



Original Article

CYP3A4 induction mechanism of polycyclic aromatic hydrocarbons differs from that of rifampicin in PXR binding element

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(Received September 22, 2017; Accepted September 27, 2017)

ABSTRACT — CYP3A4 is an important drug-metabolizing enzyme induced by various compounds causing drug-drug interactions. However, the molecular mechanism of CYP3A4 induction is not completely understood. CYP3A4 induction is caused by pregnane X receptor (PXR) through binding to some PXR binding elements. These elements comprise an everted repeat separated by six nucleotides in the promoter region and distal nuclear receptor binding element 1 (dNR-1) as well as the essential distal nuclear receptor binding element for CYP3A4 induction (eNR3A4) in the enhancer region of the *CYP3A4* gene. Recently, we found that polycyclic aromatic hydrocarbons including anthracene induce CYP3A4 in HepG2 cells with a different induction profile from that of rifampicin (RF), a typical PXR ligand. When a CYP3A4 reporter plasmid in which the eNR3A4 DNA fragment binds directly to the CYP3A4 promoter (-362 bases) was evaluated in a reporter assay, dibenz[a,h]anthracene (DBA) induced reporter activity, while RF did not. To be induced reporter activity by RF, more 14 nucleotides 5' upstream of the eNR3A4 (rifampicin eNR3A4: reNR3A4) DNA fragment were required. However, eNR3A4 and reNR3A4 did not respond to recombinant PXR without dNR-1. These results suggest that eNR3A4 and reNR3A4 are necessary for CYP3A4 induction by DBA and RF, respectively, and that dNR-1 is indispensable for full induction through PXR.

Key words: CYP3A4 induction, Pregnane X receptor, Rifampicin, Polycyclic aromatic hydrocarbons, Dibenz[a,h]anthracene, PXR binding element

INTRODUCTION

CYP3A4 is one of the most important drug-metabolizing enzymes involved in the metabolism of more than 50% of marketed drugs (Wienkers and Heath, 2005). Numerous studies have examined drug-drug interac-

tions (DDIs) mediated by CYP3A4. DDIs may cause serious problems from a clinical perspective. Particularly, CYP3A4 induction decreases concomitant drug exposure and efficacy in the human body. Therefore, the U.S. Food and Drug Administration (FDA, 2012), European Medicines Agency (EMA, 2012), and Pharmaceuti-

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icals and Medical Devices Agency (PMDA, 2014) have released guidance for DDI where required to be included in the package insert and examined through clinical trials. Many pharmaceutical companies have attempted to estimate the risk of DDI on the clinical side and develop low-risk compounds by estimating inducibility using *in vitro* tools such as hepatocytes. However, it is very difficult to precisely evaluate the induction potency of individual compounds because of their complicate mechanisms compared to CYP inhibition.

A number of studies have demonstrated that CYP3A4 inducers activate transcription of *CYP3A4* mediated by nuclear receptors such as pregnane X receptor (PXR, NR1I2) (Bertilsson *et al.*, 1998; Blumberg *et al.*, 1998; Lehmann *et al.*, 1998), constitutive androstane receptor (NR1I3) (Goodwin *et al.*, 2002), and vitamin D receptor (NR1I1) (Drocourt *et al.*, 2002). PXR is a main regulator of drug-metabolizing enzymes and transporters (Synold *et al.*, 2001; Geick *et al.*, 2001). Human PXR is activated by binding to various drugs such as rifampicin (RF), phenytoin, and carbamazepine (Kamiguchi, 2010), transferred into the nucleus, and activates *CYP3A4* transcription by binding to the regulatory region of the gene as a heterodimer with retinoid X receptor (Wan *et al.*, 2000).

New activation pathways of *CYP3A4* transcription have also been reported. For example cyclin-dependent kinase 2 negatively regulates PXR activity through phosphorylation of Ser³⁵⁰ (Lin *et al.*, 2008). Other reports indicated that promoter transactivation on *CYP3A4* and *UDP-glucuronosyltransferase 1A1* were reduced by mutation of Ser³⁵⁰ (Elias *et al.*, 2014; Sugatani *et al.*, 2010).

Several nucleotide sequences of the PXR binding element have been identified in CYP3A4, but are not fully understood. Some PXR binding elements are composed of an everted repeat separated by six nucleotides (ER-6) in the promoter region and distal nuclear receptor binding element1 (dNR-1) (Blumberg *et al.*, 1998; Lehmann *et al.*, 1998; Goodwin *et al.*, 1999), as well as the essential distal nuclear receptor binding element for CYP3A4 induction (eNR3A4) (Toriyabe *et al.*, 2009) in the enhancer region of *CYP3A4*. These elements have a cluster of TGA(A/C)CT-like sequences recognized by the nuclear receptor family of transcription factors (Goodwin *et al.*, 1999).

We recently reported that poly aromatic hydrocarbons (PAHs) and indirubin, which were thought to be aryl hydrocarbon receptor (AhR) ligands, induced CYP3A4 in HepG2 cells. Their induction is mediated through PXR, but not through AhR (Kumagai *et al.*, 2012; Kumagai *et al.*, 2016). In addition, the molecular mech-

anism of CYP3A4 induction by PAHs and indirubin differ from those by RF and clotrimazole (CTZ), typical CYP3A4 inducers, in the responsible element of the *CYP3A4* gene. In this study, the roles of PXR binding element in eNR3A4 and dNR-1 were investigated to clarify the CYP3A4 induction mechanism.

MATERIALS AND METHODS

Materials

RF, 3-methylcholanthrene, benzo[e]pyrene, and 1,2-benzanthracene were purchased from Sigma-Aldrich (St. Louis, MO, USA). The other PAHs listed Fig. 1. were obtained from Tokyo Chemical Industry (Tokyo, Japan). Dimethyl sulfoxide (DMSO) was purchased from Nacalai Tesque (Kyoto, Japan). CTZ and 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD) were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Cell culture

Human hepatoma cell line HepG2 cells were obtained from the RIKEN BRC (Tukuba, Japan) through Cell Resource Center for Biomedical Research (Tohoku University). The HepG2-derived cell line clone 3-1-20 stably expressing the CYP3A4-luciferase reporter gene was established as reported previously (Noracharttiyapong *et al.*, 2006). These cells were cultured in Dulbecco's modified Eagle's medium (Wako Pure Chemical Industries) supplemented with non-essential amino acids (Invitrogen, Carlsbad, CA, USA), 10% heat-inactivated fetal bovine serum (Biowest, Nuaille, France), and antibiotic-antimycotic (Invitrogen).

Reporter gene constructs and expression vectors

pCYP3A4-362, pCYP3A4-362-7.7k, pCYP3A4-362-m-7.7k, pCYP3A4-362-7.7km, and pCYP3A4-362m-7.7km and pCYP3A4-362-7.7ΔBsite were constructed as described previously (Takada *et al.*, 2004). pCYP3A4-362-7.7kΔeNR3A4 was reported previously (Toriyabe *et al.*, 2009). pCYP3A4-362+dNR-1, pCYP3A4-362+eNR3A4, pCYP3A4-362+rifampicin eNR3A4 (reNR3A4), and pCYP3A4-362+14αβ were prepared by inserting the dNR1, eNR3A4, reNR3A4, and 14αβ DNA fragments, which were isolated by PCR with primers (Table 1) and digested with *MluI* and *BglII*, into the *MluI* and *BglII* sites of pCYP3A4-362. pCYP3A4-362+eNR3A4+dNR-1 and pCYP3A4-362+reNR3A4+dNR-1 were made from pCYP3A4-362+eNR3A4 and pCYP3A4-362+reNR3A4 by inserting the dNR-1 DNA fragment digested with *Acc65I* and *MuII* into those of

Table 1. Primers used for construction of reporter gene plasmids

		Primer sequence
dNR1	FW	gcgggtaccATCTCAGCTGAATGAACTTGCTGAC
	RV	gcgacgcgtAGGAAAGCAGAGGGTCAGCAAGTTC
eNR3A4	FW	gcgacgcgtATTAAACCTTGTCTGTGTTGACCCAGGT
	RV	gcgagatctTGAAAAGAATATGATAGCTTGT
reNR3A4	FW	gcgacgcgtCAGTGATTATTAACCTTGTCT
	RV	gcgagatctTGAAAAGAATATGATAGCTTGT
14 α β	FW	gcgacgcgtCAGTGATTATTAACCTTGTCT
	RV	gcgagatctTTCACCTGGGGTCAACACAGG

the *Acc65I* and *MulI* sites. pCYP3A4-362-7.7k Δ α , pCYP3A4-362-7.7k Δ β , pCYP3A4-362-7.7k Δ γ , pCYP3A4-362-7.7k Δ δ , and pCYP3A4-362-7.7k Δ ϵ were prepared from reporter plasmids mutated in the α , β , γ , δ , and ϵ sites as previously reported (Toriyabe *et al.*, 2009) by digestion with *XhoI*, treatment with S1 nuclease, and self-ligation. Point-mutated reporter plasmids were constructed with the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) using pCYP3A4-362-7.7k as a template.

Luciferase assay for reporter plasmid

HepG2 cells were seeded into a 48-well tissue culture plate (BD Biosciences, Franklin Lakes, NJ, USA) at 3.0×10^4 cells per well the day before transfection. Each reporter plasmid and pGL4.82 (Promega, Madison, WI, USA) were cotransfected using Targefect F-1 (Targeting Systems, El Cajon, CA, USA) according to the manufacturer's protocols. Transfection efficiency was normalized by Renilla luciferase activity of pGL4.82. After transfection, HepG2 cells were cultured in the presence of various compounds dissolved in DMSO for 48 hr. Control cells were treated with vehicle (0.1% DMSO). After 48 hr incubation, the cells were washed with PBS and suspended in passive lysis buffer (Promega). Luciferase activities were evaluated using the Dual-Luciferase assay system and GloMax™ 96 Microplate Luminometer (Promega) according to the manufacturer's protocols. Fold-induction values were calculated from the luminescence normalized as the ratio of control samples.

Luciferase assay for PAHs

3-1-20 cells were seeded into 96-well tissue cultured plate at 1.0×10^4 cells per well the day before treatment with various compounds dissolved in DMSO. After 48 hr incubation, the cells were washed with PBS and suspended in passive lysis buffer. Luciferase activities were evaluated using the Luciferase assay system (Promega) with GloMax™ 96 Microplate Luminometer. The luminescence of each sample was normalized to the pro-

tein concentration using the Protein Assay Kit (Bio-Rad, Hercules, CA, USA).

Construction of adenovirus expression vector and infection

The human PXR-expressing adenovirus (AdhPXR) was constructed as described previously (Matsubara *et al.*, 2007). The β -galactosidase-expressing adenovirus (AdCont; AxCALacZ), used as a control adenovirus, was provided by Dr. Izumi Saito (Tokyo University, Tokyo, Japan) (Miyake, 1996). The titer of the adenoviruses, the 50% titer culture infectious dose (TCID₅₀), was determined as reported previously (Matsubara *et al.*, 2007). Multiplicity of infection was calculated as the TCID₅₀ based on the number of cells. One day before transfection, cells were seeded in 48-well plates. The adenovirus infection protocol was described previously (Kumagai *et al.*, 2012).

Statistical analysis

Data are presented as the mean \pm standard deviation (S.D.) and were evaluated by paired Student's *t*-test. A *P* value < 0.05 was considered significant.

RESULTS

Effect of PAHs on CYP3A4 reporter activity in 3-1-20 cells

First, CYP3A4 reporter activity by PAHs was investigated by using HepG2-derived cell line clone, 3-1-20 stably expressing the CYP3A4-luciferase reporter gene. Many PAHs increased CYP3A4 reporter activity. Among them, dibenz[a,h]anthracene (DBA) and dibenz[a, c]anthracene strongly increased reporter activity (20-30-fold), while benzo[e]pyrene, pyrene, naphthalene showed no activation, and dibenz[de,kl]anthracene showed minimal activation of *CYP3A4* transcription (Fig. 2).

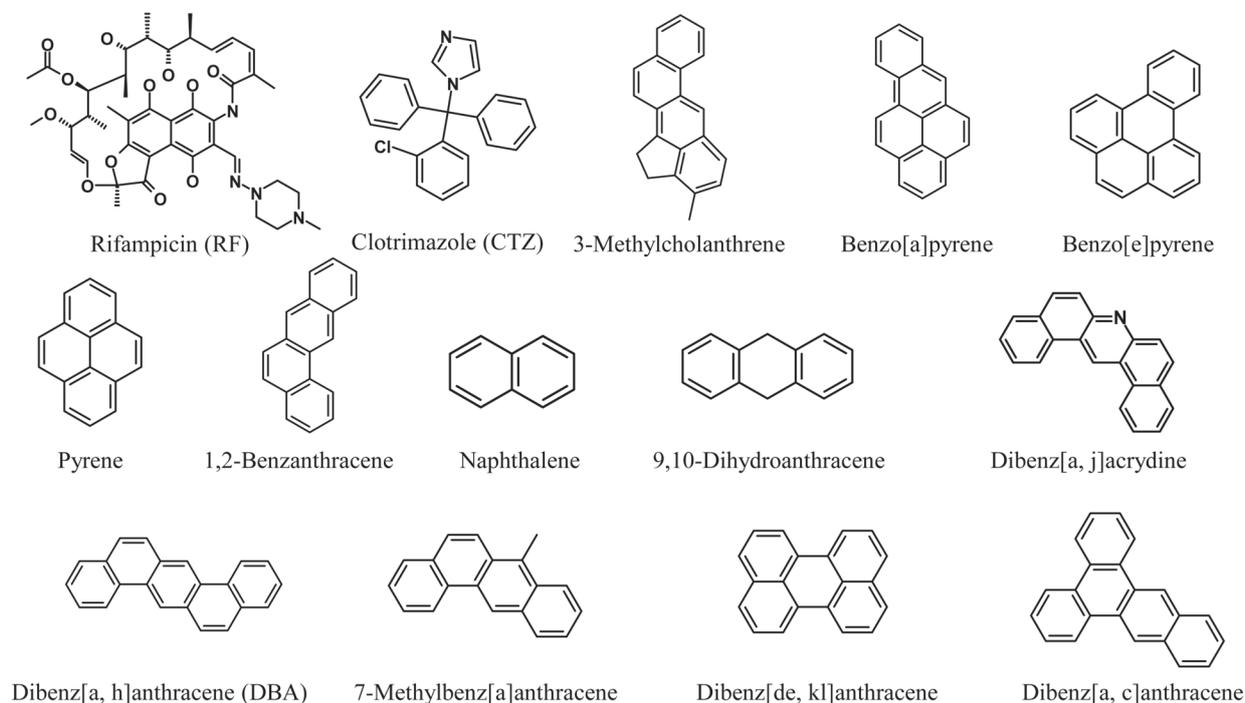


Fig. 1. Structure of RF, CTZ and PAHs.

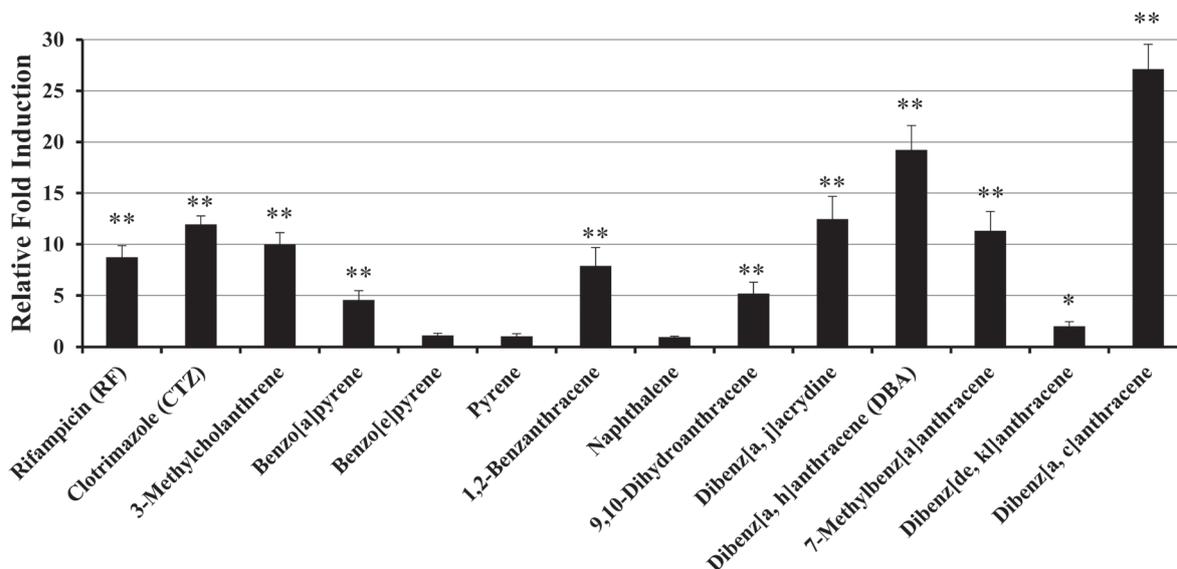


Fig. 2. Effect of PAHs on CYP3A4 reporter activity in 3-1-20 cells. Clone 3-1-20 cells were seeded at 1×10^4 cells in a 96-well tissue culture plate and pre-incubated for 24 hr before treatment with various compounds. The cells were treated with RF (10 μ M), CTZ (10 μ M) and various PAHs (1 μ M) for 48 hr and reporter activity was measured by the luciferase assay. Reporter activities are expressed as the fold to that in the vehicle-treated cells. Data are shown as the mean \pm S.D. from three different samples. * $P < 0.05$, ** $P < 0.005$, differences from vehicle-treated cells.

Effect of mutation in prER-6 and dNR-1 on reporter activity

Next, transient transfection assays were conducted by using CYP3A4 luciferase reporter plasmids containing three elements (dNR-1, eNR3A4, and prER-6), which bind to PXR (pCYP3A4-362-7.7k). The role of these elements was investigated using reporter plasmids containing mutations in dNR-1 and/or prER-6 (pCYP3A4-362-7.7km, pCYP3A4-362m-7.7k, pCYP3A4-362m-7.7km). Reporter activity was compared among the reporter plasmids using PXR ligands (RF and CTZ) and AhR ligands (DBA and TCDD) (Fig. 3). All inducers increased reporter activity (8.2-, 7.9-, 11.0-, and 9.1-fold, respectively) in cells transfected with pCYP3A4-362-7.7k. The construct mutated in prER-6, pCYP3A4-362m-7.7k, showed nearly the same activity as pCYP3A4-362-7.7k for all inducers. In contrast, the constructs mutated in dNR-1, pCYP3A4-362-7.7km, in dNR-1 and prER-6, pCYP3A4-362m-7.7km showed very low responses to RF and CTZ (pCYP3A4-362-7.7km: 2.7- and 1.8-fold and pCYP3A4-362m-7.7km: 1.5- and 2.3-fold, respectively). However, DBA and TCDD showed relatively high responses compared to those of RF and CTZ in pCYP3A4-362-7.7km (10.4- and 6.2-fold) and pCYP3A4-362m-7.7km (5.0- and 5.1-fold). Similar results were observed in cells treated with indirubin (data not shown).

Effect of deletion in enhancer region on the reporter activity

To verify the involvement of eNR3A4 for CYP3A4 induction, transient transfection assays were conducted by using several CYP3A4 luciferase reporter plasmids with deletions in various enhancer regions (Fig. 4). RF did not increase reporter activity in cells treated with deleted plasmids in this experiment, pCYP3A4-362-7.7kΔeNR3A4, pCYP3A4-362-7.7kΔBsite, pCYP3A4-362+dNR-1, pCYP3A4-362+eNR3A4, and pCYP3A4-362, although RF increased reporter activity in cells treated with pCYP3A4-362-7.7k (17.9-fold). In contrast, DBA increased reporter activity to some extent in all plasmids used in this experiment. Of all deleted plasmids, pCYP3A4-362-7.7kΔBsite and pCYP3A4-362+eNR3A4 showed a high reporter activity (5.4- and 16.3-fold, respectively) following treatment with DBA.

Effect of overexpressing PXR on the reporter activity

It is widely known that PXR is a major transcription factor involved in CYP3A4 induction. The effect of PXR on CYP3A4 induction by RF and DBA was investigated by overexpressing human PXR in HepG2 cells using adenovirus (Fig. 4). The induced reporter activity (17.9-fold) by RF was dramatically increased (51.3-

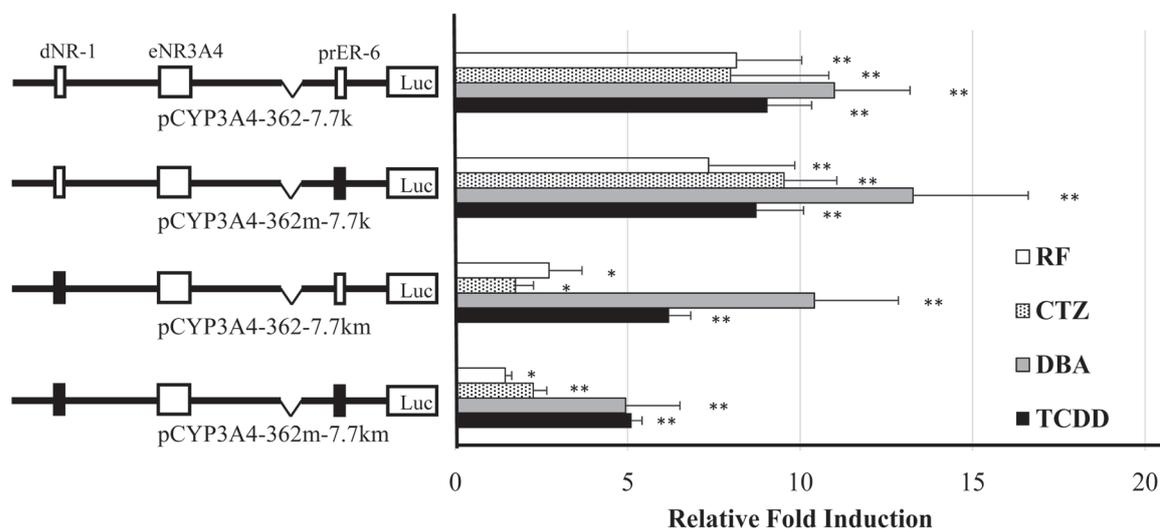


Fig. 3. Effect of mutation in CYP3A4 promoter and/or enhancer region on reporter activity induced by various compounds. HepG2 cells were seeded at 3×10^4 cells in a 48-well tissue culture plate. The cells were incubated for 24 hr before transfection. The transfection protocol is described in the "Materials and Methods". Closed boxes represent mutated PXR-binding elements. The cells were treated with RF (10 μ M), CTZ (10 μ M), DBA (10 μ M), or TCDD (10 nM) for 48 hr. Luciferase activity was normalized to Renilla luciferase activity. The values were expressed as the fold induction of the vehicle control (DMSO) group. Data are shown as the mean \pm S.D. from six different samples. * $P < 0.05$, ** $P < 0.005$, differences from vehicle-treated cells.

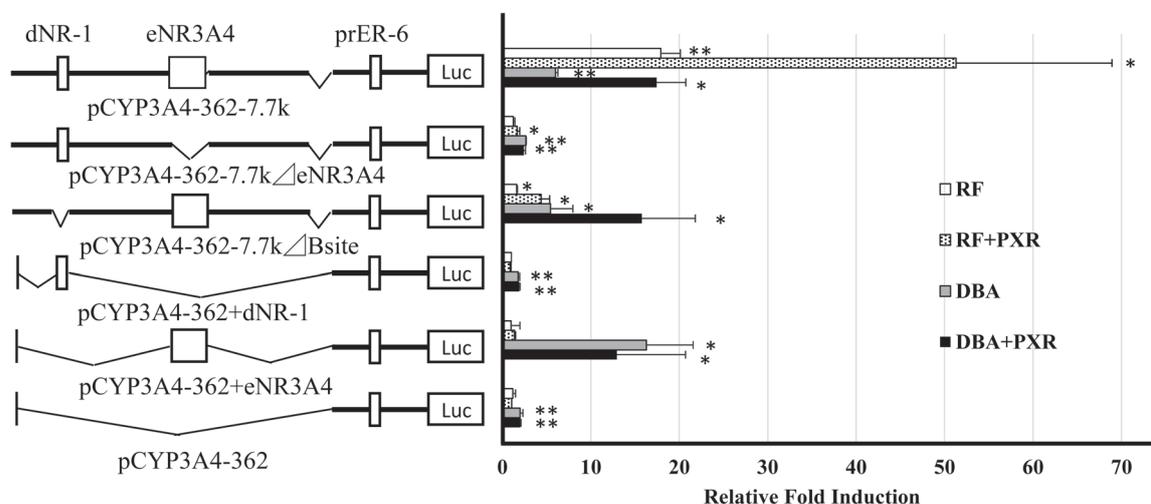


Fig. 4. Effect of overexpressing PXR on CYP3A4 reporter activity in HepG2 cells by RF and DBA. HepG2 cells were seeded at 3×10^4 cells in a 48-well tissue culture plate. The cells were incubated for 24 hr before transfection. The transfection protocol is described in the “Materials and Methods”. After 24 hr of Ad-PXR (3 MOI) infection, the cells were treated with RF (10 μ M) or DBA (10 μ M) for 48 hr. Luciferase activity was normalized to Renilla luciferase activity. The values were expressed as the fold induction of the vehicle control (DMSO) group. Data are shown as the mean \pm S.D. from three different samples. *P < 0.05, **P < 0.005, differences from vehicle-treated cells.

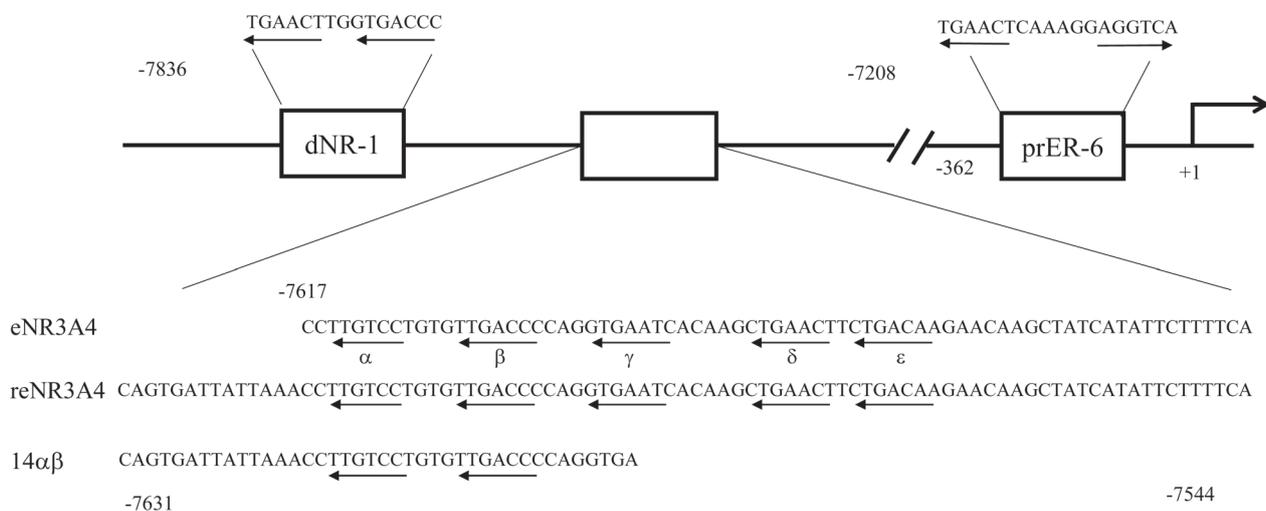


Fig. 5. Sequence of eNR3A4 and reNR3A4 of CYP3A4 and reporter constructs containing mutations and or deletions. These elements contain a cluster of TGA(A/C)CT-like sequences recognized in the nuclear receptor family of transcription factors.

fold) in cells by overexpressing PXR after transfection of pCYP3A4-362-7.7k. In cells transfected with pCYP3A4-362-7.7kΔBsite, reporter activity also increased by overexpressing PXR. Similarly, DBA highly increased the reporter activity (6.0- and 5.4-fold, respectively) in cells transfected with pCYP3A4-362-7.7k and pCYP3A4-

362-7.7kΔBsite, and these activities were dramatically increased (17.5- and 15.8-fold, respectively) by overexpressing PXR. In contrast, increased reporter activity by treatment with DBA was not activated by overexpressing PXR in cells transfected with pCYP3A4-362+eNR3A4 in which the eNR3A4 element binds directly to the promot-

er region (-362).

Effect of 14 bases 5'-upstream of eNR3A4 on reporter activity

Because increased reporter activity was not observed in pCYP3A4-362+eNR3A4 treated with RF, pCYP3A4-362+reNR3A4 containing 14 additional bases 5'-upstream of eNR3A4 was transfected into HepG2 cells. The results are shown in Fig. 6. RF and DBA increased reporter activity in cells transfected with pCYP3A4-362+reNR3A4 (5.1- and 7.2-fold, respectively), but these reporter activities were not significantly increased by overexpressing PXR (8.3- and 7.3-fold, respectively). Similar results were observed in cells transfected with pCYP3A4-362+14 $\alpha\beta$, although the reporter activity was increased to some extent without overexpressing PXR.

Role of dNR-1 for CYP3A4 induction

In the study above shown, we suggest that eNR3A4 and reNR3A4 are essential elements for *CYP3A4* gene activation by DBA and RF. However, it remained unclear how dNR-1, eNR3A4, and reNR3A4 function in CYP3A4 induction. To verify the involvement of dNR-1, eNR3A4, and reNR3A4, further transient transfection assays were conducted using CYP3A4 luciferase reporter plasmids with or without dNR-1 (Fig. 7). RF did not increase reporter activity in cells treated with pCYP3A4-362-eNR3A4 and pCYP3A4-362+dNR-1+eNR3A4. Howev-

er, RF induced reporter activity (4.5-fold) was dramatically increased (29.7-fold) in cells overexpressing PXR after transfection of pCYP3A4-362+dNR-1+reNR3A4. In contrast, DBA increased reporter activity in cells transfected with any reporter constructs and the induced activity was increased by overexpressing PXR in cells transfected with pCYP3A4-362+dNR-1+eNR3A4 and pCYP3A4-362+dNR-1+reNR3A4 (8.4- and 18.1-fold).

Effect of deletion in eNR3A4 region on reporter activity

Several TGA(A/C)CT-like sequences binding to nuclear receptor family members were observed in the eNR3A4 region. To verify the role of half-sites in eNR3A4 for CYP3A4 induction, several CYP3A4 luciferase reporter plasmids with mutated and deleted half-sites were constructed. The results are shown in Fig. 8. RF did not increase reporter activity in cells treated with pCYP3A4-362-7.7k $\Delta\alpha$ and pCYP3A4-362-7.7k $\Delta\beta$, even by overexpressing PXR. However, DBA increased reporter activity in pCYP3A4-362-7.7k $\Delta\alpha$ and pCYP3A4-362-7.7k $\Delta\beta$ and the activity was not increased by overexpressing PXR. Moreover, reporter activity increased by RF and DBA was lower in pCYP3A4-362-7.7k $\Delta\gamma$ and pCYP3A4-362-7.7k $\Delta\delta$ than in pCYP3A4-362-7.7k, although these activities were increased by overexpressing PXR.

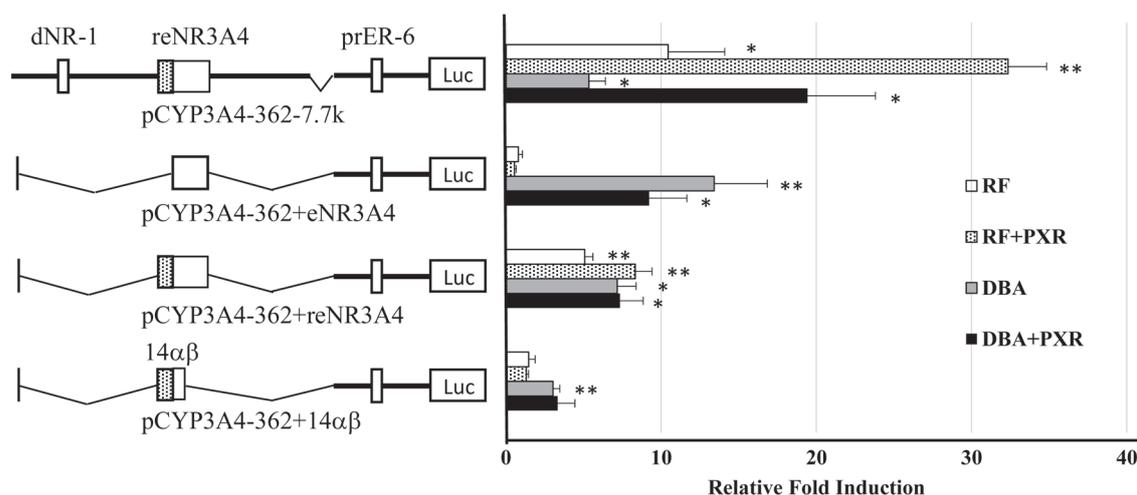


Fig. 6. Effect of DNA deletion in CYP3A4 enhancer region and overexpressing PXR on reporter activity in HepG2 cells induced by RF and DBA. HepG2 cells were seeded at 3×10^4 cells in a 48-well tissue culture plate. The cells were incubated for 24 hr before transfection. The transfection protocol is described in the "Materials and Methods". After 24 hr of Ad-PXR (3 MOI) infection, the cells were treated with RF (10 μ M) or DBA (10 μ M) for 48 hr. Luciferase activity was normalized to Renilla luciferase activity. The values were expressed as the fold induction of the vehicle control (DMSO) group. Data are shown as the mean \pm S.D. from three different samples. * $P < 0.05$, ** $P < 0.005$, differences from vehicle-treated cells.

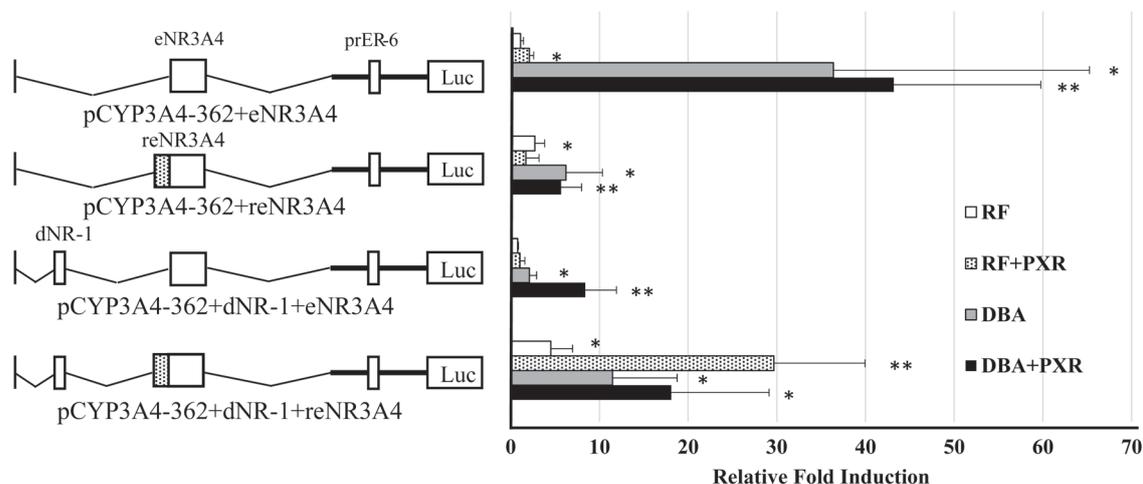


Fig. 7. Effect of DNA deletion in CYP3A4 enhancer region and overexpressing PXR on reporter activity in HepG2 cells induced by RF and DBA. HepG2 cells were seeded at 3×10^4 cells in a 48-well tissue culture plate. The cells were incubated for 24 hr before transfection. The transfection protocol is described in the “Materials and Methods”. After 24 hr of Ad-PXR (3 MOI) infection, the cells were treated with RF (10 μ M) or DBA (10 μ M) for 48 hr. Luciferase activity was normalized to Renilla luciferase activity. The values are expressed as the fold induction of the vehicle control (DMSO) group. Data are shown as the mean \pm S.D. from six different samples. * $P < 0.05$, ** $P < 0.005$, differences from vehicle-treated cells.

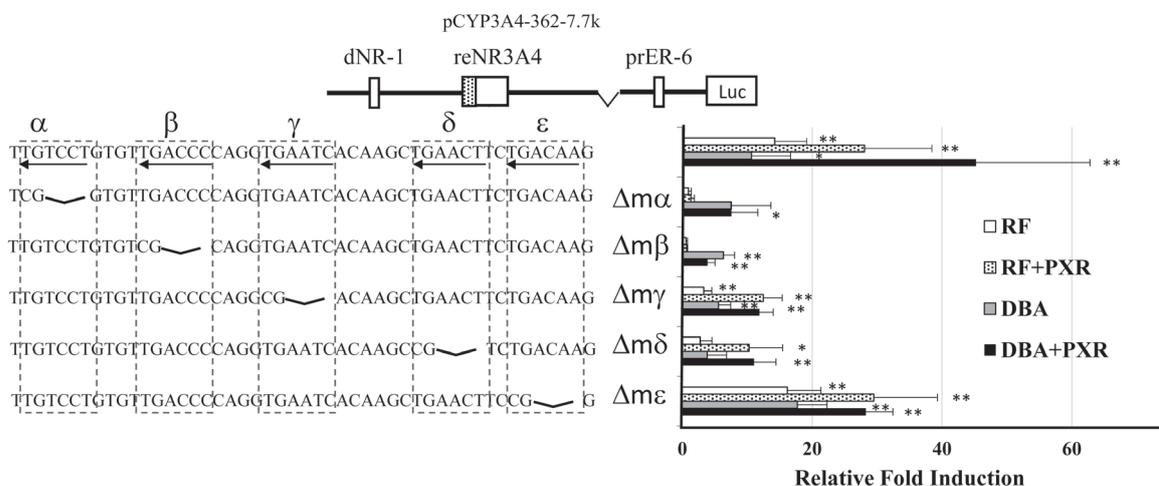


Fig. 8. Effect of DNA deletion in eNR3A4 and overexpressing PXR on reporter activity in HepG2 cells induced by RF and DBA. HepG2 cells were seeded at 3×10^4 cells in 48-well tissue culture plate. The cells were incubated for 24 hr before transfection. The transfection protocol is described in the “Materials and Methods”. After 24 hr of Ad-PXR (3 MOI) infection, the cells were treated with RF (10 μ M) or DBA (10 μ M) for 48 hr. Luciferase activity was normalized to Renilla luciferase activity. The values are expressed as the fold induction of the vehicle control (DMSO) group. Data are shown as the mean \pm S.D. from five different samples. * $P < 0.05$, ** $P < 0.005$, differences from vehicle-treated cells.

Effect of point mutation in eNR3A4 region on reporter activity

To confirm the role of the half-site in eNR3A4 in detail, point mutations were introduced into pCYP3A4-

362-7.7k at several bases of the eNR3A4 DNA fragment as indicated with an arrow by changing to A. Mutation No. 3, 4, 8, and 9 showed low activity compared to that of pCYP3A4-362-7.7k by RF and DBA.

DISCUSSION

We first evaluated CYP3A4 induction by PAHs using 3-1-20 cells stably expressing the CYP3A4-luciferase reporter gene (Noracharttiyapot *et al.*, 2006). The results showed that many PAHs increased CYP3A4 reporter activity (Fig. 2). PAHs are known as AhR ligands, and activated AhR binds to a cluster of CACGC-like sequences (Watson and Hankinson, 1992), but a CACGC-like sequence was not identified in the promoter and enhancer region of *CYP3A4* used in this experiment. As shown previously, *CYP3A4* activation by PAHs such as 3-methylcholoranthrene and TCDD was increased by overexpressing PXR and decreased by PXR-siRNA (Kumagai *et al.*, 2012). One of the PAHs, DBA, increased reporter activity in cells transfected with pCYP3A4-362-7.7k by overexpressing PXR (Fig. 4). PAHs including anthracene in their chemical structure likely activate the CYP3A4 reporter gene in HepG2 cells, but compounds such as benzo[e]pyrene, pyrene and dibenz[de, kl]anthracene categorized as condensed-ring annulation types only minimally activated the CYP3A4 reporter gene. However, it is unclear whether PXR binds to these compounds. In this study, because of its high induction potency, we used DBA to evaluate the CYP3A4 induction mechanism of PAHs.

It has been reported that dNR-1, eNR3A4, and prER-6 are very important for CYP3A4 induction (Blumberg *et al.*, 1998; Lehmann *et al.*, 1998; Goodwin *et al.*, 1999; Toriyabe *et al.*, 2009). In this study, we investigated the role of these PXR binding elements on CYP3A4 induction in detail by using several reporter plasmids, including CYP3A4 enhancer region into which we introduced deletion and mutations. The results indicate strong involvement not only of dNR-1, but also of eNR3A4 in the transcriptional activation of *CYP3A4* by RF and CTZ, while eNR3A4 appeared to be important for transcriptional activation of *CYP3A4* by DBA and TCDD (Fig. 3). To confirm the involvement of eNR3A4, the reporter activities of each plasmid containing deletions in the enhancer region were measured. The results showed that eNR3A4 is more important than dNR-1 in activating *CYP3A4* transcription by DBA, as demonstrated by the pCYP3A4-362-7.7kΔBsite and CYP3A4-362+eNR3A4 containing an eNR3A4 DNA fragment (Fig. 4).

We previously reported that indirubin, a ligand of AhR, induces CYP3A4 through PXR (Kumagai *et al.*, 2012; Kumagai *et al.*, 2016) and other group also reported PAHs induce CYP3A4 (Luckert *et al.*, 2013). In addition, indirubin showed a similar profile of transcriptional activation of *CYP3A4* to that of TCDD and DBA (data

not shown) in HepG2 cells. To confirm the involvement of PXR in DBA, CYP3A4 reporter activity was examined by overexpressing PXR in HepG2 cells. As a result, plasmids containing eNR3A4, pCYP3A4-362-7.7k, and pCYP3A4-362-7.7kΔBsite showed increased reporter activity by overexpressing PXR. pCYP3A4-362+eNR3A4 showed increased the reporter activity by DBA, but not by RF. The increased reporter activity was not enhanced by overexpressing PXR (Fig. 4). These results suggest that CYP3A4 induction through eNR3A4 by DBA does not depend only on PXR. There are some reports of new activation pathways of *CYP3A4* transcription. For example, cyclin-dependent kinase2 negatively regulates the activity of PXR through phosphorylation of Ser³⁵⁰ (Lin *et al.*, 2008) and cell proliferation in Huh7 cells (Sivertsson *et al.*, 2013), but the mechanism of CYP3A4 induction by DBA remains unclear. Therefore, further studies are needed to determine the CYP3A4 induction mechanism by DBA.

In addition, RF did not increase reporter activity in cells transfected with pCYP3A4-362+eNR3A4 (Fig. 4). The present results suggest that eNR3A4 is not sufficient for the *CYP3A4* transcription. We previously reported that the α and β sites in the eNR3A4 DNA fragment are key elements for induction of CYP3A4 (Toriyabe *et al.*, 2009). Therefore, we predicted that a specific DNA fragment is required for CYP3A4 induction other than the α and β sites. Reporter plasmids containing an extra 14-base pair DNA fragment 5'-upstream of eNR3A4 named as reNR3A4 were constructed. In addition, we constructed a reporter plasmid, pCYP3A4-362+14 $\alpha\beta$, with the extra 14-base pair DNA fragment and α and β sites. The results of transient transfection assays indicated that the 14-base pair DNA fragment is necessary for CYP3A4 induction by RF other than the PXR binding element (Fig. 5). However, overexpressing PXR did not increase reporter activity in either pCYP3A4-362+eNR3A4 or pCYP3A4-362+reNR3A4. Therefore, to verify the role of dNR-1, pCYP3A4-362+dNR-1+reNR3A4 and pCYP3A4-362+dNR-1+eNR3A4 were prepared. The increased reporter activity of pCYP3A4-362+dNR-1+reNR3A4 by DBA and RF was dramatically enhanced by overexpressing PXR, but the reporter activity of pCYP3A4-362+dNR-1+eNR3A4 was enhanced by overexpressing PXR following DBA treatment. These results indicate that for full CYP3A4 induction, dNR-1 is indispensable and the 14-base pair DNA fragment 5'-upstream of eNR3A4 is necessary for RF other than binding elements of dNR-1, but not for DBA.

We further verified the role of each half-site (α , β , γ , δ , ϵ) in eNR3A4 using CYP3A4 reporter constructs in

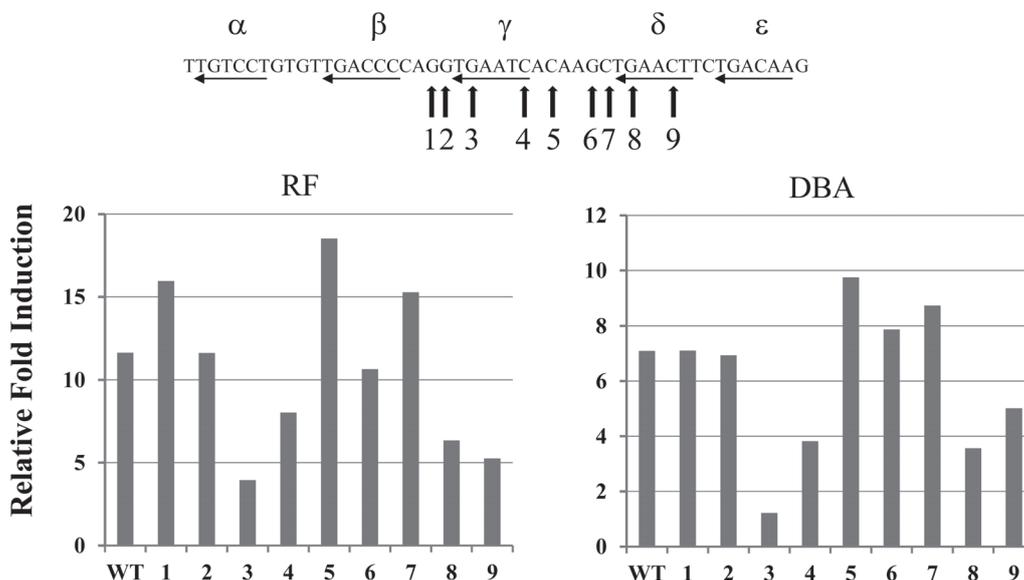


Fig. 9. Effect of DNA point mutation in γ and δ half-sites of eNR3A4 on reporter activity induced by RF and DBA. HepG2 cells were seeded at 3×10^4 cells in a 48-well tissue culture plate. The cells were incubated for 24 hr before transfection. The transfection protocol is described in the “Materials and Methods”. After 24 hr of Ad-PXR (3 MOI) infection, the cells were treated with RF (10 μ M) or DBA (10 μ M) for 48 hr. Luciferase activity was normalized to Renilla luciferase activity. The values are expressed as the fold-induction of the vehicle control (DMSO) group. Data are shown as the mean from two different samples.

which various DNA deletions and single mutations were introduced. As previously reported, α and β sites are very important for CYP3A4 induction by RF (Fig. 8) (Toriyabe *et al.*, 2009), but this study showed that α and β sites are not essential for CYP3A4 induction by DBA. Similar results were also observed by mutation of α and β sites (data not shown). Deletion of the γ and δ sites decreased reporter activity, which was increased by DBA and RF, but did not disappear. These results suggest that the γ and δ sites are also important for CYP3A4 induction. This idea was confirmed by mutation analysis of γ and δ sites as shown in Fig. 9.

In conclusion, we demonstrated that the molecular mechanism of CYP3A4 induction differs between RF and DBA in the PXR binding element. While reNR3A4 is an important DNA fragment for CYP3A4 induction by RF, eNR3A4 is an important DNA fragment for CYP3A4 induction by DBA. Furthermore, our results might suggest that CYP3A4 induction by DBA and RF use different transcription factors, although we did not confirm transcription factor binding to eNR3A4 and reNR3A4. The results of our study revealed the role of each half-sites in eNR3A4. The α and β sites are essential for CYP3A4 induction by RF, but not by DBA; in addition to the α and

β sites, the γ and δ sites are necessary for CYP3A4 induction. Moreover, to obtain full CYP3A4 induction through PXR by RF and DBA, dNR-1 was indispensable.

ACKNOWLEDGMENTS

This work was supported by a Health and Labor Sciences Research Grant for Research on Food Safety (No. 22230301) from the Ministry of Health, Labor and Welfare of Japan; Grant-in-Aid for Scientific Research (B) (No. 23790193); and Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT)-Supported Program for the Strategic Research Foundation at Private Universities.

Conflict of interest---- The authors declare that there is no conflict of interest.

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