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Unveiling a New Essential Cis Element for the Transactivation of the *CYP3A4* Gene by Xenobiotics[S]

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ABSTRACT

Pregnane X receptor (PXR) has been shown to form a heterodimer with retinoid X receptor α (RXRα) and to bind to the distal nuclear receptor-binding element 1 and an everted repeat separated by six nucleotides in the proximal promoter of the *CYP3A4* gene. In the present study, a new rifampicin-responsive region, located at −7.6 kilobases 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 them α–η half-sites. Introduction of a mutation into either an α or β half-site of *CYP3A4* reporter genes almost completely diminished the rifampicin-induced transcription. In electrophoretic mobility shift assays, PXR/RXRα 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 direct repeat separated by four nucleotides-type PXR binding element of the *CYP3A4* gene for xenobiotic induction of CYP3A4.

Hepatic and intestinal cytochromes P450 (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; Shimada et al., 1994; Paine et al., 2006). In addition, significant interindividual variations in hepatic and intestinal levels of CYP3A4 have been reported previously (Wolbold et al., 2003). The hepatic and intestinal expression levels of CYP3A4 are increased in vivo after the exposure to a variety of drugs (Schuetz et al., 1993; Kocarek et al., 1995; Michalets, 1998), 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 is also a risk factor associated with adverse drug-drug interactions in patients receiving the combination drug therapy.

A number of independent studies have established that chemicals so-called "CYP3A4 inducers" activate the transcrip-

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ABBREVIATIONS: P450, cytochrome P450; AdCont, control adenovirus; AdhPXR, adenovirus expressing human pregnane X receptor; 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; prER6, everted repeat separated by six nucleotides in the *CYP3A4* proximal promoter; PXR, pregnane X receptor; kb, kilobase(s); MOI, multiplicity of infection; RXRα, retinoid X receptor α; $TCID_{50}$, 50% titer culture infectious dose; PCR, polymerase chain reaction; RU486, 11β-(4-dimethylamino)phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one; SR12813, tetraethyl 2-(3,5-di-*tert*-butyl-4-hydroxyphenyl)ethenyl-1,1-bisphosphonate; T0901317, *N*-(2,2,2-trifluoroethyl)-*N*-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl]-benzenesulfonamide.

tion 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 (Xie et al., 2000; Geick et al., 2001; Synold et al., 2001). PXR is abundantly and selectively expressed in the liver and intestine, in which 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 binding to its cognate response element(s) within the regulatory region of this gene after dimerization with retinoid X receptor α (RXRα) (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. (1996) demonstrated that these half-sites, composed of an everted repeat separated by six nucleotides (ER6), 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) after the dimerization with RXRα 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. (1999) demonstrated the existence of another xenobiotic-responsive enhancer module located at −8 kb upstream from the transcription start point and identified distal nuclear receptor-binding element 1 (dNR1). 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 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 (Ipswich, 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 BD Biosciences (Heidelberg, Germany). KHEM5310 medium was from KAC Co., Ltd. (Kyoto, Japan). Fetal bovine serum, rifampicin, RU486, SR12813, and nifedipine were purchased from Sigma-Aldrich (St. Louis, MO). T0901317 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, pCYP3A4-362-7.7kΔSpeI, pCYP3A4-362-7.7km, pCYP3A4-362m-7.7k, pCYP3A4-362m-7.7km, and 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 PvuII and BglII, and then the fragment was inserted into SmaI and BglII sites of pCYP3A4-362 (pCYP3A4-362-7.7kΔPvuII). pCYP3A4-362-7.7k was digested with MluI and SpeI, 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 KpnI and HindIII sites of pBluescriptII-SK+ (Stratagene, La Jolla, CA), was digested with HincII and self-ligated. This construct was further digested with KpnI and HindIII, and then the fragment was inserted into the same restriction sites of pGL3-Basic (pCYP3A4-362-7.7kΔHincII).

Mutated constructs targeted on each putative half-site at approximately 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-

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	GCGCTCGAGGTGTTGACCCCAGGTGAATC
Antisense	GCGCTCGAGAGGTTTAATAATCACTG
β	
Sense	CGCCTGGAGCAGGTGAATCACAAGCTGAAC
Antisense	CGCCTCGAGCAACACAGGACAAGG
γ	
Sense	GCGCTCGAGACAAGCTGAACTTCTGACAAGAACAAGC
Antisense	GCGCTCGAGCTTGTGATTCACCTGGGGTCAACACAGG
δ	
Sense	CGCCTCGAGTCTGACAAGAACAAGC
Antisense	CGCCTCGAGGCTTGTGATTCACCTGG
ϵ	
Sense	GCGCTCGAGTGTCAGAAGTTCAGCTTGTG
Antisense	CGCCTCGAGGAAGTTCAGCTTGTGATTCAC
η	
Sense	CGCCTCGAGGCTTGTGATTCACCTGG
Antisense	GCGCTCGAGTGTCAGAAGTTCAGCTTGTG

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

The hPXR cDNA, in which the CTG initiation codon was modified to ATG, was isolated by PCR with primers 5′-GGACTCGAGATGGAGGTGAGACCCAAAGAAAGC-3′ and 5′-GGATCTAGACTTTCAGCTACCTGTGATGCCGAA-3′ and inserted into the pGEM-T vector (Promega). The plasmid was digested with XhoI and NotI, and then the cDNA was inserted into the same restriction sites of pT_NT vector (Promega) for in vitro translation. The hRXRα 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 pT_NT-hPXRΔATG and pT_NT-hRXRα, respectively.

Cell Culture and Transfections. HepG2 cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, minimum essential medium nonessential amino acids, and antibiotic-antimycotic (Invitrogen). Cells were seeded onto 12-well plates (BD Biosciences) at a density of 1×10^5 cells/well, and 24 h later, the cells were transfected with 1 μg of luciferase reporter gene construct and 1 μg of pSV-β-Galactosidase Control Vector (Promega) for each well by using CellPhect Transfection Kit (GE Healthcare Bio-Sciences). After the transfection, cells were cultured in serum-free DMEM in the presence of various chemicals for 48 h. Control cells were cultured with vehicle [0.1% dimethyl sulfoxide (DMSO)] alone. Subsequently, the cells were harvested and suspended in 1× 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 hRXRα were synthesized in vitro from pT_NT-hPXRΔATG and pT_NT-hRXRα, 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-^{32}P]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 μl) containing 10 mM Tris-HCl, pH 8.0, 5% glycerol, 100 mM KCl, 2 mM dithiothreitol, 1 μg of poly(dI-dC) (GE Healthcare Bio-Sciences Inc.), and 1 μl each of synthesized hPXR- or hRXRα-containing lysate, or control lysate. Reactions were preincubated on ice for 1 h before the addition of ^{32}P-labeled probe (35 fmol). Competitor oligonucleotides were included at various concentrations as indicated in each figure. Samples were kept on ice for an additional 30 min and then separated on 4% polyacrylamide gel in 0.5× Tris/borate/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 pCYP3A4-362-7.7k$\Delta\beta\gamma$ were digested with KpnI and XbaI, 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 human embryonic kidney 293 cells according to the manual from Quantum Biotechnologies.

Adenovirus that expresses hPXR (AdhPXR) was reported previously (Norachartiyapot 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 ($TCID_{50}$) was determined as reported previously (Matsubara et al., 2007).

Human Hepatocyte Culture and Infection of Recombinant Adenovirus. Cryopreserved human hepatocytes (lot. H704; white, female, 49 years old) were purchased from XenoTech LLC (Lenexa, KS) and thawed using Hepatocytes isolation Kit (XenoTech LLC) according to the manufacturer's protocol. The cells were plated onto collagen-coated 24-well plate (BD Biosciences) 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 h in an atmosphere of 5% CO_2/95% 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 h. After removal of the medium, the hepatocytes were incubated with 0.1 ml of the recombinant adenovirus-containing medium for 1 h followed by the addition of 0.4 ml of the medium containing a chemical. After 24 h, the medium was changed to the fresh medium containing the chemical, and the cells were cultured for an additional 24 h. 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 $TCID_{50}$ with the number of cells.

In Vivo Reporter Assay in Mice. Male ICR mice were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan) were used at 6 weeks old weighing 30 to 33 g. After 20 h of fasting, the mice were injected intraperitoneally with adenoviruses (1×10^{10} $TCID_{50}$/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 3 consecutive days and sacrificed 24 h 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. Prism version 4.0 (GraphPad Software, Inc., San Diego, CA) was used for all of 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 μM 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 with pCYP3A4-362-7.7k (9- and 6.7-fold, respectively, versus 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- 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 the possibility that hPXR/hRXRα heterodimer binds to the DR4 formed with α and β half-sites. Thus, we termed the DR4 as eNR3A4 (the *e*ssential distal *n*uclear *r*eceptor-binding element for CYP*3A4* induction).

Binding of hPXR/hRXRα Heterodimer to eNR3A4. To examine the direct binding of hPXR/hRXRα heterodimer to eNR3A4, EMSAs were carried out with in vitro synthesized hPXR and hRXRα 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/hRXRα heterodimer bound to both dNR1 and prER6 with high affinities (Fig. 4B). hPXR/hRXRα 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/hRXRα 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/hRXRα 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 unlabeled probes at various concentrations (1, 2, 4, 10, or 20 times that of the labeled probe). The results of these assays demonstrated that the affinity of hPXR/hRXRα for eNR3A4 is comparable with or slightly lower than those for dNR1 (Supplemental Data). When a specific antibody against hPXR was included in binding reactions, supershifted complexes were observed with both elements (Fig. 4, C and D).

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

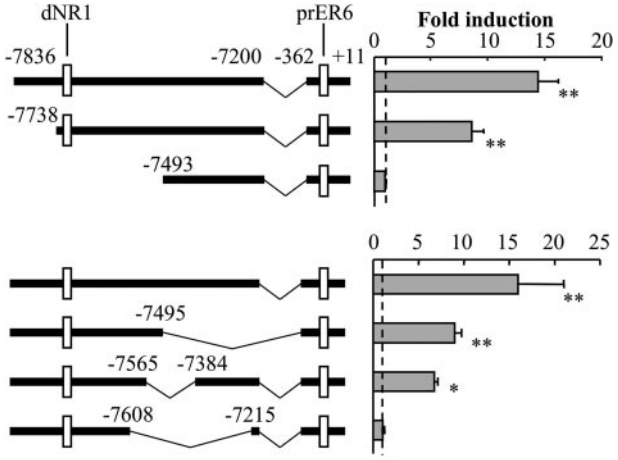

Fig. 1. Influence of partial deletion of the distal promoter on rifampicin-induced transcription of the *CYP3A4* gene. A series of *CYP3A4* luciferase reporter gene constructs was prepared as described under *Materials and Methods* and are shown on the left. Numbers indicate the positions relative to the transcriptional start site. These reporter gene constructs were transiently transfected into HepG2 cells. The cells were treated with rifampicin (10 μM) or vehicle (0.1% DMSO) for 48 h 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 ± 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.

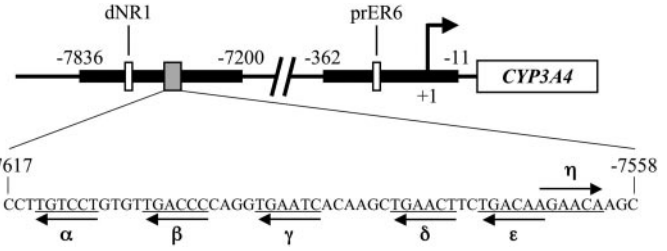

Fig. 2. Sequence of the novel rifampicin-responsive region of the *CYP3A4* gene. ▨, the newly identified rifampicin-responsive region, which includes a cluster of AG(G/T)TCA-like sequences. Of these putative half-sites, six half-sites underlined and indicated by arrows were named α–η half-sites from the 5′ to 3′ direction. Numbers indicate the positions relative to the transcriptional start site. ■, sequences used for the construction of reporter genes. □, dNR1 and prER6.

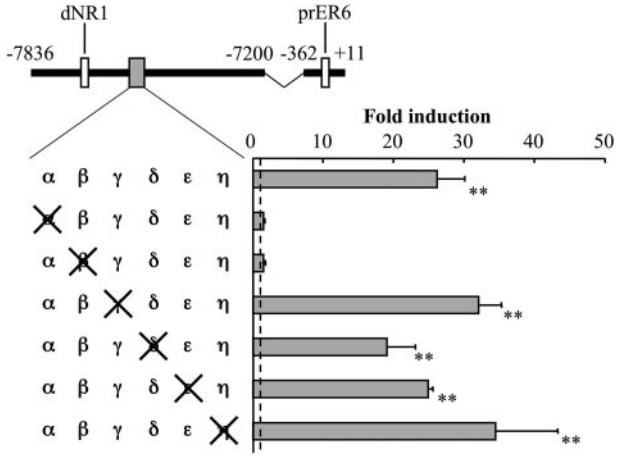

Fig. 3. Influence of mutation of putative nuclear receptor-binding half-site on the response to rifampicin. Left, a schematic representation of the mutated reporter gene constructs is shown. Using the *CYP3A4* luciferase reporter gene construct (pCYP3A4-362-7.7k), each putative half-site contained in the cluster was mutated to CTCGAG (indicated by X) as described under *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 μM) or vehicle (0.1% DMSO) for 48 h 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 ± 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.

and the mutation of both elements did not abolish the rifampicin response completely (4.8-, 7.4-, and 3.5-fold, respectively, versus 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 μM rifampicin for 48 h 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

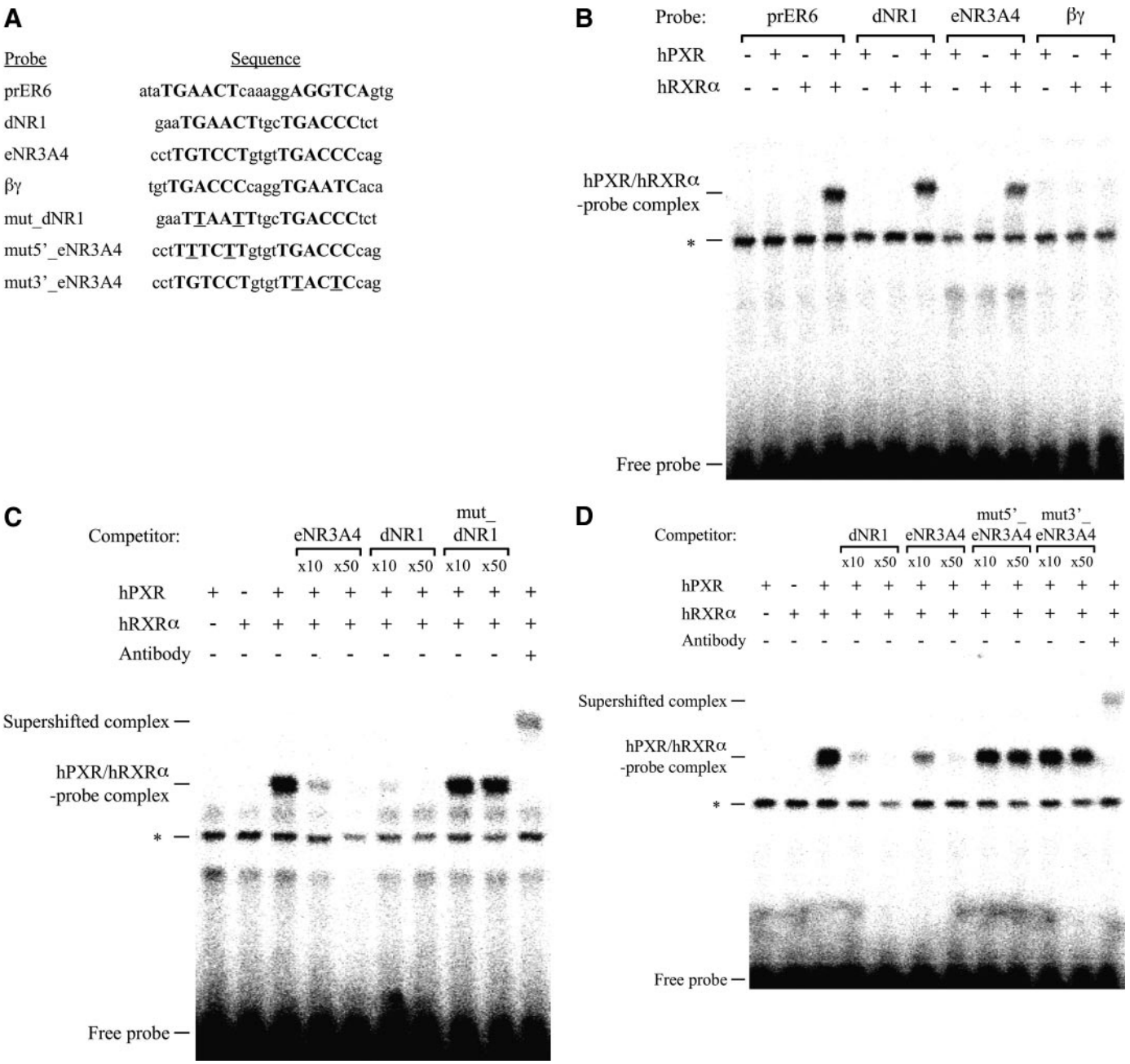

Fig. 4. Binding of hPXR/hRXRα to eNR3A4 in electrophoretic mobility shift assays. A, sequences of the oligonucleotides used for EMSA are shown. Putative nuclear receptor-binding half-sites are shown in uppercase and boldface letters. Mutated nucleotides are underlined. B, EMSAs were performed with radiolabeled prER6, dNR1, eNR3A4, and $\beta\gamma$. Incubations were carried out with in vitro synthesized hPXR and/or hRXRα 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 50-fold molar excess of unlabeled competitors was added to the reaction, as indicated. Supershift analyses were carried out with 1 μg of polyclonal antibody against hPXR protein.

CYP3A4 luciferase reporters (AdCYP3A4-362-7.7k, AdCYP3A4-362m-7.7km, and AdCYP3A4-362-7.7kΔβγ) 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.

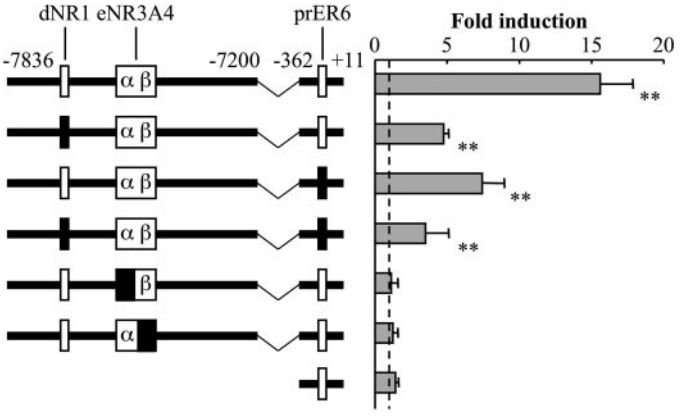

Fig. 5. Evaluation of the role of three PXR binding elements in the rifampicin-induced transactivation of the *CYP3A4* gene in HepG2 cells. PXR binding elements in the pCYP3A4-362-7.7k were mutated as described under *Materials and Methods*, and their rifampicin-responsiveness was analyzed in transient 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 μM) or vehicle (0.1% DMSO) for 48 h 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 ± 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.

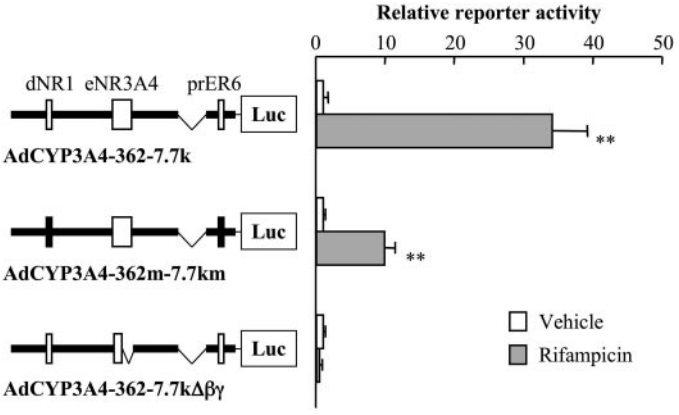

Fig. 6. Reporter assays with adenoviruses expressing *CYP3A4* luciferase reporter genes in human hepatocytes. The schematic structures of the *CYP3A4* reporter adenoviruses are shown on the left. Open and closed boxes represent wild-type and mutated PXR binding elements, respectively. Human hepatocytes were infected with each reporter adenovirus (MOI of 60) and β-galactosidase-expressing AdCont (MOI of 20). The cells were treated with rifampicin (10 μM) or vehicle (0.1% DMSO) for 48 h 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 ± 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.

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 pre-

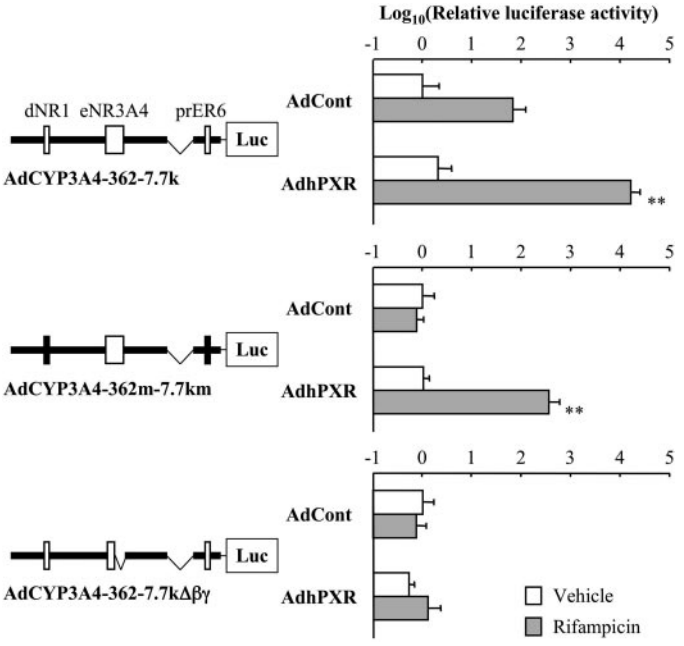

Fig. 7. In vivo reporter assays with adenoviruses expressing *CYP3A4* luciferase reporter genes in mice. AdCYP3A4-362-7.7k, AdCYP3A4-362m-7.7km, or AdCYP3A4-362-7.7kΔβγ (5.0×10^9 $TCID_{50}$/mouse) in combination with AdCont or AdhPXR (5.0×10^9 $TCID_{50}$/mouse) was intraperitoneally injected into mice. These mice were administered rifampicin (100 mg/kg/day, i.p.) or vehicle for 3 days before sacrifice. The animals were sacrificed on the 10th day, and livers were collected. Luciferase activities were measured as described under *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 ± 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.

vious 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 hRXRα.

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. It is noteworthy that the introduction of a mutation into eNR3A4 completely abolished the rifampicin-induced transcriptional activation, whereas 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 a clear disappearance of reporter gene activations in response to phenobarbital, RU486, SR12813, pyrbuticarb, T0901317, hyperforin, nifedipine, and rifampicin (Fig. 8). These compounds were reported previously to activate the transcription of the *CYP3A4* gene through hPXR (Bertilsson et al., 1998; Lehmann et al., 1998; Jones et al., 2000; Moore et al., 2000; Drocourt et al., 2001; Luo et al., 2002; Duniec-Dmuchowski et al., 2007; Matsubara et al., 2007). These findings further support the general function of eNR3A4 in hPXR-mediated CYP3A4 induction.

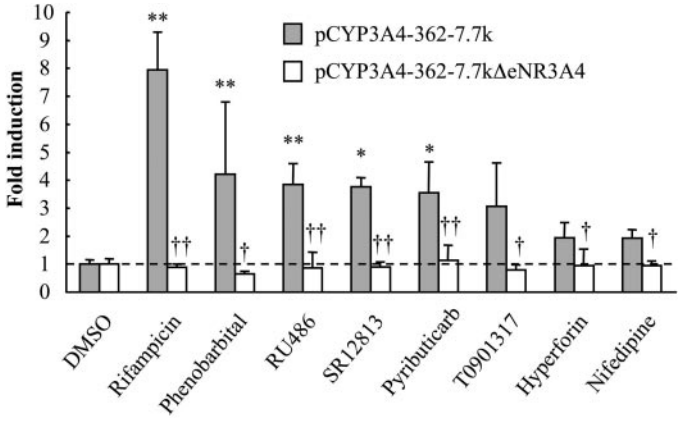

Fig. 8. Role of eNR3A4 in the xenobiotic-induced transcriptional activation of the *CYP3A4* gene. The ability of various CYP3A inducers to transactivate pCYP3A4-362-7.7k through eNR3A4 was tested. HepG2 cells were transiently transfected with pCYP3A4-362-7.7k or pCYP3A4-362-7.7kΔeNR3A4, in which eNR3A4 was deleted. The cells were treated with rifampicin (10 μM), phenobarbital (1 mM), RU486 (10 μM), SR12813 (10 μM), pyributicarb (3 μM), T0901317 (100 nM), hyperforin (100 nM), nifedipine (10 μM), or vehicle (0.1% DMSO) for 48 h 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 ± 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.

These results propose the hPXR-mediated transcriptional regulation of the *CYP3A4* gene through eNR3A4, as well as through 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 (Wang et al., 2003; Chen et al., 2004). 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. (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 approximately 7.6 kb upstream from the transcription initiation site of the *CYP3A4* gene, to which hPXR/hRXRα 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.

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