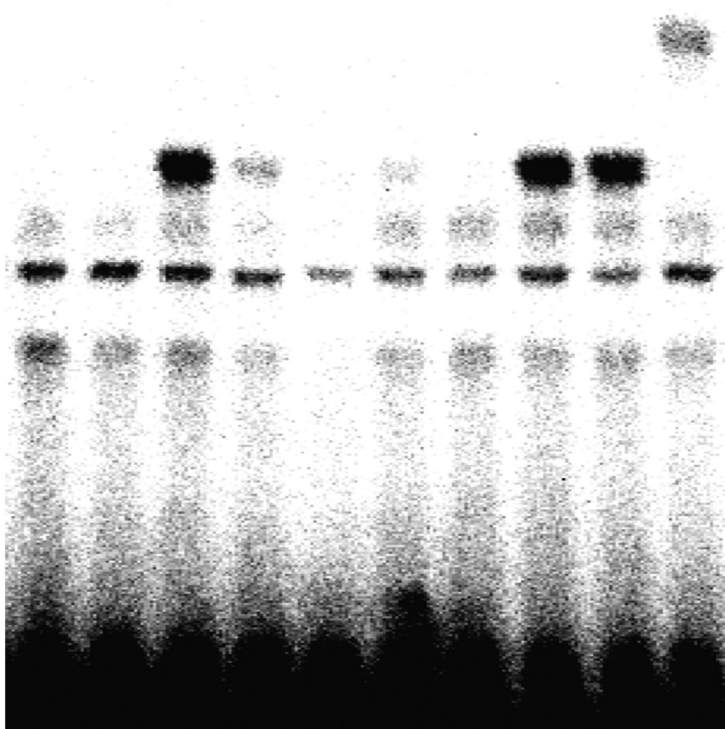


Fig. 4.

D

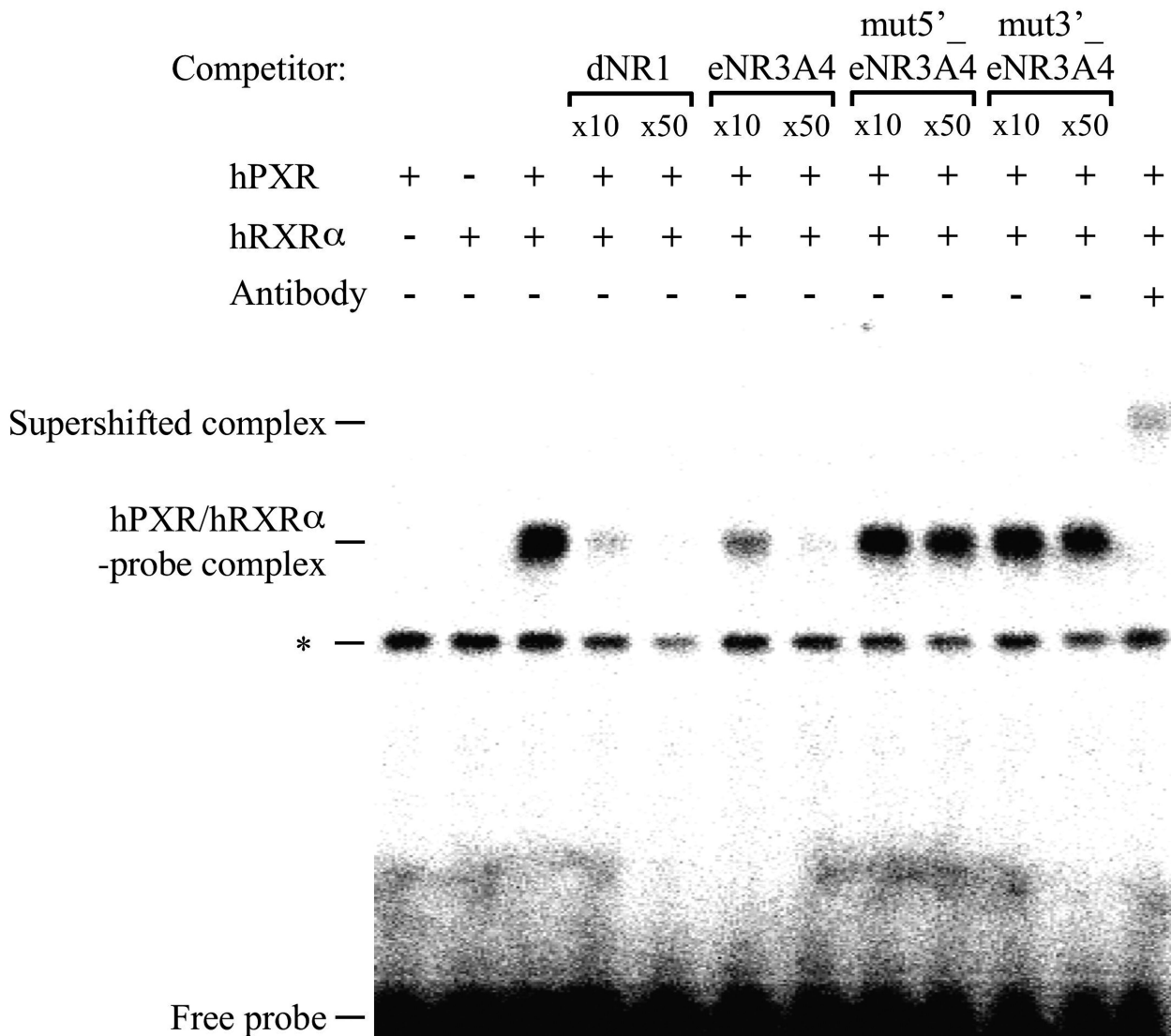


Fig. 5.

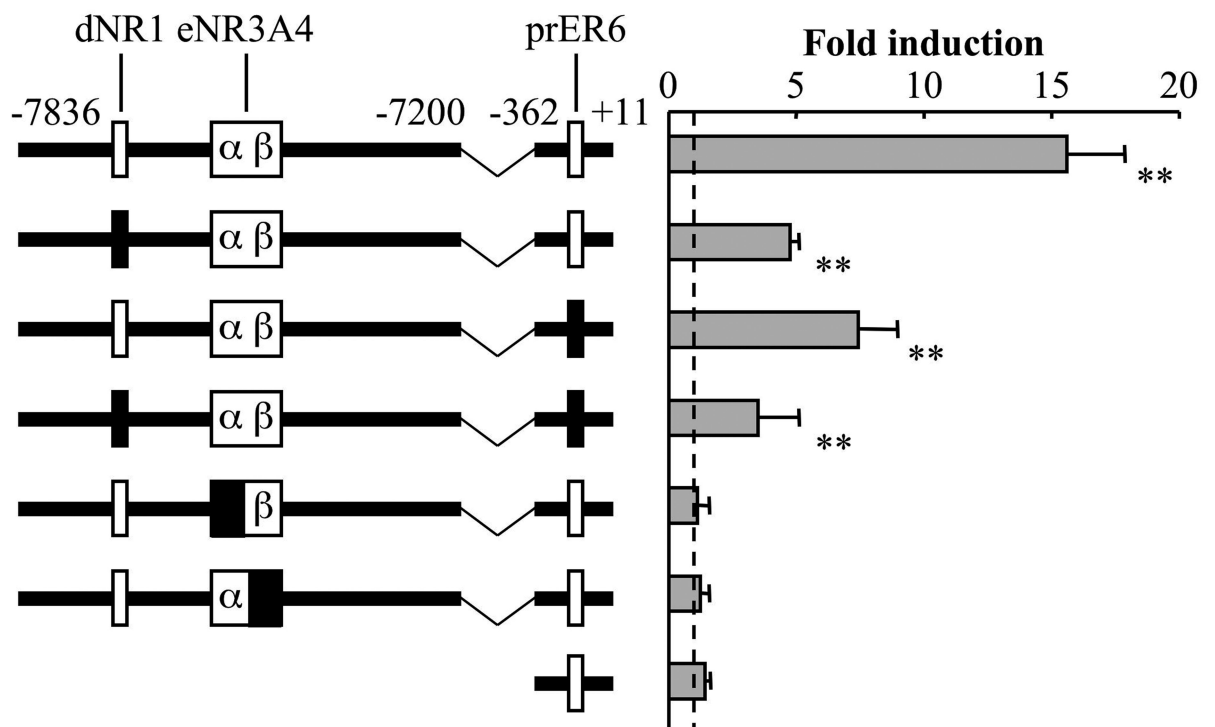


Fig. 6.

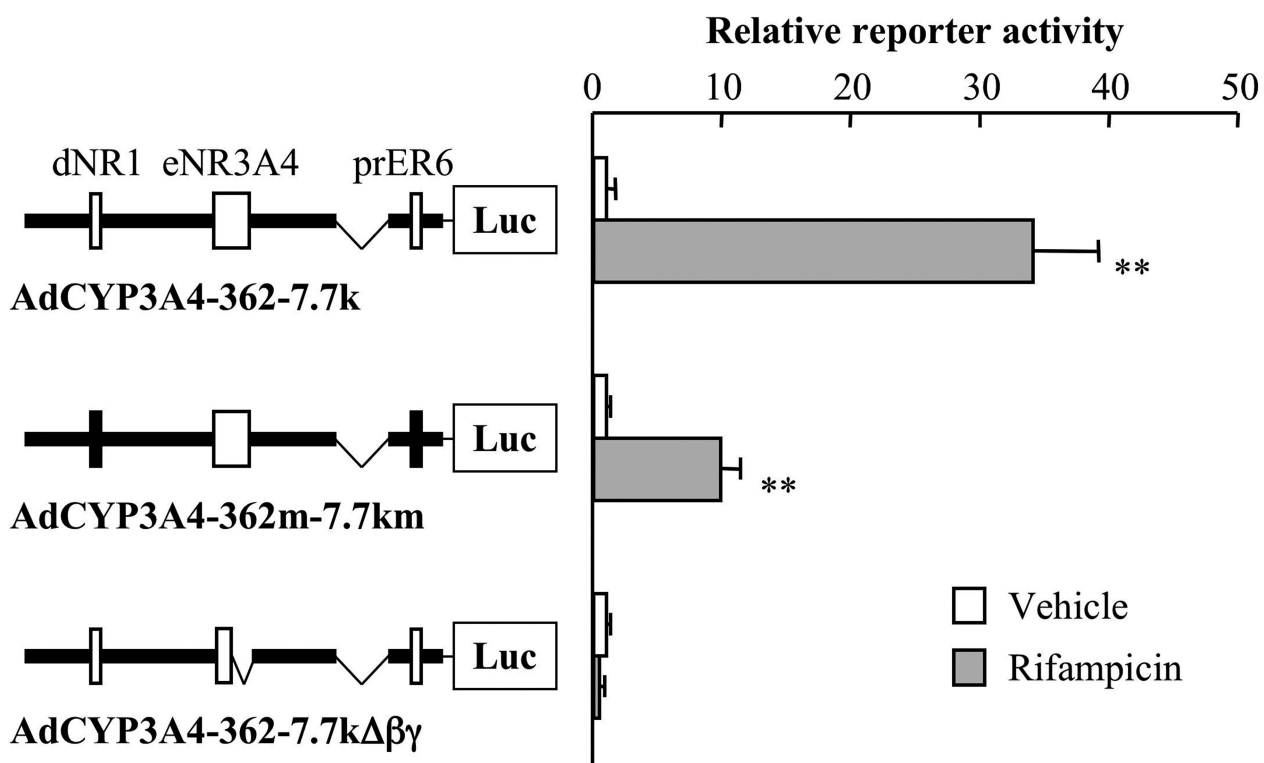


Fig. 7.

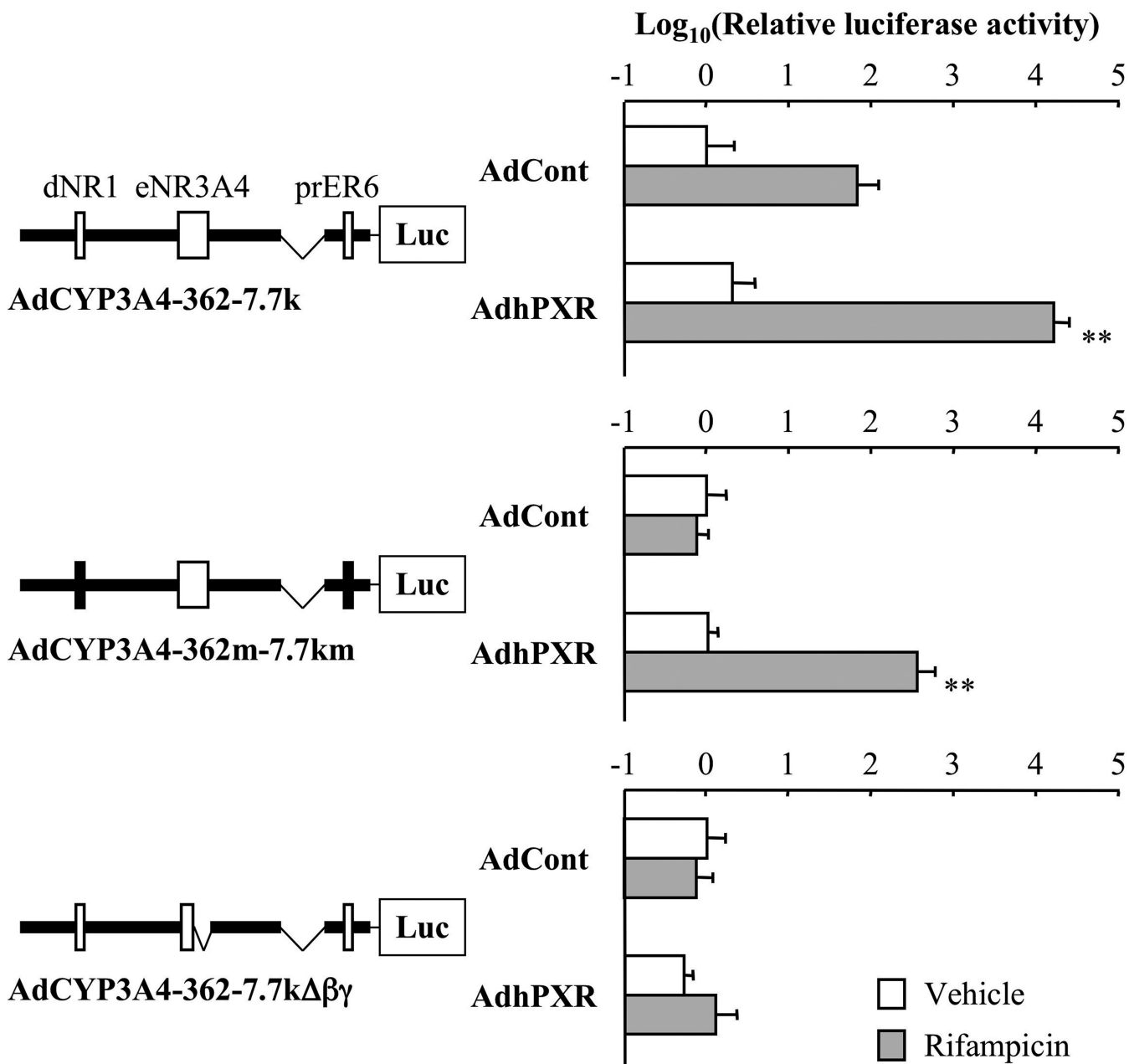


Fig. 8.

