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Transcriptional regulation of the *PXR* gene: Identification and characterization of a functional PPARα-binding site within the proximal promoter of *PXR*

Sihem Aouabdi, Gordon Gibson and Nick Plant

School of Biomedical and Molecular Sciences, University of Surrey, Guildford, Surrey, GU2 7XH, UK (SA, GG, NP)

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Running Title:

Gene Regulation of the Pregnane-X- Receptor

Corresponding Author:

Dr Nick Plant

School of Biomedical and Molecular Sciences, University of Surrey, Guildford, Surrey, GU2 7XH, UK

Tel: +44 (0)1483 686412

Fax: +44 (0)1483 300374

Email: N.Plant@Surrey.ac.uk

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Abbreviations:

CAR, Constitutive active receptor; C/EBPα, CAAT-enhancer binding protein alpha; COUP-TF, Chicken ovalbumin upstream promoter transcription factor; EMSA, Electromobility shift assay; FXR, Farsenoid-X-receptor; GRα, Glucocorticoid receptor alpha; HNF, Hepatic nuclear factor; LATF, Ligand-activated transcription factor; LXR, Liver-X-receptor; PPARα, Peroxisome proliferator-activated receptor alpha; PXR, Pregnane-X-receptor; RXRα, Retinoid-X-receptor alpha

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Abstract

The Pregnane-X-receptor (PXR, NR1I2) is widely regarded as a central factor in the body's response to changes in the fluxome, the overall metabolite profile in the body. *PXR* expression is regulated by a number of chemicals at the transcriptional level; the majority of these chemicals are ligands for PXR and substrates for PXR target genes. However, transcriptional activators of *PXR* such as clofibrate do not appear to be PXR ligands, or substrates for its target genes. Understanding the molecular mechanisms underlying both these expected, and more importantly unexpected, transcriptional activations is central to fully understanding the roles of PXR in the human body.

We have carried out an *in silico* analysis of the human *PXR* proximal promoter, identifying putative protein:DNA interaction sites within the 2Kb 5' to the putative transcription start site. These sites included several for liver-enriched transcription factors such as the HNFs and C/EBPα, and COUP-TF, commensurate with the high expression of PXR in liver. Further, we identified putative binding sites for a number of ligand activated transcription factors, suggesting these factors may regulate *PXR* gene expression. Further analysis of this regulatory region has shown that transcriptional activation of *PXR* by PPARα is via a binding site located approximately 1.3kb upstream of the putative transcription start site, with ablation of this site preventing PPARα-mediated activation of *PXR* gene expression. We present a model of how regulation of PXR gene expression by ligand-activated transcription factors may play a central role in the body's response to xenobiotic exposure.

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Introduction

Chemical levels within the body are constantly fluctuating. This may be as a result of circadian rhythms, normal or pathophysiological processes, or the exposure of the body to foreign chemicals such as pollutants or therapeutic medicines. The body responds to these changes by altering chemical flow through metabolic pathways (the fluxome (Sauer, 2004)), aiming to maintaining the *status quo* and ensuring normal/homeostatic physiology. Proteins involved in this process include active transport pumps (e.g. MDR1 and OATP2) to regulate cellular influx/efflux of chemicals and Phase I (e.g. cytochrome P450s) and Phase II (e.g. glutathione *S*-transferase) metabolic enzymes, which catalyse chemical alterations to increase rates of excretion (Plant, 2004). To respond effectively to fluxome alterations a feedback mechanism exists, whereby levels of drug transporters and metabolic enzymes are regulated by a superfamily of ligand-activated transcription factors (LATFs). These LATFs generally possess large ligand binding domains and show promiscuity in their activation profile (Watkins et al., 2001). Due to the overlapping nature of these activation profiles and the complex chemical pool within the body at any one time, it is perhaps not surprising that an interaction network exists between these LATFs, with the sum of the interactions/activations elicited by a chemical determining the exact profile of transporters and/or drug metabolising enzymes activated to respond.

The pregnane X-receptor (PXR, alternate names SXR, PAR or NR1I2) is an LATF that has emerged as a transcriptional activator of at least 40 genes, including several biologically important drug transporters and metabolic enzymes; including CYP3A4 (El-Sankary et al., 2000), CYP2B6 (Goodwin et al., 2001), GST-A2 (Falkner et al., 2001), OATP2 and MDR1a (Maglich et al., 2002). This allows PXR to act as a xenobiotic/metabolite sensor, responding to alterations in the fluxome. Furthermore,

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evidence is beginning to accrue that PXR may function as a *master* xenobiotic/metabolite sensor; integrating inputs from other LATFs into the final output it places on the body. This integration is achieved through the interactions of these LATFs with PXR at both the transcriptomic (Pascussi et al., 2000a) and proteomic (Ourlin et al., 2003) levels. To understand how the body responds to alterations in the fluxome it is therefore imperative that we delineate this highly refined interaction network.

Whereas many studies have been undertaken on the transcriptional regulation of PXR target genes (e.g. *CYP3A4* (El-Sankary et al., 2001; El-Sankary et al., 2002)), little research has been directed at understanding the transcriptional regulation of *PXR* itself. Several chemicals have been shown to regulate PXR mRNA levels, both in primary human hepatocytes (dexamethasone (Pascussi et al., 2000a), lithocholic acid (Kliewer and Willson, 2002)) and rat liver (clofibrate, perfluorodecanoic acid, isoniazid and troleandomycin (Zhang et al., 1999)). As these chemicals are known ligands for other metabolite sensors (e.g. GRα, FXR, LXR, PPARα) this is consistent with PXR acting as a master metabolite sensor, coordinating body responses to changes in the fluxome. In the case of glucocorticoids such as dexamethasone the reason behind such an interaction is clear; glucocorticoids are ligands for both GRα and PXR (Ekins and Erickson, 2002) and are metabolised by PXR target genes (e.g. CYP3A4 (Gibson et al., 2002)). Hence, increased levels of PXR will ultimately lead to increased metabolism of the stimulating glucocorticoid. Activation of PXR expression by bile acids such as lithocholic acids may also be explained as they are ligands for the LATFs FXR and LXR, which undergo protein:protein interactions with PXR (Edwards et al., 2002). By comparison, the activation of *PXR* gene expression by clofibrate and perfluorodecanoic acid, ligands for the fatty acid sensor

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PPARα, is more difficult to explain. These chemicals do not appear to be PXR ligands, nor does their metabolism appear to be dependent upon PXR target genes, although some more potent PPARα agonists do appear to be able to activate PXR. Such data is suggestive that the role of PXR as a master metabolite sensor extends beyond what is currently understood; delineation of the interaction network of metabolic sensing LATFs will thus greatly increase our knowledge on this key biological molecule. Currently, several assays have been developed to measure PXR activation (Kawana et al., 2003; Vignati et al., 2004), and research into PXR target genes/pathways is extensive (Handschin and Meyer, 2003; Bhalla et al., 2004; Uppal et al., 2005). However, the majority of research on transcriptional activation of *PXR* has been descriptive (Zhang et al., 1999; Pascussi et al., 2000a; Pascussi et al., 2000b; Kliewer and Willson, 2002) rather than mechanistic, and the work presented herein aims to provide novel mechanistic insights into the transcriptional regulation of the master xenobiotic/metabolite sensor PXR.

Materials and Methods

In silico binding site analysis. 2.2Kb of proximal promoter immediately upstream of the putative transcription start site for PXR was extracted from the NCBI database, and MatInspector used to interrogate the TRANSFAC database (Wingender et al., 1996). Putative binding sites for transcription factors were identified using a stringent search setting, with the matrix similarity at >0.75 and core similarity at >0.85 in order to minimise the identification of false positives. The matrix represents the DNA binding profile for individual or groups of transcription factors, with the matrix similarity being the quality of a match between the matrix and the input sequence. core similarity represent the quality of a match between the core sequence of a matrix

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(the four most conserved position within a matrix) and the input sequence (Quandt et al., 1995).

Chemicals: Fugene-6 transfection reagent was purchased from Roche Diagnostics, Lewes, UK. Unless otherwise stated all other chemicals were of molecular biology grade and obtained from Sigma Chemical Co. (Poole, UK)

Plasmids: Expression plasmids for LATFs were kindly provided as follows: PXR, Dr Kliewer (University of Texas, Dallas, USA); CAR, Prof M. Negishi (NIEHS, USA); VDR, Dr R. Kim (University of Vanderbilt, Tennessee, USA); PPARα and GRα, Dr J. Tugwood (Astrazeneca, Macclesfield, UK); and RXRα, Prof. P. Chambon (INSERM, Strasbourg, France),

Cell Culture: All cell culture medium and supplements were purchased from Invitrogen (Paisley, UK).

Primary human hepatocytes were obtained from the UK Human Tissue Bank, (Leicester, UK) and cultured in William's Medium E (containing 2mM L-glutamine, 10 % heat-inactivated bovine serum, penicillin/streptomycin (50 U/ml, 50 μg/ml, respectively) and insulin (1 mg/ml)) in collagen-coated 24-well plates (Becton Dickinson) at 3 x 10^5 cells/well. Cells were allowed to attach for 24 hours and then exposed, in triplicate, for 48 hours to 100μM clofibrate or Wy-14,643.

The Huh7 human hepatocellular carcinoma cell line (Nakabayashi et al., 1982) was a kind gift from Dr Steve Hood (GSK, Ware, UK). All cells were routinely cultured in 75 cm^2 vented tissue culture flasks (Nunc, UK) using minimal essential medium with Earle's salts supplemented with 1 % non-essential amino acids, 2 mM L-glutamine, 100U/ml penicillin, 100 μg/ml streptomycin and 10 % foetal bovine serum. In order to maintain phenotypic consistency, Huh7 cells were only used for three weeks (approximately 5 passages) following recovery from liquid nitrogen.

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Transfection: Huh7 cells were seeded into 96-well plates (Nunc International, Leicestershire, UK) at a concentration of 10,000 cells/well and incubated at 37 °C for 24 hrs in a humidified container for attachment. FuGENE 6-mediated DNA co-transfections, using 75ng/well PXR reporter gene construct, were performed as previously described previously (Goodwin et al., 1999), using serum-free medium for the six-hour transfection period; this was then replaced with fresh, complete medium for the remaining culture period. For co-transfection experiments, 25ng/well of the expression plasmid for each ligand-activated transcription factor, or the empty expression plasmid as a control, was included in the transfection mix. Transfections were allowed to proceed for 48 hours, and secretory alkaline phosphatase (SEAP) activity measured.

Alkaline phosphatase activity assay and data analysis: Aliquots of cell culture medium (25 µl/well) were transferred into 96-well optiplates (Canberra Packard, UK). Endogenous alkaline phosphatase activity was deactivated by heat-treatment of the medium at 65 °C for 30 minutes. SEAP activity was then assayed using the AURORA system (ICN, Thame, UK), according to the manufacturer's protocol. Chemiluminescent output was measured using a LumiCount automated plate reader (Canberra Packard, UK).

SEAP activity following 48 hours culture was calculated for both reporter constructs and blank, control, plasmid, and a fold induction relative to vehicle control calculated.

Quantitative PCR. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Crawley, UK), quantified using RiboGreen (Invitrogen, Paisley, UK), and the 1 µg of total RNA treated with RNase-free DNase I (Promega, Southampton, UK) at 37°C for

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30 min. Following heat inactivation, cDNA was produced using Superscript II (Invitrogen) according to the manufacturer's protocol.

Q-PCR reactions were set up using FAM reporter dye/TAMRA quencher dye labelled probes in conjunction with appropriate primer sets as given below (MWG Biotech, Milton Keynes, UK).

Forward PXR primer: 5' - CGAGCTCCGCAGCATCA – 3'

Reverse PXR primer: 5' – TGTATGTCCTGGATGCGCA – 3'

PXR probe: 5' - FAM-TGCTCAGCACACCCAGCGGCT-TAMRA – 3'

QPCR Rox Mastermix (Abgene, Epsom, UK) was used and 25µl reactions set up according to the manufacturer's instructions; Q-PCR results were quantified using the ABI proprietary software against a standard curve generated from human genomic DNA (Promega).

Site-directed Mutagenesis. Specific mutations for disruption of the putative PPARα binding site were created using a PCR-based methodology. Primers were designed to amplify the fragments upstream and downstream of the putative PPRE, with alterations in the primer sequence producing the desired mutation (see below). Engineering of an *Age* I restriction site into these mutant sequences allowed the two fragments to then be joined via ligation to form the full PXR proximal promoter sequence (shown in bold below). The second primer for each amplicon was derived from the SEAP plasmid, thus allowing easy cloning of the mutated construct in to the reporter gene system. All mutation constructs were sequenced on both strands to ensure that the desired mutations had been incorporated.

Mutant A upstream fragment primer: 5'– CCATAGAG**AC**C**G**GTCCTTTTTCCA –3'

Mutant B upstream fragment primer: 5'– CAGCCATA**CC**G**G**TCTGTCCTTTTT –3'

Downstream fragment primer 5'– AGGACAGA**C**C**GG**TATGGCTGTGG –3'

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SEAP upstream fragment primer: 5'– ATAAGGGATTTTGCCGATTTCGG –3'

SEAP downstream fragment primer: 5'– CACAGGTAGGCCGTGGCTGTG –3'

Preparation of nuclear extracts. Nuclear protein extracts were isolated according to the protocol of Dignam (Dignam et al., 1983). Briefly, Huh7 cells were grown to approximately 90% confluence and then collected by trypsinization. Cells were pelleted by centrifugation (1300 g for 5 minutes) and washed twice with PBS. After the second wash, cells were resuspended in 5 x packed cell volume of ice-cold PBS. Cells were pelleted, resuspended in 2 x packed cell volume of buffer A (10 mM Hepes-KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCI, 0.5 mM DTT) and allowed to swell on ice for 10 minutes before disruption using a Dounce homogenizer. Nuclei were pelleted (2000 g for 15 minutes) and resuspended 0.5 x packed nuclear volume (homogenate volume-supernatant volume) of buffer C (25 % glycerol, 20 mM Hepes-KOH pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20 mM NaCl, 0.5 mM DTT, 0.5 mM PMSF). 0.5 x packed nuclear volume of high salt buffer (buffer C containing 1.2 M NaCl) was then added drop wise with swirling, and the suspension homogenized with a Dounce homogenizer. The resulting homogenate was centrifuged at 16000 g for 30 minutes and supernatant (nuclear protein) aliquots stored at -80 °C. Protein concentration was determined by a modification of the method of Stoscheck (Stoscheck, 1990) and integrity assessed by SDS-PAGE. Each aliquot was taken through only three freeze/thaw cycles to maintain protein integrity.

Electromobility shift assay (EMSA): Sense oligomers for wild type and mutated binding sites were labelled at their dephosphorylated 5' ^{32}P ATP, using T4 polynucleotide kinase (Promega). Oligomer sequences were as follows (PPRE sequence underlined, mutated bases in bold),

Putative PPRE: tggaaaa<u>aggacagagctct</u>atggc
Mutated PPRE#1: tggaaaa<u>aggacaga**cc**g**g**t</u>atggc

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Mutated PPRE#2: ctggaaaaaggac**cg**<u>**gtat**</u>**gg**<u>**c**</u>tgt

EMSA binding reactions were carried out at room temperature (22 °C) and consisted of 10 µl of 2x binding buffer (40 mM Tris-HCl, pH 7.9, 100 mM NaCl, 20 % glycerol and 0.2 mM DTT), 1 µg of poly-dI:dC, 1-10 µl of protein extracts (representing 4 to 40 µg nuclear proteins) in a total volume of 20 µl. After 10 minutes incubation, 2 µl of oligomer probe were added and the reaction was further incubated for 30 minutes, followed by separation by polyacrylamide electrophoresis. Competition experiments used between 1x, and 100x excess of unlabelled putative PPRE probe in addition to the labelled probe. In vitro translated PPARα was produced using the PPARα expression plasmid coupled to the TNT T7 Quick system (Promega) according to the manufacturer's protocol.

Results

To begin dissection of the molecular mechanisms underlying transcriptional regulation of the *PXR* gene we initially used web-based resources to predict protein:DNA interaction sites within the PXR proximal promoter. The reference mRNA sequence for *PXR* was aligned against human chromosome 3, and 2.2 kb of genomic DNA 5' to this taken from the human genomic contig. MatInspector Professional was used to interrogate the TRANSFAC database and identify putative protein:DNA interaction sites (Quandt et al., 1995). Figure 1 details those sites identified with a high match and hence represent good possibilities of representing functional sites. A TATAA box was identified 30 bp from the putative transcription site, consistent with previous studies that have indicated that approximately 25-30 bp upstream of the transcription start site is the optimal location for a TATAA box

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(Latchman, 2001). In addition, we have identified putative DNA:protein interaction sites for both auxiliary/tissue specific transcription factors such as HNFs, C/EBPα and Sp1, and a large number of ligand activated transcription factors, including VDR, GRα, PRE and PPARα. This suggests that the PXR proximal promoter is a complex promoter with binding sites for many regulatory transcription factors, consistent with the paradigm of PXR as a master xenobiotic/metabolite sensor, capable of responding to many different stimuli.

Whereas identification of putative protein:DNA interaction sites by *in silico* data mining provides the basis for an investigative hypothesis, it does not form the basis for proven molecular mechanisms of action. Hence, we next examined the regulation of *PXR* using *in vitro* methodology. The 2.2 Kb region of *PXR* proximal promoter was cloned from human genomic DNA, confirmed by sequencing and a reporter gene construct prepared: From this nine daughter constructs were made (Figure 2a). Transfection of these constructs into Huh7 cells was used to determine the input of each region into the basal expression of *PXR.* Figure 2b shows the basal reporter expression from each of these constructs and from these both positive and negative regions of regulation can be inferred. It can be seen that the 2.2 kb proximal to the PXR transcription start site has a number of both positive and negative regulatory regions, consistent with the observation of this region as a complex regulatory region.

As *in silico* analysis identified putative protein:DNA interactions sites for a number of ligand-activated transcription factors within the *PXR* proximal promoter we next undertook co-transfection experiments with expression plasmids for a number of these factors to examine how they altered basal expression. Figure 2c shows the results of this analysis; ER, GRα and PPARα over-expression had a positive effect on *PXR* reporter expression when the whole 2.2 Kb construct was used, suggesting that

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ligands for these receptors may act to increase *PXR* expression and hence activation of PXR target genes. However, it is interesting to note that over-expression of PXR itself, or CAR, significantly decreases basal expression of the *PXR* reporter construct, and indeed this suppression occurs across most of the tested deletion constructs (Figure 3a and 3b). This would suggest that PXR may act in a negative-feedback mode and prevent over-expression of both PXR and its target genes.

The increase in *PXR* gene expression by ligand activated transcription factors such as the glucocorticoid receptor may be readily understandable as GRα ligands are also ligands for PXR itself (El-Sankary et al., 2001), and are metabolised in the body by protein products of PXR target genes (Gibson et al., 2002). By contrast, the large transcriptional activation of *PXR* gene expression by over-expression of the ligand activated transcription factors PPARα is less readily understandable as PPARα ligands have not previously been shown to be PXR ligands or to be metabolised by PXR target genes. In all co-transfection experiments carried out herein it should be noted that no exogenous ligands are added, with activation of receptors via the endogenous ligand pool being presumed. Whereas this may, to some extent, limit the extent of the responses observed, it should not alter their effect/presence. Indeed, previous evidence has shown that many of the ligand-activated and liver-enriched transcription factors are expressed in Huh7, albeit at reduced levels compared to in vivo, supportive of the presence of all the necessary factors within these cells for functioning of these transcription factors (Phillips et al., 2005).

To further investigate this phenomenon we examined which region of the PXR proximal promoter was involved in mediating activation by PPARα. Figure 4a shows that the region -1514 to -1321bp (relative to the putative transcription start site) bounded by the -1.5 Kb and -1.3 Kb daughter constructs was significantly activated

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by over-expression of PPARα; examination of other fragments showed no significant induction, thus localising the PPARα-mediated activation of PXR to this region (Figure 4b). In addition, we examined the role of RXRα, the heterodimerisation partner of PPARα, in this response (Figure 4b). Over-expression of both PPARα and RXRα in the system resulted in an increased degree of activation of the 1.5 Kb fragment (data not shown). Thus we have experimentally localised the PPARα-mediated activation of *PXR* to the -1514 to -1321bp region, a localisation that is consistent with the identification of a putative PPARα binding site (PPRE) within this region (Figure 2a).

To test the functionality of this putative PPRE we have used electromobility shift assay and site directed mutagenesis. Figure 4c shows EMSA of the putative PPRE with Huh7 nuclear protein extract, demonstrating a protein:DNA interaction: Competition with unlabelled probe demonstrates this interaction to be specific, and the binding of *in vitro* translated PPARα provides strong evidence that it is indeed PPARα that is the protein that interacts with this site *in vitro*. Finally, mutagenesis of the putative PPRE resulted in disrupted protein:DNA interactions (Figure 4d), and reduced activation of the 1.5kb reporter gene construct to PPARα over-expression (Figure 4e). To examine if the observed in vitro effects were likely to translate into an in vivo effect, we next examined the level of PXR transcripts in primary human hepatocytes exposed to the PPARα ligands clofibrate and Wy-14,643. As can be seen from Figure 4f, clofibrate elicited a statistically significant increase in PXR transcript level, 189 % of control levels, whereas no significant change was observed with Wy-14,643 (160 % of control levels). For comparison, these changes are significantly less that that caused by dexamethasone (316 % of control), a chemical previously shown to increase human PXR gene expression (Pascussi et al., 2000a), although the use of

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only a single dose cannot rule out the possibility of larger effects with increased doses.

We have thus demonstrated that PPARα-mediates it's activation of PXR gene expression via a PPRE located -1346bp upstream of the putative transcription start site, and that this activation is mirrored in primary human hepatocytes.

Discussion

There has existed for a long time the accepted but unexplained paradigm that the body was capable of producing the most effective response to any individual chemical stimulus, or indeed mixture of chemicals. For such a paradigm to be realistic it is necessary for the body to not only interpret and react to stimuli, but to assimilate multiple stimuli into a single response, probably through interaction networks (Plant, 2004). The ligand-activated transcription factors represent one of the best examples of such an interaction network, with the 57 members of this family found in man (Zhang et al., 2004) interacting both at the transcriptional (Pascussi et al., 2000a) and protein (Edwards et al., 2002) levels. The outcomes of such interactions are twofold: First, it is possible for a single stimulus to elicit tissue-specific responses, activating a gene set to produce the most effective response in each tissue (Hartley et al., 2004). Second, that redundancy in the response system allows for a metabolic safety net, ensuring correct cellular homeostasis is maintained even under extreme external stimulation (Xie et al., 2000b). The PXR appears to be a prime candidate for this last role, with increasing evidence demonstrating that it can be stimulated (at both the gene and protein level) by a wide-range of both xenobiotic and endogenous chemical (Lehmann et al., 1998; Kliewer and Willson, 2002).

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Based upon the findings described herein, we propose a model by which *PXR* may be regulated both by itself and other LATFs (Figure 5). Xenobiotic exposure results in the activation of LATFs, which in turn may stimulate PXR gene expression. If the stimulating chemical is a ligand for PXR then PXR is activated and two endpoints are seen: feedback inhibition of *PXR* gene expression and activation of PXR target gene expression. The latter would result in increased metabolism of the stimulating chemical and reducing its level. However, if the stimulating chemical is *not* a ligand for PXR then we hypothesise that the increased expression of *PXR* would only result in increased *activity* of PXR if sufficient levels of endogenous PXR ligands were present within the cell. Activation of PXR target genes in this latter case would presumably not result in metabolism of the stimulating chemical, but may have consequences for co-exposed chemicals or endogenous metabolism.

An interesting implication from our studies is the potential transcriptional activation of *PXR* by chemicals that are not direct ligands for the gene product, PXR. Previous work has shown that PPARα ligands such as clofibrate increase PXR transcript levels in rat hepatocytes (Zhang et al., 1999), despite the fact that molecular modelling suggests that clofibrate is not a ligand for PXR, with a theoretical EC50 in excess of 100μM (D.Lewis, University of Surrey, UK, *pers. comm.*). In this study we have shown that there exists a molecular rationale for a transcriptional activation by PPARα, enabled by the presence of a functional PPRE within the proximal promoter of the human *PXR* gene. In addition, this suggests that transcriptional activation of *PXR* by non-ligands such as clofibrate may be a cross-species event, as the initial observations were made in rat liver. This is of potential interest in terms of extrapolation from rodent models to the human situation, and may go some way to explaining the apparent conflict between the high species selectivity of the PXR

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ligand binding domain (Xie et al., 2000a) and the cell-type specific responses observed in vivo (Barwick et al., 1996; Swales et al., 2003).

If the stimulating chemical is not a direct ligand of PXR, the question as to the biological relevance of such activations is raised. It is possible that such activation may be effectively a 'bystander effect', whereby *PXR* gene activation is not the desired biological endpoint, but merely a silent side-effect of chemical stimulation (Butte, 2002; Cajiao et al., 2004; Jansen and Gerstein, 2004). However, as described in figure 5, an increase in PXR protein levels may result in increased PXR activation by endogenous ligands, or co-administered chemicals. This increased biological activity could result in disruption of endogenous metabolic processes, leading to a loss of cellular homeostasis. Such events could therefore represent a mechanism by which adverse side effects could occur; indeed, these events may become increasingly relevant as the potency of chemicals against *PXR*-activating LATFs increases, and hence their activation of *PXR* gene expression.

In summary, we have undertaken an examination of the proximal promoter of *PXR* and provide a molecular rationale for the activation of PXR gene expression by LATFs, including PPARα. We have proposed a model of PXR-regulation whereby PXR levels are controlled through the activation of both PXR and other LATFs, placing PXR at the centre of a regulatory network designed to sense, assimilate and respond to chemical stimulus.

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Figure Legends

Figure 1: *In silico* analysis of the PXR proximal promoter. 2.2 Kb of PXR proximal promoter was identified *in silico* and MatInspector used to interrogate the TRANSFAC database and identify putative transcription factor binding sites within this sequence. Boxes represent approximate positions of identified putative DNA:protein interaction sites with matrix and core similarity scores greater than 0.75 and 0.85 respectively. tss = putative transcription start site. AhR=aryl hydrocarbon receptor binding element; CEBPα=CAAT enhancer binding protein element; COUPTF=chicken ovalbumin upstream promoter binding element; ER=oestrogen receptor binding element; GRE=glucocorticoid receptor binding element; HNF1/3/4α=hepatic nuclear factor binding element; PPARα=peroxisome proliferator-activated receptor binding element; PRE = progesterone receptor binding element; VDR=vitamin D receptor binding element

Figure 2: Positive and negative transcriptional elements in the PXR proximal promoter. A deletion construct series was made from 2.2kb of the PXR proximal promoter, anchored at the 3' end, inserted into a secretory alkaline phosphatase reporter gene: Major putative transcription factor binding sites unique to each construct are shown (A). This PXR proximal promoter reporter gene series was then transfected into Huh7 human hepatoma cells, and expression measured 48 hours later, demonstrating regions of positive and negative regulation for *PXR* basal gene expression (B). Co-transfection of the 2.2Kb *PXR* reporter gene construct with expression plasmids for ligand-activated transcription factors is shown in (C). * = $p<0.01$, ** = $p<0.05$, *** = $p<0.001$ relative to previous fragment (B) or no co-

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transfection control (C) using one-way ANOVA with Bonferroni post hoc analysis. Data is representative of experiments undertaken on at least two separate occasions.

Figure 3: Repression of *PXR* expression by PXR and CAR protein. The *PXR* reporter genes deletion construct series was transfected into Huh7 human hepatoma cells, and expression measured 48 hours later. Co-transfection with PXR (A) or CAR (B) resulted in a decrease in PXR gene expression. ** = $p<0.05$, *** =$p<0.001$ relative to no co-transfection control by one-way ANOVA with Bonferroni post hoc analysis. Data is representative of experiments undertaken on at least two separate occasions.

Figure 4: PPARα-mediated increases in *PXR* gene expression occur via a PPRE within the proximal promoter. The 1.5Kb *PXR* reporter genes construct was transfected into Huh7 human hepatoma cells, and expression measured 48 hours later. Co-transfection with expression plasmids for ligand activated transcription factors demonstrated a positive effect by PPARα (A), and this was further increased by over-expression of its heterodimer partner RXRα (B). EMSA demonstrated that a specific protein:DNA interaction occurs at a putative PPARα binding site via competition with excess unlabelled PXR PPRE and binding of in vitro translated PPARα protein (C), and that ablation of the site via mutagenesis could remove this binding (D). Transfection of 1.5 Kb PXR reporter gene construct containing the mutated PPARα binding site resulted in a loss of transcriptional activation by PPARα over expression

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(E). An arrow indicates specific protein:DNA interactions, and a dagger unbound probe. Primary human hepatocytes were exposed to xenobiotics for forty-eight hours and then RNA extracted and PXR transcript levels measured using TaqMan (F)
* = $p<0.01$, ** = $p<0.05$, *** = $p<0.001$ relative to indicated control by one-way ANOVA with Bonferroni post hoc analysis. Data is representative of experiments undertaken on at least two separate occasions.

Figure 5: Proposed model for the role of PXR as a central regulator of body responses to LATF stimulation

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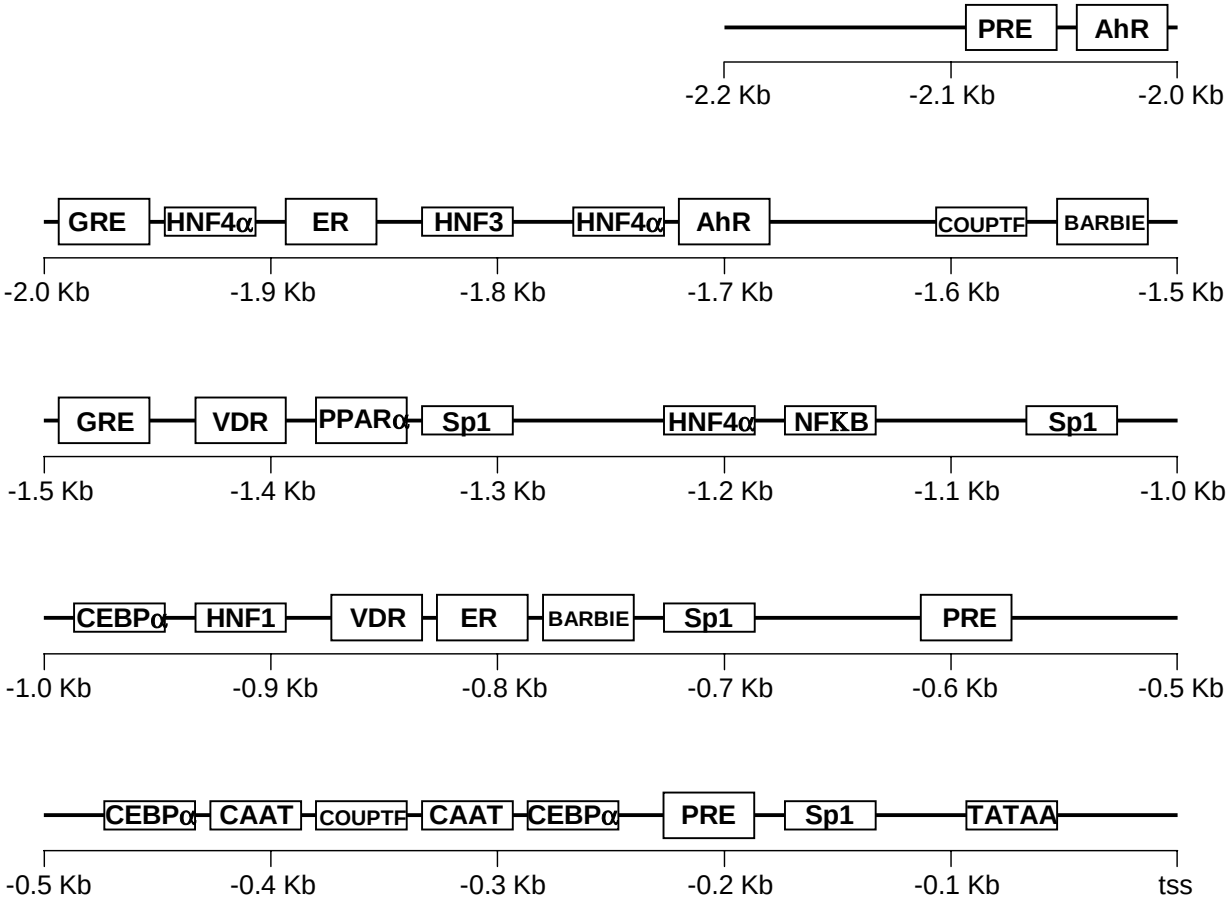

Figure 1

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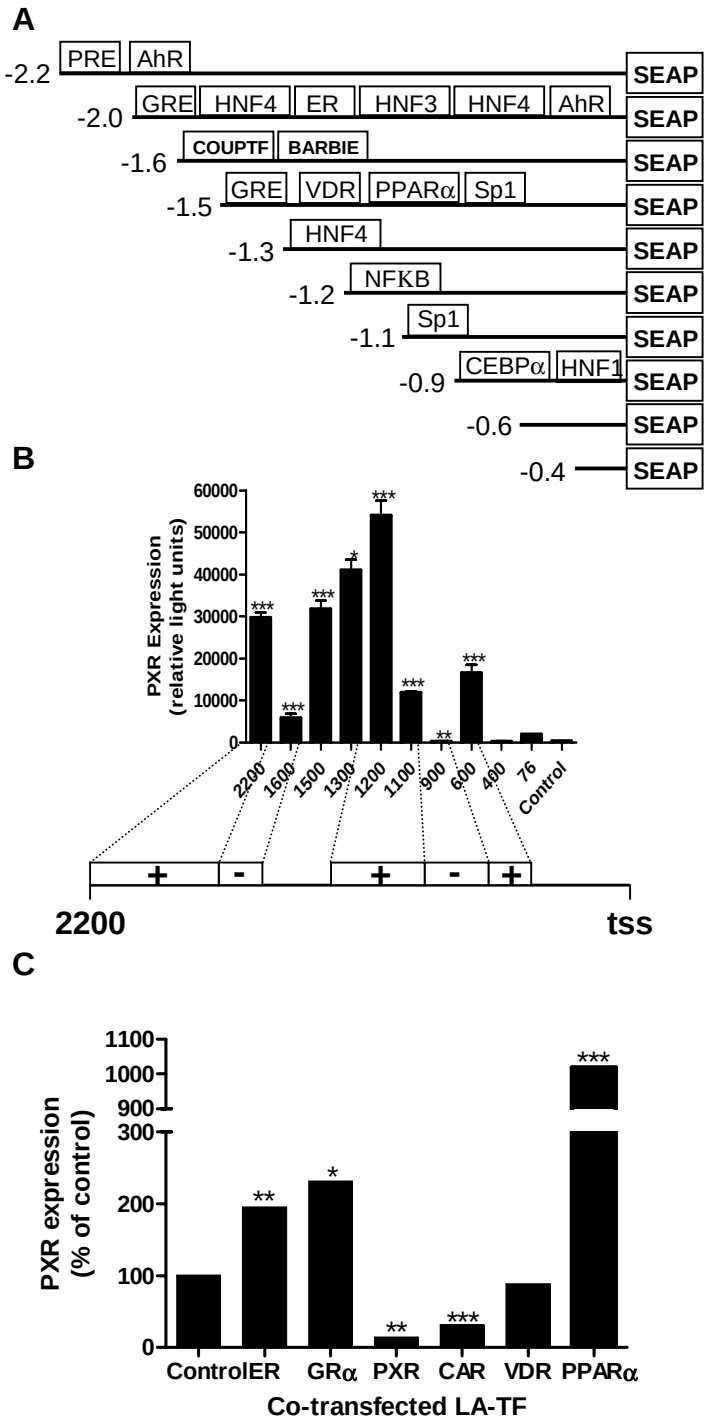

Figure 2

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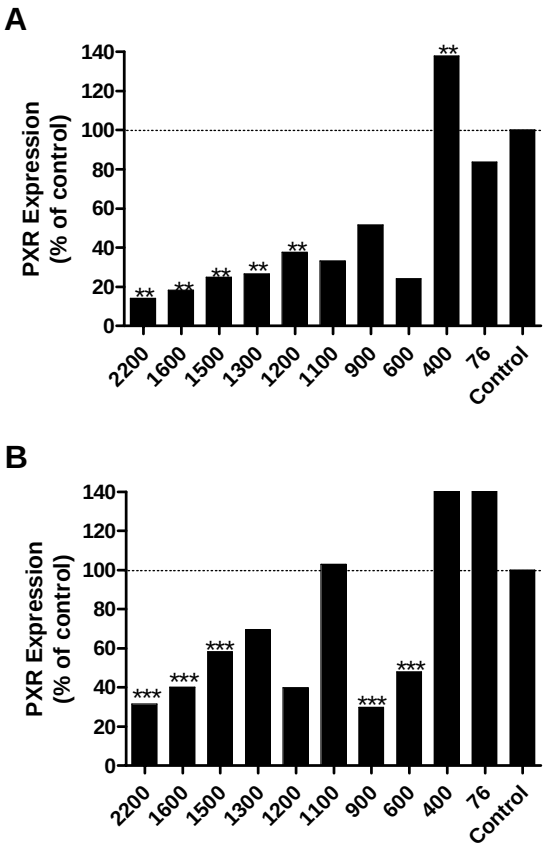

Figure 3

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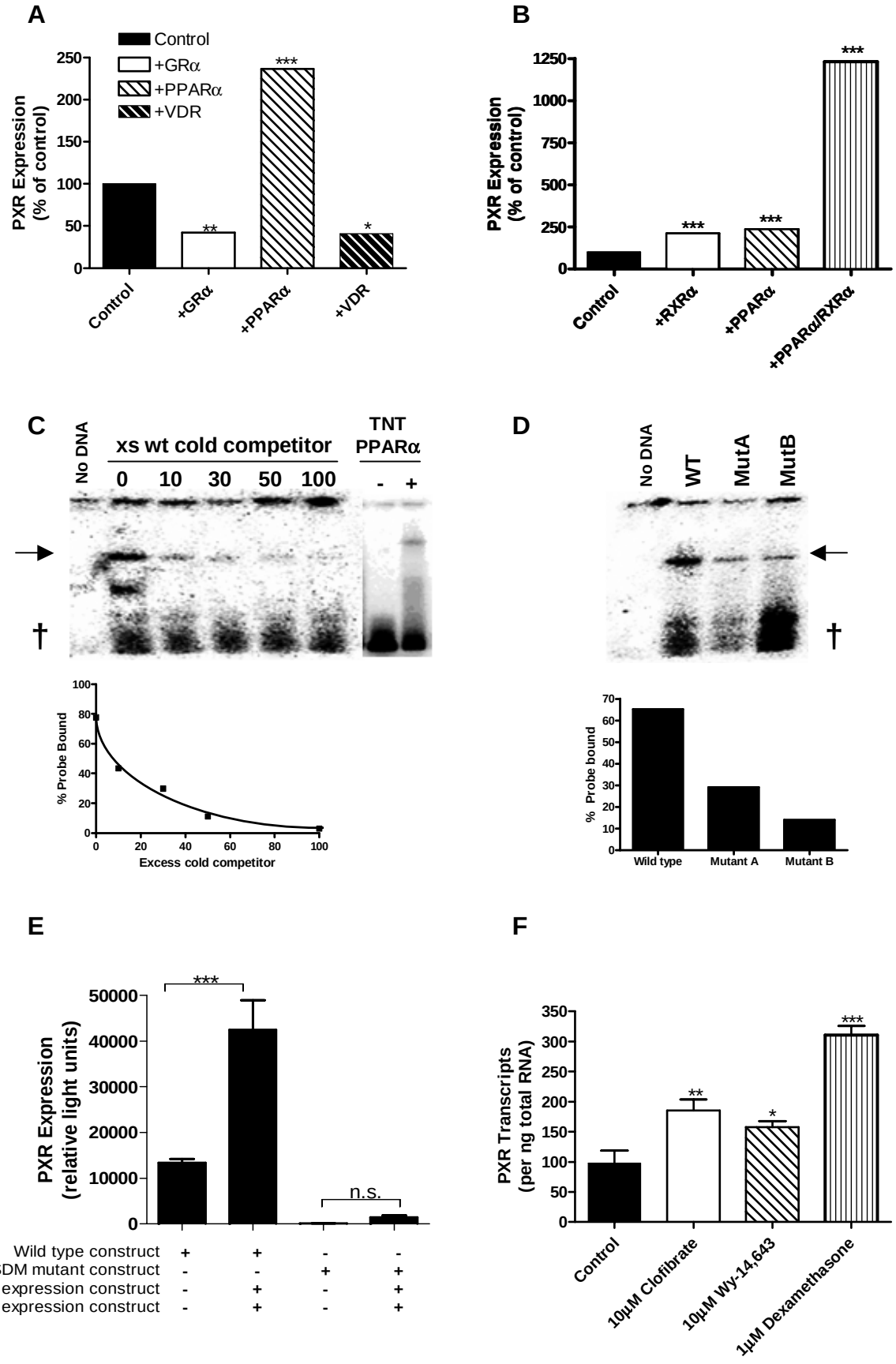

Figure 4

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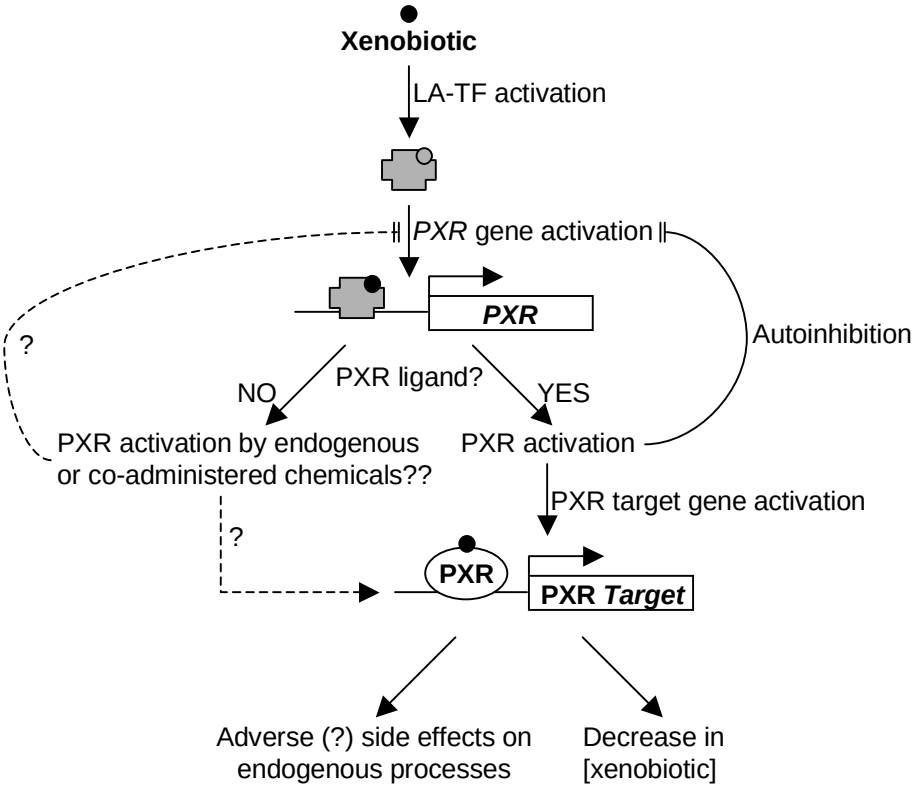

Figure 5

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