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The far and distal enhancers in the *CYP3A4* gene co-ordinate the proximal promoter in responding similarly to the pregnane X receptor but differentially to hepatocyte nuclear factor-4 α

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Abstract

CYP3A4 (cytochrome P450 3A4) is involved in the metabolism of more than 50 % of drugs and other xenobiotics. The expression of CYP3A4 is induced by many structurally dissimilar compounds. The PXR (pregnane X receptor) is recognized as a key regulator for the induction, and the PXR-directed transactivation of the *CYP3A4* gene is achieved through a coordinated mechanism of the distal module with the proximal promoter. Recently, a far module was found to support constitutive expression of CYP3A4. The far module, like the distal module, is structurally clustered by a PXR response element (F-ER6) and elements recognized by HNF-4 α (hepatocyte nuclear receptor-4 α). We hypothesized that the far module supports PXR transactivation of the *CYP3A4* gene. Consistent with the hypothesis, fusion of the far module to the proximal promoter of CYP3A4 markedly increased rifampicin-induced reporter activity. The increase was synergistically enhanced when both the far and distal modules were fused to the proximal promoter. The increase, however, was significantly reduced when the F-ER6 was disrupted. Chromatin immunoprecipitation detected the presence of PXR in the far module. Interestingly, HNF-4 α increased the activity of the distal-proximal fused promoter, but decreased the activity of the far-proximal fused promoter. Given the fact that induction of CYP3A4 represents an important detoxification mechanism, the functional redundancy and synergistic interaction in supporting PXR transactivation suggest that the far and distal modules ensure the induction of CYP3A4 during chemical insults. The difference in responding to HNF-4 α suggests that the magnitude of the induction is under control through various transcriptional networks.

Keywords

chromatin immunoprecipitation; cytochrome P450 3A4 (CYP3A4); hepatocyte nuclear receptor-4 α (HNF-4 α); liver tissue; pregnane X receptor; transcription regulation

INTRODUCTION

CYP (cytochrome P450) enzymes are a superfamily of haem-containing proteins and rank first among the Phase I biotransformation enzymes in terms of catalytic versatility and the

number of xenobiotics they metabolize [1–3]. In each mammalian species, four families of P450 enzymes with a total of ~ 20 members are involved in xenobiotic metabolism [4]. Among them, the CYP3A subfamily enzymes are the most abundant P450s in the liver [4]. In humans, the CYP3A subfamily has four members including CYP3A4, CYP3A5, CYP3A7 and CYP3A43 [5]. CYP3A4 is the predominant form of CYP3A enzymes and is responsible for the metabolism of approximately half of commonly used drugs [1]. As a result, CYP3A4 activity is a major pharmacokinetic determinant and serves as an important source for drug–drug interactions.

Many drugs induce the expression of CYP3A4 and thus they accelerate the elimination of drugs that are CYP3A4 substrates. Induction of CYP3A4, in some cases, may have profound clinical consequences. For example, the antidepressant herbal remedy St John's wort is a potent inducer of CYP3A4, and concurrent use of this remedy increases the clearance of the anticancer agent imatinib mesylate by as much as 43 % [6]. Induction of CYP3A4 is largely due to transcriptional activation [7,8]. The PXR (pregnane X receptor) is recognized as a key regulator that mediates the induction [9,10]. This receptor binds to various DNA elements containing repeats of half-site AG(G/T)TCA arranged as different configurations such as DR3 (direct repeat separated by 3 nt) and ER6 (everted repeat separated by 6 nt) [11–13]. In some cases, the two half-sites (a repeat) are slightly different and thus they are called imperfect repeats [12].

Two PXR elements in the *CYP3A4* gene have been shown to support transactivation by PXR. The proximal promoter contains an imperfect ER6 [14], while the distal module contains an imperfect DR3 [12]. Co-transfection with reporter constructs demonstrates that both elements are required for maximum PXR transactivation. Selective disruption of either element decreases the activation by as much as 46 % [12]. A minimal promoter containing the proximal element, although supporting the basal transcription, shows little ability to confer PXR transactivation [12,15]. ChIP (chromatin immunoprecipitation) detects the presence of PXR in the distal module and the proximal promoter [16,17].

A far polymorphic enhancer module has been reported to be involved in the constitutive expression of CYP3A4 [18]. This module is located –11.5 kb from the transcription initiation site and even further upstream compared with the distal module (–8 kb). More importantly, the far and distal modules share some important features in terms of binding sites by transcription factors. While the distal module contains a PXR element DR3, the far enhancer module contains a perfect ER6 element [12,18]. In addition, both the far and distal modules contain numerous *cis*-elements potentially recognized by such transcription factors as HNF-4 α (hepatocyte nuclear factor-4 α) [12,18]. Interestingly, both far and distal PXR elements are flanked by one or more HNF-4 α -binding sites. It has been shown in the distal module that HNF-4 α synergistically acts on PXR transactivation of the *CYP3A4* gene [15,17].

The aim of the present study was to test the hypothesis that the far module, like the distal module, supports PXR transactivation of the *CYP3A4* gene. Consistent with the hypothesis, fusion of the far module to the proximal promoter markedly conferred responsiveness to the PXR activator RIF (rifampicin). The responsiveness was synergistically enhanced when

both the far and distal modules were fused to the proximal promoter. The responsiveness, however, was significantly reduced when the ER6 element in the far enhancer module was disrupted. Both EMSA (electrophoresis mobility-shift assay) and ChIP assay detected PXR binding towards F-ER6. Interestingly, HNF-4 α increased the activity of the distal-proximal fused promoter, but decreased the activity of the far-proximal fused promoter. These findings functionally establish the far module as a critical regulatory component in the transcription of the *CYP3A4* gene.

MATERIALS AND METHODS

Chemicals and supplies

RIF was purchased from Sigma–Aldrich (St. Louis, MO, U.S.A.). Biotinylated probes, DMEM (Dulbecco's modified Eagle's medium), Lipofectamine™ and Plus Reagent™ were from Invitrogen (Carlsbad, CA, U.S.A.). Fetal bovine serum was from Hyclone Laboratories (Logan, UT, U.S.A.). Kits for luciferase assays were from Promega (Madison, WI, U.S.A.). Reaction kits and Biodyne® nylon membrane for EMSA were purchased from Pierce (Rockford, IL, U.S.A.). Protein G–agarose was purchased from Millipore (Temecula, CA, U.S.A.). Unless otherwise specified, all other reagents were purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.).

Plasmid constructs and site-directed mutagenesis

hPXR (human PXR) expression plasmid was isolated with a cDNA trapping method as described previously [19]. The pDGT26.1-HNF-4 α expression construct was a gift from Dr Todd Leff, Department of Pathology, Wayne State University School of Medicine, Detroit, MI, U.S.A., and the RXR α (9-*cis* retinoic acid receptor- α) expression construct was kindly provided by Dr Ronald M. Evans of the Salk Institute. Construction of CYP3A4-P-luc reporter (–362/+53) and CYP3A4-DP-luc reporter (–7836/–6093 to –362/+35) has been described elsewhere [16]. The CYP3A4-FP-luc reporter was prepared by fusing the far module (–11382/–10421) to the proximal promoter (–362/+53). The genomic fragment was generated by PCR with the following primers: forward primer, 5'-cttggtaccgtagtcgttagaatctgaac-3'; reverse primer, 5'-cttacgcgtctccagagacatctcgttctgtag-3'. The fused fragment was inserted into the pGL3 basic vector through KpnI and BglII. To selectively disrupt a PXR response element (e.g. ER6), site-directed mutagenesis was conducted with a QuikChange® kit from Stratagene (La Jolla, CA, U.S.A.). The sequence of the mutagenic primer for the D-DR3 (distal DR3 element) was 5'-gatctcagctgaaagcattgctgaccctctgc-3', and the sequence for the F-ER6 (far ER6 element) was 5'-tagtcgttagaat-ctgttctcctgaagaacatgtgcaaagttgag-3'. To prepare mutant reporters, complementary oligonucleotides containing multiple nucleotide substitutions were annealed to the CYP3A4-DP-luc, CYP3A4-DP-luc or the CYP3A4-FDP-luc construct, and amplified for a total of 16 cycles. The resultant PCR-amplified constructs were then digested with DpnI to remove the non-mutated parent construct. The mutated PCR-amplified constructs were used to transform Epicurian Coli® XL1-Blue bacteria. All constructs were subjected to sequence analysis.

Transient co-transfection and chemical treatment

Human hepatocarcinoma cells (Huh7) were obtained from the A.T.C.C. (Rockville, MD, U.S.A.) and were cultured in DMEM supplemented with 10 % (v/v) fetal bovine serum, 1 % non-essential amino acids and 1 % penicillin/streptomycin (Sigma–Aldrich). Huh7 cells were seeded on 48-well plates at a density of 6×10^4 cells/well. After 24 h, transfection was performed by lipofection with Plus ReagentTM and LipofectamineTM. The transfection mixture contained 50 ng of hPXR or HNF-4 α expression construct, 50 ng of reporter (wild-type or mutant) and 5 ng of the null-*Renilla* luciferase plasmid as an internal control. In some cases, co-transfection was conducted with both PXR and HNF-4 α constructs, and the vector was used to equalize the total amount of constructs. After an overnight incubation, the transfected cells were treated with RIF for 24 h and then collected for the determination of luciferase activities by a dual luciferase assay system as described previously [16,20].

EMSA

Cells {HEK-293T cells [HEK-293 cells (human embryonic kidney cells) expressing the large T-antigen of SV40 (simian virus 40)]} were co-transfected with hPXR and hRXR α (human RXR α) expression constructs, and nuclear extracts were prepared with a nuclear extraction kit (Active Motif, Carlsbad, CA, U.S.A.). The probes (wild-type and mutants) containing proximal, distal and far PXR response elements of the *CYP3A4* gene are shown in Figure 1. The wild-type probes were biotinylated at the 3'-end. In EMSA, nuclear protein (4.5 μ g) was incubated with a double-stranded biotinylated probe (0.1 pmol) at room temperature (25 °C) for 20 min. In competition assays, nuclear extracts were first incubated with an unlabelled probe at a 50 times excess for 5 min before addition of a labelled probe. For supershift assays, the nuclear extracts were first incubated with an anti-hPXR antibody on ice for 20 min and then with a labelled probe. The protein–DNA complexes were resolved by non-denaturing PAGE (5 %) and transferred on to a Biotodyne[®] nylon membrane. The biotinylated probes were detected with streptavidin-conjugated horseradish peroxidase and chemiluminescent substrate (Pierce). The reciprocal inhibition assays were conducted as described for the competition assays, but various amounts of an unlabelled probe (5, 10 and 15 times excess) were used. The chemiluminescent signal was captured by a Kodak Image Station 2000, and the relative intensities were quantified by Kodak 1D Image Analysis Software (Kodak Molecular Imaging Software, Version 4.0).

ChIP

Normal human liver tissues (two individual livers) were obtained from the Liver Tissues Procurement and Distribution System (University of Minnesota). Upon arrival, liver tissues were frozen in liquid nitrogen and pulverized into powder. The pulverized tissues were cross-linked for 15 min by 1.0 % formaldehyde at room temperature (1 g of tissue/30 ml of fixative), and the cross-linking was terminated with glycine (final concentration of 125 mM). The soluble chromatin was prepared as described previously [16]. For ChIP experiments, chromatin was precleared for 2 h at 4 °C with Protein G beads pretreated with herring sperm DNA (0.2 mg/ml) and BSA (0.5 mg/ml). A fraction of the precleared chromatin was stored at –80 °C for later use as an input. A polyclonal antibody against hPXR [21] was added into the precleared chromatin, and an overnight incubation at 4 °C

was performed. As a negative control, the agarose beads were pre-incubated with lysates from non-cross-linked cells. The antibody-bound chromatins and DNA input were analysed by PCR for the presence of the genomic fragments containing the far ER6 element, the D-DR3 and the P-ER6 (proximal ER6 element). Sequences of the PCR primers are listed in Table 1.

Protein analysis and statistical analysis

Protein determination was conducted with a Pierce BCA (bicinchoninic acid) assay kit. Preparation of a polyclonal antibody against hPXR has been described elsewhere [21]. Statistical analyses were performed using R software (version 2.1.1; Free Software Foundation, Boston, MA, U.S.A.). Probability values of $P < 0.05$ were considered indicative of statistically significant differences. The results were expressed as means $\pm$ S.D. for at least three separate experiments, except where results of blots are shown, in which case a representative experiment is depicted in the Figures. All statistical tests were two-sided.

RESULTS

PXR binds to the ER6 element in the far module

To functionally link the ER6 element in the far module to PXR-directed transactivation, we first tested whether this element (designated F-ER6) supports PXR binding. As controls, the binding assays were also conducted with the D-DR3 and the P-ER6. Both D-DR3 and P-ER6 have been shown to serve as PXR-binding sites and support the transactivation by this transcription factor [12,21]. Biotinylated probes (F-ER6, D-DR3 or P-ER6) were incubated with nuclear extracts from PXR/RXR α -transfected cells, and mobility shift was determined by electrophoresis. As shown in Figure 1 (bottom), incubation of the biotinylated F-ER6 probe resulted in the detection of a shifted band (lane 2). The shifted band was eliminated by an excess amount of unlabelled F-ER6 probe (50 times) (lane 3) and was supershifted by addition of the anti-hPXR antibody (lane 4). In contrast, the oligonucleotides with a mutated F-ER6 showed no competitive activity (lane 5). As expected, both D-DR3 and P-ER6 were bound by PXR/RXR α , and the binding was completely competed by respective unlabelled probes and supershifted by the anti-hPXR antibody (Figure 1, top and middle). This antibody caused no changes in the formation of the shifted band by the liver receptor homologue (results not shown), a transcription factor structurally related to PXR.

F-ER6 is less competitive against D-DR3 or P-ER6 in PXR binding

The formation of a supershifted band by the anti-PXR antibody provided direct evidence that the F-ER6 element, like the D-DR3 and P-ER6 elements, supports PXR binding (Figure 1). Among these elements, F-ER6 is a perfect repeat, whereas D-DR3 and P-ER6 elements are imperfect repeats [12,18]. Therefore it was hypothesized that PXR preferably binds to F-ER6 over D-DR3 and P-ER6. In order to test this possibility, a reciprocal inhibition for PXR binding was tested. In these assays, one biotinylated probe such as biotin-F-ER6 was subjected to PXR binding in the presence of a non-biotinylated probe (e.g. D-DR3, P-ER6 or F-ER6). The unlabelled probe was assayed at a 5-, 10- and 15-fold excess of a labelled probe. The relative signal intensities were quantified and plotted as a function of the amounts of an unlabelled probe. It was expected that an unlabelled probe should compete for

PXR binding most effectively against its own labelled probe (e.g. F-ER6 against biotin-F-ER6).

The results on the reciprocal inhibition study are summarized in Figure 2. As expected, all labelled probes produced the highest intensity in the absence of unlabelled probes (lane 4: LP, labelled proximal ER6; LD, labelled distal ER3; and LF, labelled far ER6). The binding intensity was proportionally decreased with increasing amounts of unlabelled probes (Figure 2). The magnitude of the decreases, however, varied among these probes. Unlabelled D-DR3 and P-ER6 probes caused comparable decreases in the binding intensities against all three labelled probes (Figure 2, right). Approx. 75 % decreases were detected when the unlabelled D-DR3 and P-ER3 probes were used at a 10-fold excess against all three labelled probes (Figure 2). In contrast, the decrease was markedly less with the unlabelled F-ER6, even against biotin-F-ER6 (Figure 2, bottom). At a 10-fold excess, the unlabelled F-ER6 decreased the binding against all three labelled probes by only ~ 25 %, although a 65 % decrease was detected against biotinylated F-ER6 when unlabelled F-ER6 was used at a 15-fold excess (Figure 2, bottom).

The far module co-ordinates the proximal promoter in PXR transactivation

The lower effectiveness for the competitive binding by F-ER6 suggests that PXR preferably binds to the D-DR3 and the P-ER6 over the F-ER6 element intracellularly. In order to directly test this possibility, a ChIP experiment was performed with human liver tissues. In addition to the genomic fragments containing F-ER6, D-DR3 and P-ER6, a genomic fragment derived from the *DEC2* (differentially expressed in chondrocytes 2) gene was monitored as a negative control. When chromatin input was used as the template, PCR amplifications produced a band corresponding to the respective genomic fragment including the *DEC2* sequence (Figure 3A, top). The sizes of these bands were the same as predicted based on a particular set of primers (Table 1). In contrast, when the chromatins precipitated by the anti-PXR antibody were used as the template, PCR amplifications produced bands corresponding to genomic fragments harbouring a PXR element but not the fragment derived from the *DEC2* gene. It appeared that the signal on the far module was slightly less than that on the distal module or the proximal promoter (Figure 3A), although the ChIP experiment is generally less quantitative. It should be noted that similar results were obtained with two individual liver tissues.

The ChIP experiment clearly demonstrated that the far module serves as an intracellular binding site for PXR, most likely through the F-ER6 element. We next tested whether this module supports PXR transactivation. The far module was fused to the proximal promoter of CYP3A4 to produce a far-proximal reporter (3A4-FP-luc), and to 3A4-DP-luc (distal-proximal fused reporter) to produce a far-distal-proximal reporter (3A4-FDP-luc) (Figure 3B). As controls, 3A4-P-luc (the proximal promoter reporter) and 3A4-DP-luc were included as well. As expected, the 3A4-P-luc reporter responded negligibly to PXR activator RIF (Figure 3B, right). In contrast, the 3A4-FP-luc and 3A4-DP-luc reporters were markedly activated by RIF. The 3A4-FP-luc reporter was activated slightly less than the 3A4-DP-luc (14-fold versus 11-fold). The 3A4-FDP-luc reporter exhibited marked increases in responding to RIF compared with the 3A4-FP-luc or 3A4-DP-luc, although its basal

activation was also increased (Figure 3B). More importantly, disruption of the F-ER6 element in the 3A4-FP-luc or 3A4-FDP-luc reporter markedly decreased the reporter activity (Figure 3B), providing direct evidence that the F-ER6 element supports PXR transactivation. As expected, disruption of D-DR3 in the 3A4-DP-luc reporter decreased PXR-mediated transactivation, and the decrease was even more profound (~ 90 %). Such a high magnitude of decrease with the 3A4-DP-luc mutant (disrupted D-DR3) was unexpected because disruption of the same DR3 element in the p3A4-362 (–7836/–7208ins) reporter caused a decrease by only 36 % [12]. However, the 3A4-DP-luc contains the distal module sequence from –7836 to –6038 bp, whereas the p3A4-362 (–7836/–7208ins) reporter contains the sequence from –7836 to –7208 bp [12,21].

We next examined the transactivation of the 3A4-FP-luc, 3A4-DP-luc and 3A4-FDP-luc reporters as a function of the increasing concentrations of RIF (0.05–50 μ M). All three reporters were activated in a concentration-dependent manner (at least up to 25 μ M). Overall, the 3A4-FP-luc and 3A4-DP-luc reporters exhibited a similar activation pattern (Figure 3C). The 3A4-FP-luc reporter was activated slightly more at lower concentrations (0.05–0.5 μ M), whereas at higher concentrations (5–50 μ M), the 3A4-DP-luc reporter was activated slightly more (Figure 3C). The activation of the 3A4-FDP-luc reporter, on the other hand, was much higher than either 3A4-FP-luc or 3A4-DP-luc reporter, although the 3A4-FDP-luc reporter exhibited higher basal activation as well (Figure 3C, inset). It should be noted that both 3A4-FP-luc and 3A4-DP-luc reporters showed similar basal activity (DMSO treatment, Figure 3B).

HNF-4 α inversely regulates PXR transactivation between 3A4-FP-luc and 3A4-DP-luc reporters

In addition to the PXR elements, the far and distal modules share several other elements, notably those recognized by HNF-4 α [12,18]. We next tested whether 3A4-FP-luc and 3A4-DP-luc reporters respond similarly to HNF-4 α . In the absence of PXR, HNF-4 α caused few changes in the transactivation of either reporter (Figure 4A). However, the reporters differed markedly in responding to HNF-4 α in the presence of PXR. HNF-4 α markedly enhanced PXR transactivation of the 3A4-DP-luc reporter, but decreased the transactivation of 3A4-FP-luc by ~ 50 % (Figure 4A).

3A4-FP-luc and 3A4-DP-luc differentially respond to certain PXR natural variants

We previously reported that some PXR variants showed altered activity towards the 3A4-DP-luc reporter [22]. We next tested whether these variants show a similar effect on the transactivation of the 3A4-FP-luc reporter. As shown in Figure 4(B), the wild-type PXR transactivated the 3A4-DP-luc reporter to a greater extent than the 3A4-FP-luc reporter in response to RIF, but the opposite was true with PXR variants R98C and R148Q (Figure 4B). Interestingly, R98C and R148Q variants also caused higher basal activation of the 3A4-FP-luc reporter than that of the 3A4-DP-luc reporter (treated with DMSO). In contrast, variants F326Y and R381W caused higher basal but lower RIF-dependent activation of the 3A4-FP-luc reporter (Figure 4B). Overall, some of the differences in responding to PXR variants were relatively small. Nevertheless, these differences suggest that the far and distal modules differ in co-ordinating the proximal promoter, even on PXR-directed transactivation.

DISCUSSION

The far module contains many *cis*-elements putatively recognized by transcription factors such as activator protein-1, HNF-1 α , HNF-4 α or upstream stimulatory factor-1 [18]. EMSA studies on some of these elements have demonstrated that they serve as DNA-binding sites for respective transcription factors. Selected disruption on some of the elements results in decreased basal activation [18], suggesting that the far module supports constitutive transcription of the *CYP3A4* gene. In the present study, we have demonstrated with a ChIP experiment that PXR is present in the far enhancer module (Figure 3A). Fusion of the far module to the *CYP3A4* proximal promoter confers responsiveness to RIF, a potent activator of PXR (Figure 3B). The responsiveness, however, is markedly decreased when the ER6 element is selectively eliminated. These findings establish that the far module supports induced expression of *CYP3A4* as well.

The established role of the far module presents an alternative to the co-ordinated mechanism involving the distal module in supporting PXR transactivation. Like the far module, the distal module confers the responsiveness of the proximal promoter of *CYP3A4* to PXR activators [12]. In spite of co-ordinating with the proximal promoter, both modules are located far upstream of the *CYP3A4* gene. The far module is located at -11.4 to -10.5 kb [18], whereas the distal module is located at -7.8 to -7.2 kb [12]. More importantly, both modules contain numerous *cis*-elements, which are potentially recognized by liver-enriched transcription factors (e.g. HNF-4 α). Interestingly, the PXR element in both modules is flanked by one or more HNF-4 α elements. In the distal module, the HNF-4 α element flanks the PXR element from the 5'-end [12]. In contrast, the PXR element in the far module is flanked by two HNF-4 α elements, which are located downstream of the 3'-end of the PXR element [18].

The differences in the physical location of the PXR element relative to the HNF-4 α elements is likely to contribute to the opposite effect of HNF-4 α on the transactivation of the 3A4-DP-luc and 3A4-FP-luc reporters. We and other investigators have shown that HNF-4 α markedly enhances PXR-transactivation of the 3A4-DP-luc reporter (Figure 4A; [15,17]). In contrast, transfection of HNF-4 α decreases the transactivation of the 3A4-FP-luc reporter by more than 40 % (Figure 4A). The precise mechanisms of the modulation of PXR transactivation by HNF-4 α remain to be determined. It has recently been reported that selective disruption of the HNF-4 α element in the distal module causes few changes in the HNF-4 α enhancement of PXR transactivation, suggesting that DNA binding is not required for the enhancement [17]. In contrast, Tirona et al. [15] have reported that disruption of the HNF-4 α almost completely eliminates the enhancement of PXR transactivation.

The repression of HNF-4 α against PXR transactivation of the 3A4-FP-luc reporter, on the other hand, is probably due to interference with PXR binding in the far enhancer module. One of the HNF-4 α elements in this module is immediately next to the PXR-binding site. Because of such a close proximity, HNF-4 α and PXR are likely to interfere with each other for binding to their respective element. This HNF-4 α -binding site is an imperfect element for this transcription factor; nonetheless, EMSA with an anti-HNF-4 α antibody has established that this element is indeed bound by HNF-4 α [18]. Clearly, the binding

interference mechanism operates largely depending on the relative abundance or even relative binding affinity between PXR and HNF-4 α . Nevertheless, the repression by HNF-4 α of PXR transactivation in the far module probably represents a fine-tuning mechanism in the induced expression of CYP3A4. In addition to the difference in responding to HNF-4 α , the 3A4-DP-luc and 3A4-FP-luc reporters exhibit several other differences. For example, PXR_{R98C} causes markedly higher transactivation of the 3A4-FP-luc reporter, but the opposite is true with PXR_{F326Y} (Figure 4B). Finally, disruption of the PXR element DR3 in the distal module results in decreased basal activation of the 3A4-DP-luc reporter (Figure 3B). In contrast, no changes in basal activity are detected when the ER6 element in the 3A4-FP-luc reporter is disrupted (Figure 3B).

It is interesting to notice that the F-ER element is the least competitive for PXR binding among all elements tested. At a 10-fold excess, the unlabelled F-ER6 probe reduces the binding by only ~ 25 % against all three labelled probes (Figure 2). In contrast, the unlabelled D-DR3 and P-ER6 probes reduce the binding by as much as 75 % (Figure 2). F-ER6 is a perfect repeat, whereas D-DR3 and P-ER6 are imperfect repeats (Figure 1). All three elements have the same 5' half-site (TGAAC). PXR usually heterodimerizes with RXR α ; thus imperfect repeats such as D-DR3 and P-ER6 are likely to support better binding for heterodimers such as PXR–RXR α . Conversely, perfect repeats such as F-ER6 probably support better binding for homodimers. It has been shown by analytical ultracentrifugation experiments that PXR forms homodimers in solution [23]. However, it remains to be determined whether PXR homodimers bind to F-ER6. It should be noted that, in the absence of RXR α , PXR alone does not bind to D-DR3 or P-ER6 [12].

Based on the normalized luciferase activity, co-presence of both the far and distal modules in the heterologous promoter synergistically enhances the reporter activity under both basal (DMSO-treated) and ligand-stimulated conditions (RIF-treated) (Figures 3A and 3C). Interestingly, the increased basal activity by the far module occurs only with the 3A4-FDP-luc but not 3A4-FP-luc reporter, although Matsumura et al. [18] reported increased basal activity of the 3A4-FP-luc as well. It should be noted that the 3A4-FP-luc reporter contains a longer genomic sequence than the one described by Matsumura et al. [18] (three bases at the 5'-end and 46 bases at 3'-end). Nevertheless, the basal activity of the 3A4-FDP-luc reporter is only 20–30 % lower than the ligand-stimulated activity of the 3A4-FP-luc and 3A4-DP-luc reporters, and disruption of the F-ER6 element in the far module abolishes the increase (Figure 3B). Such a magnitude of increase associated with the F-ER6 element suggests that PXR bound to this element, even without ligand-occupancy, confers profound transactivation activity. Such a ligand-independent transactivation is probably achieved through multiple interactions among the far module, the distal module and the proximal promoter. Given the fact that CYP3A4 is involved in the metabolism of more than 50 % of drugs and other xenobiotics, the increased basal expression through the F-ER6 element may ensure the realization of such a functionality.

In summary, we report that PXR is intracellularly present in the far enhancer module and this module supports PXR transactivation. Therefore the far module is functionally redundant to the distal module. The physiological significance of such redundancy remains to be elucidated. CYP3A4 metabolizes more than 50 % of drugs and other xenobiotics; thus

induction of CYP3A4 represents an important detoxification mechanism. The functional redundancy by two regulatory modules ensures that induced expression of CYP3A4 takes place during chemical insults. On the other hand, the far and distal modules differ markedly in responding to HNF-4 α , suggesting that both modules are engaged in a fine-tuning co-ordination in the transcription regulation of the *CYP3A4* gene.

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Abbreviations used

ChIP	chromatin immunoprecipitation
CYP	cytochrome P450
DMEM	Dulbecco's modified Eagle's medium
DR3	direct repeat separated by 3 nt
D-DR3	distal DR3 element
EMSA	electrophoresis mobility-shift assay
ER6	everted repeat separated by 6 nt
F-ER6	far E6 element
HEK-293T cells	HEK-293 cells (human embryonic kidney cells) expressing the large T-antigen of SV40 (simian virus 40)
HNF	hepatocyte nuclear factor
P-ER6	proximal ER6 element
PXR	pregnane X receptor
hPXR	human PXR
RIF	rifampicin
RXRα	9- <i>cis</i> retinoic acid receptor- α
hRXRα	human RXR α

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Figure 1. Binding of PXR to the proximal ER6 (P-ER6), distal DR3 (D-DR3) and far ER6 (F-ER6)

Nuclear extracts (4.5 μ g) from HEK-293T cells co-transfected with hPXR and hRXR α were incubated with a biotinylated probe (0.1 pmol) for 20 min. In the competition assay, nuclear extracts were pre-incubated with an excess (50 times) of an unlabelled probe (lane 3) or a mutant (lane 5) for 5 min, and then incubated with the corresponding biotinylated probe. In the supershift assay, nuclear extracts were incubated first with an anti-PXR antibody on ice for 20 min and then with a biotinylated probe (lane 4). The protein–DNA complexes were electrophoretically resolved and transferred to a Biotodyne[®] nylon membrane. The biotinylated probes were located with streptavidin-conjugated horseradish peroxidase and chemiluminescent substrate. The chemiluminescent signal was captured by Kodak Image Station 2000. WT, wild-type; MT, mutant.

Figure 2. Reciprocal inhibition of PXR element binding among P-ER6, D-DR3 and F-ER6

Nuclear extracts (4.5 μ g) from HEK-293T cells co-transfected with hPXR and hRXR α were pre-incubated with 0–15-fold excess of an unlabelled probe (P-ER6, D-DR3 or F-ER6) and then incubated with a biotinylated probe (e.g. biotin-F-ER6). The PXR/RXR α -bound probes were detected as described in Figure 1. The signal intensity was quantified and expressed as a percentage of the intensity in the absence of an unlabelled probe. Photographs (left) are representative of EMSA from each biotinylated probe, and the relative intensity (right) is plotted as a function of the amount of unlabelled probe. Each data point represents the mean and S.D. for three individual experiments. P, unlabelled P-ER6; LP, labelled P-ER6; D, unlabelled D-DR3; LD, labelled D-DR3; F, unlabelled F-ER6; LF, labelled F-ER6.

Figure 3. Presence of PXR in various regions of the CYP3A4 regulatory sequence and PXR-directed transactivation

(A) ChIP assay for the binding of PXR towards various response elements in the CYP3A4 genomic sequence. Human liver tissues were cross-linked and soluble chromatin was prepared as described in the Materials and methods section. The chromatin was selectively precipitated by an anti-PXR antibody. The input and antibody-precipitated chromatin were analysed by PCR for the presence of genomic fragments harbouring the P-ER6 element (P), D-DR3 (D), F-ER6 (F) and DEC2 promoter. M, marker. (B) Mutational analysis of F-ER6 and D-DR3 in PXR transactivation. Huh7 cells were transfected with the PXR expression construct, along with a reporter (wild-type or mutant), and the null-*Renilla* luciferase plasmid. The transfected cells were treated with RIF at 10 μ M for 24 h. Cell lysates were analysed for luciferase activity. (C) Dose-dependent transactivation of 3A4-DP-luc, 3A4-FP-luc and 3A4-FDP-luc reporters by PXR. Huh7 cells were processed in the same way as mentioned above but exposed to RIF at various levels (0.05, 0.5, 5, 10, 25 and 50 μ M DMSO). The reporter activities were normalized based on *Renilla* luciferase activity. Each data point represents the mean and S.D. for three individual experiments. *Significant difference between 3A4-DP-luc and 3A4-FP-luc reporter activity ($P < 0.05$).

Figure 4. Transactivation of the 3A4-DP-luc and 3A4-FP-luc reporters by PXR, PXR natural variants, or along with HNF-4α

(A) Transactivation of 3A4-DP-Luc and 3A4-FP-luc reporters by PXR, HNF-4α or both. Huh7 cells were transfected with a reporter (3A4-DP-luc or 3A4-FP-luc), the null-*Renilla* luciferase plasmid, along with PXR, HNF-4α or both. The transfected cells were treated with RIF (10 μM) for 24 h. Cell lysates were collected and analysed for luciferase activities. (B) Transactivation of 3A4-DP-luc and 3A4-FP-luc reporters by PXR variants. Huh7 cells were transfected with PXR or a variant, a reporter (3A4-DP-luc or 3A4-FP-luc), and the null-*Renilla* luciferase plasmid. The transfected cells were treated with RIF (10 μM) for 24 h. Cell lysates were collected and analysed for luciferase activities. The reporter activities were normalized according to *Renilla* luciferase activity. Each data point represents the

mean and S.D. for three individual experiments. *Significant difference between DMSO and RIF treatment on the same reporter or two bars connected by a line ($P \leq 0.05$).

Table 1

PCR primer sequences and the predicted size in ChIP assays

Primer	Sequence	Position	Predicted size (bp)	Accession number
P-ER6				
Sense	5'-acaagggaagagagaggcga-3'	10171–10191	200	AF185589
Antisense	5'-aagaggcttctccaccttgga-3'	10370–10350		
D-DR3				
Sense	5'-gagagatgggtcattccttc-3'	2637–2647	374	AF185589
Antisense	5'-ttaccatgtgcacatattacc-3'	3110–2990		
F-ER6				
Sense	5'-ggtaagcctactcatattctc-3'	50518–50538	248	AF280107
Antisense	5'-ttccaaggttcgagaccacga-3'	50765–50745		
DEC2				
Sense	5'-ttcgtccgttggtacgggtgca-3'	47171–47191	240	AC022509
Antisense	5'-aatcctcaaggagcggattg-3'	47410–47390		