Examensarbete

Development of a reporter gene assay for PXR mediated CYP3A4 induction

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Examensarbetet utfört vid Medivir AB
2008-05-22
LITH-IFM-EX--08/1945—SE
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PXR mediated elevation of CYP3A4 expression is a costly problem in drug development as well as a clinical problem due to clinically important drug interactions caused by the enzyme induction. CYP3A4 is responsible for the metabolism of more than 50% of the drugs commonly used today. Many of these, as well as other compounds e.g. in herbal medicines can induce transcription of CYP3A4 and thereby enhance the metabolism of other drugs, rendering them ineffective or more toxic. By using an in vitro assay for CYP3A4 induction, tests can be performed on candidate drugs early in development and thereby save time and resources since CYP3A4 inducers are eliminated from further development. A reporter gene assay was constructed by inserting three modules, which includes PXR binding sites isolated from the CYP3A4 sequence, in front of a luciferase gene. This construct was transfected together with PXR into HEK 293 cells. Induction was evoked by adding rifampicin, a known CYP3A4 inducer, to the medium. After lysis of the HEK cells and addition of luciferase substrate, luminescence intensity was recorded as a measure of induction. The construct worked and consistently showed induction by rifampicin, but could be further improved to yield higher sensitivity.
### Abbreviations.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>AUC</td>
<td>Area under curve</td>
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<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CMV promoter</td>
<td>Human cytomegalovirus promoter</td>
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<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagles medium, for culturing of cells</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>DR-3</td>
<td>Direct repeat with a three nucleotide spacer</td>
</tr>
<tr>
<td>ER-6</td>
<td>Everted repeat with a six nucleotide spacer</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
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<tr>
<td>FD</td>
<td>Construct of the far and distal modules</td>
</tr>
<tr>
<td>FDA</td>
<td>U.S. Food and drug administration</td>
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<tr>
<td>FDP</td>
<td>Construct of the far, distal and proximal modules</td>
</tr>
<tr>
<td>FDP-Luc</td>
<td>Construct with far, distal and proximal modules merged with the luciferase gene</td>
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<tr>
<td>Flp</td>
<td>A recombinase for site specific recombination</td>
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<tr>
<td>Flp-in</td>
<td>System for site specific stable transfections</td>
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<tr>
<td>FRT</td>
<td>Flp recombinase target</td>
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<tr>
<td>GOI</td>
<td>Gene of interest</td>
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<tr>
<td>HEK 293</td>
<td>Cell line derived from human embryonic kidney cells</td>
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<tr>
<td>HNF-4α</td>
<td>Hepatocyte nuclear factor-4α</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>LB</td>
<td>Lysogeny broth medium, for culturing bacteria</td>
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<tr>
<td>LBD</td>
<td>Ligand binding domain</td>
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<tr>
<td>MCS</td>
<td>Multiple cloning site</td>
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<tr>
<td>MFO</td>
<td>Mixed-function oxygenases</td>
</tr>
<tr>
<td>pcDNA</td>
<td>Plasmid complementary DNA</td>
</tr>
<tr>
<td>pCMV6-XL4</td>
<td>Vector containing the PXR gene</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEST</td>
<td>A mixture of penicillin and streptomycin</td>
</tr>
<tr>
<td>pGL3</td>
<td>Vector containing the luciferase gene</td>
</tr>
<tr>
<td>PXR</td>
<td>Pregnane X receptor</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real time polymerase chain reaction</td>
</tr>
<tr>
<td>RIPA</td>
<td>Buffer used for cell lysis</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SMRT</td>
<td>Silencing mediator of retinoid and thyroid hormone receptors</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-Buffered Saline Tween-20</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region of mRNA</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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</table>
1. Introduction.

1.1 Background.
In the 60’s, scientists noticed that certain chemicals administered before short-acting hypnotic drugs, such as pentobarbitone, shortened the sleeping time for test animals. It was concluded that this must be because of induction of metabolizing enzymes by the first substance. Further studies since then have shown that the metabolizing enzymes belong to the cytochrome P450 (CYP) family. The cytochrome P450 is a large family of heme-containing enzymes. Their name is derived from their characteristic absorbance peak at 450 nm. CYPs are found among all branches of life and the official number of CYPs in human today is 57 (as of may, 2008 http://drnelson.utmem.edu/cytochromep450.htm). Approximately 15 of these play the major part in our bodies’ defense against unwanted chemicals. Some are highly selective while others interact with a wide variety of substrates. They are also called monooxygenases or mixed-function oxygenases (MFO) because of their ability to insert an oxygen atom, according to this overall formula: R-H + O\(_2\) + 2H\(^+\) + 2e\(^-\) → ROH + H\(_2\)O were R-H can be one of many substrates.

In an average human liver the CYP3A subfamily is the most abundant, with CYP3A4 (Figure 1) as the top xenobiotic metabolizing enzyme. More than half of the commonly used drugs are metabolized by CYP3A4. (Li et al 1995)

The kinetics of CYP3A4 is rather slow in accordance with its promiscuous ligand binding domain (LBD) and its efficiency in vivo is the result of its abundance rather than kinetics. The economy of this unspecific binding site can be discussed, but it is probably more suitable to have one unspecific protein than millions of specific, especially as the xenobiotics vary and may only be present during short time periods. CYP3A4 is known to be up- and down regulated, e.g. transcription regulation, as well as activated or inhibited at the protein level, by a large number of xenobiotics (Montebello 2005) and thus plays a pivotal role when testing for drug-drug interaction and determination of pharmacokinetic parameters. Induction can have severe clinical consequences when drugs are metabolized too fast, keeping the drugs beneath therapeutic level, while inhibition causes accumulation of the drug to toxic levels. (Figure 2)

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**Figure 1.** Structure of CYP3A4 with heme group at center, adapted from the protein data bank, PDB. (2008) Structure id 1W0E.
The most common pathway of CYP3A4 induction is pregnane X receptor (PXR) mediated. PXR is a member of the nuclear receptor superfamily, characterized by both a ligand and a DNA binding domain. (Lehmann, McKee et al. 1998; Moreau, Vilarem et al. 2008)

Inducing agents bind to PXR which dimerises with another nuclear receptor called retinoid X receptor (RXR). Together they bind to DNA-enhancer elements in the 5’ flanking region of the CYP3A4 gene (Figure 3).

The elements consist of repeats with different configuration of the following sequence AG(G/T)TCA. These are placed in various ways giving rise to different names, such as direct repeat-3 (DR-3, spaced by three nucleotides) and everted repeat-6 (ER-6, spaced by six nucleotides). Three modules have been found so far, (Liu, Song et al. 2008) called the far and distal modules and the proximal promoter. These three enhancer modules have all been shown to support transactivation by PXR in a synergistic manner. The far and proximal modules have an ER-6, the distal a DR-3. Sometimes there is a small difference between the sequences in the repeat; this is the case for the distal and proximal modules and they are then named imperfect repeats.

![Figure 3. Schematic image of PXR mediated CYP3A4 induction. Inducer binds to PXR which in turn dimerises with RXR. Together they bind to the recognition sites in the far, distal and proximal modules and enhance transcription.](image-url)
FDA guidelines (CBER 2006) recommend that CYP3A4 induction should be evaluated for all new drugs. A drug, however potent it is, will not be further developed if its pharmacokinetic profile makes it necessary to administer the drug too often or at too high doses. Because of the high costs of drug development, it would be preferable to test for CYP3A4 induction at an early stage as it is one of the most common reasons for termination of a project. Figure 4 shows percentages of reasons why drug development programs were terminated after clinical trials between 1964 and 1985. The number of projects terminated due to poor pharmacokinetics has been reduced somewhat since 1985, but is still responsible for a significant number of drug failures (Terelius 2008).

![Figure 4. “The reasons for failure of drug development programs by seven UK-based pharmaceutical companies in the period 1964–1985” (Walker 2004)](image)

There are *in vitro* assays that can give some indication whether the candidate drug will interact with PXR and activate transcription of *CYP3A4*. These tests can be performed at an early stage of development and save vast amounts of time and money. Methods used today are of various kinds; some detect induced mRNA or protein levels, some look for an elevated catalytic activity. Other methods use a reporter gene construct, and most of these are based on transient transfection systems (Figure 20, APPENDIX D) Because the only really good experiment for induction is with primary hepatocytes, and they are not easy to come by, the experiments are usually done late in development. *In vivo* animal experiments are not a good way to go either because animal studies can very seldom be extrapolated to humans. The amino acid sequence of the ligand binding domain in PXR only has 80% identity between such species as rabbit, rat and human. For example rifampicin, an antibiotic often used as a positive control for CYP3A4 induction in humans gives a very low activation in mouse and rat. (Jones, Moore et al. 2000) Rifampicin is used for treating tuberculosis and is perhaps the most documented P450 inducer and it is known to induce both human CYP3A4 and CYP2C9 *in vivo*. (Heimark, Gibaldi et al. 1987; Backman, Kivisto et al. 1998)

Today Medivir AB sends their project compounds to a contract research organization for these *in vitro* tests.
The negative side of this is of course the high cost, the long turnaround time and less control. Medivir AB would therefore like to have the means of testing for CYP3A4 induction in house. This also would make it possible to test for induction on a far larger scale. Today, only compounds that have reached a certain level of development are tested. If done earlier and on more substances then resources could be used more efficiently.

1.2 The main objective.

The main objective of this study was to produce a stable mammalian expression cell line containing a construct of human PXR followed by the far and distal enhancer modules and the proximal promoter. They should control a luciferase gene for easy detection. The general idea is that PXR shall be expressed and then interact with inducing agents. PXR will then bind to the CYP3A4 enhancer modules and the luciferase gene will be expressed and fluorescence can be detected (Figure 20, APPENDIX D).

If the first objective did not work a second strategy could be used, where the stable transfection would be performed in two steps. First hPXR would be inserted via pcDNA3.1 and then the FDP-Luc construct via the Flp™-in system. As a last resort, only transient co-transfections would be performed.

2 Materials and methods.

2.1 Chemicals.

Omeprazole, troglitazone, carbamazepine, phenytoin, dexamethasone, caffeine, simvastatin, losartan, verapamil, diazepam, chlorzoxazone, sertraline and warfarin used for induction studies were bought from Sigma-Aldrich, Sweden. The 1 kb plus DNA ladder from Invitrogen was used as standard for agarose-gels. RIPA-buffer used for lysis of cells prior to western blot contained 25 mM Tris•HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS. SDS-PAGE with western blot was used for detecting PXR. The separation gel consisted of 2.5 mL 40% acrylamide-bisacrylamide mix, 2.5 mL 1.5 M Tris (pH 8.8), 0.1 mL 10% SDS, 0.1 mL 10% ammonium persulfate; 0.004 mL TEMED and 4.8 mL water. The stacking gel contained 1.25 mL 40% acrylamide-bisacrylamide mix, 1.25 mL 1.0 M Tris (pH 6.8), 0.1 mL 10% SDS, 0.1 mL 10% ammonium persulfate, 0.004 TEMED and water to a total of 10 mL.

The electrophoresis buffer (5x) was made of 15.1 g Tris base (0.125 M final), 72.0 g glycine (0.96 M final), 5.0 g SDS (0.5% final); H₂O to 1000 mL. Dilute to 1x for working solution.

SDS and TEMED was bought from BioRad. The transfer buffer was made from 2.9 g Glycine, 5.8 g Tris, 0.37 g SDS in 200 mL methanol, and the volume adjusted to 1 liter with water. The buffer was stored at 4°C. TBST buffer consisted of 6.05 g Tris, 8.76 g NaCl in 800 mL water. The pH was adjusted to 7.5 with 1M HCl. 1 mL Tween was added and the volume adjusted to 1 liter with water.
The blocking solution consisted of 5% nonfat dried milk in TBST. Ponceau S solution was made by adding Ponceau S (0.1%) and glacial acetic acid (1%) to water. PXR antibodies (H-160, rabbit polyclonal IgG) for western blot were bought from Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA. Super signal West Pico Chemiluminescent substrate, from Pierce, was used for detection of the goat secondary antibody in the western blot. Dulbecco’s Modified Eagle’s Medium (DMEM) used for cell cultures were bought from Invitrogen. It was supplemented with 10% fetal bovine serum (FBS) and antibiotics (PEST). Cell lysis buffer used prior to the luciferase readings consisted of: 0.65% NP40, 10 mM Tris pH 8 and 1 mM EDTA. All restriction enzymes were purchased from New England Biolabs. For transfections we used Lipofectamine 2000 from Invitrogen. All primers and both the Elongase- and Taq-polymerase used for PCR reactions were purchased from Invitrogen. pGL3-basic was provided by Professor Magnus Ingelman-Sundberg at KI. For total protein concentration measurements we used the BCA protein assay kit from Pierce. All other chemicals were of analytical grade.

2.2 Equipment.
All equipment and solutions used for bacterial and cell work were sterile. NanoDrop, a UV spectrograph, for DNA concentration measurements from Saveen o Werner AB (Limhamn, Sweden). A Berthold MicroLumat Plus LB 96 V was used for luminescence measurements at Medivir AB and a luminometer from Turner Designs at the Karolinska Institute. The reaction from the western blot was detected using a gel documentation system from Fujifilm (LAS-1000). HEK 293 (Human embryonic kidney) cells were used for transfections (Passage 10-17) and they were cultured in an incubator at 37°C and with 5% CO₂. Bacteria were cultured at 37°C under rigorous shaking.

2.3 Plasmid constructs.
Human PXR in a pCMV6-XL4 plasmid was purchased from Origene. The far (-11382/-10421), distal (-7836/-7208) and proximal (-362/53) modules were generated from human genomic DNA, derived from liver cells, by PCR with the following primers: Far-sense: 5’-GGTAC^C-CAA CCT GTC TGC AGT AGT CGT TAG AAT CTG-3’ Far-antisense: 5’-A^CGCGT-GTT CTG TAG AGA GAA GAC AGT GAG TAG GGA-3’ Distal-sense: 5’-A^CGCGT-GGT TGC TGG TTT ATT CTA GAG AGA TGG TTC-3’ Distal-antisense: 5’-G^CTAGC-AAG GAG AAT GGT TAT AAG ATC ATC TCA ATG G-3’ Proximal-sense: 5’-G^CTAGC-AGA TCT GTA GGT GTG CTC TGT TGG GAT G-3’
Proximal-antisense: 5´- C^CATGG-TGTTGCTCTTTGCTGGGCTATGTGC-3´

Bold text indicates added restriction sites and “^” indicates cleavage site. Restriction enzymes do not recognize their restriction site efficiently if it is on the far end of the sequence. Therefore a few bases were added to enlarge the binding surface and for convenience the three bases CAT were added to the 5´ end of all primers. For the PCR reaction we used Elongase Enzyme Mix from Invitrogen and the reaction was performed according to their protocol (APPENDIX C). Several PCR amplifications were performed using different Mg\(^{2+}\) concentrations and different sources of genomic DNA. PCR products containing the correct fragments were re-amplified when a sufficient yield was not reached. Molecular weights of the fragments were analyzed on an agarose gel against a molecular weight standard.

The PCR products were then washed and concentrated according to the manufacturers protocol in the QiaQuick\(^{®}\) PCR purification kit from Qiagen. The PCR products were digested (APPENDIX A Digestion protocol) with the corresponding restriction enzymes (see below) and analyzed on an agarose gel together with three controls. Controls were created by partial digestion of the vector used for insertion, i.e. one vector digested with the first enzyme, and one digested with the second enzyme and one undigested. Controls are needed because the very small nucleotide sequence cut off will not be recognized on an agarose gel. Because of the difference in migrated distance between circular and linearized DNA, together these three controls, will tell you whether the digestion has worked properly or not.

The vector of choice was also digested in order to insert the fragments. After digestion, the products were purified with QiaQuick\(^{®}\) PCR Purification Kit. This removed most of the small fragments cut from vectors and PCR fragments. It also removed buffers and enzymes from the digestion.

Another possible way to remove the undesired DNA fragments would have been to load the digested samples on a 1% low-melting point agarose gel. Desired bands could be cut out and the DNA collected with the QIAquick® Gel Extraction Kit. To extract bands from the gel is a much more efficient way to remove DNA residues, although there is a big loss of DNA because of among other things the degradation by UV-light when cutting from the UV-board. We tried this method but did not get a single colony after ligation. Therefore the first alternative was used.

The samples were mixed and ligated with T4 ligase (APPENDIX B). The ligated samples were then transformed into E. Coli and cultured over night on agar-ampicillin plates. Control ligations without the insert were also performed; several colonies grew and could not be ignored. Therefore a colony screening was performed. Several colonies were picked and for recognition reasons they were re-plated in a numbered grid. They were allowed to grow over night and from this plate, bacteria was plucked and perched into water. These bacteria suspensions were heated to 99°C for five minutes. Taq-polymerase and primers for the fragment were added and PCR reactions were performed (APPENDIX C). After the PCR, the reaction mixtures were run on an agarose-gel and positives containing the correct fragment, e.g. correct size of the fragment, were selected for plasmid preparation.

The FDP-Luc construct was prepared by fusing the far, distal and proximal modules to the Luciferase gene in a pGL3-basic vector from Promega.
The proximal module was inserted through NheI and NcoI, the distal through MLuI and NheI and the far through KpnI and MLuI. 
*PXR* was cut by flanking NotI sites and inserted into the pcDNA5/FRT vector. Correct orientation of insert was controlled by restriction enzyme digestion. The FDP-Luc construct was cloned out of the pGL3 vector by PCR using the following primers:

**FDP-Luc-sense:** 5´- C^TCGAG^-CAA CCT GTC TGC AGT AGT CGT TAG AAT CTG -3´

**FDP-Luc-antisense:** 5´- GG GCC^C^- TTA CAC GGC GAT CTT TCC GCC CTT C -3´

The FDP-Luc construct was inserted behind *PXR* in the pcDNA5/FRT vector through XhoI and ApaI.

A pcDNA5/FRT vector containing only FDP-Luc construct was also prepared and it was introduced with the same restriction sites as mentioned above.

A pcDNA3.1 vector containing the *human PXR* gene was constructed by ligating the *PXR* into the NotI site.

All restriction enzymes were chosen from the vectors multiple cloning sites. (MCS)

For ensuring that the fragments were inserted a PCR screening of the bacterial colonies was performed using the primers corresponding to each fragment.

All vector constructs were also sent to MWG Biotech AG, Germany, for sequencing.

### 2.4 Primer design.

To get a successful PCR reaction, forward and reverse primers need to have approximately the same melting temperatures, Tm, and a GC content of approximately 50% is preferable. This can be verified in various bioinformatic tools. We used a web-based program from Promega for these calculations. ([http://www.promega.com/biomath/calc11.htm](http://www.promega.com/biomath/calc11.htm), 2008)

The sequence for the *CYP3A4* gene was obtained from www.ensembl.org (2008).

### 2.5 The Flp™-in system.

This system uses three vectors to achieve the stable system through Flp™ recombinase-mediated integration.

Often a stable transfection disrupts normal cell activity by insertion into the genome at a sensitive spot and thereby disrupting normal activity crucial for cell viability. With the Flp™-in system this can be circumvented. The system uses three vectors to produce the stable cell line with your choice of insert.

The first vector is used to create a cell line where the insert will not destroy any crucial functions. It is then possible to select for viability and growth rate. The cell line can then be sequenced from the insert, giving the location in the genome, as well as the number of inserts. The cell line which meets the setup criteria can then be used for an infinite number of experiments.

The other two vectors were co-transfected, one, called pcDNA5/FRT, containing a MCS with your cloned gene of interest. The other one, called pOG44, contains the sequence for an enzyme which enables integration of the pcDNA5/FRT vector into the genome at the origin of the stably transfected vector. (Figure 5)
The first vector contains a gene for Zeocin resistance and the second one for Hygromycin. When the second vector is inserted, the Zeocin resistance gene is displaced from the initiation site and replaced by the Hygromycin resistance gene, thereby giving a good selection pathway.

1. pFRT/lacZeo is stably transfected into the mammalian cells of interest to generate the Zeocin™-resistant Flp-In™ Host Cell Line(s)

2. The pcDNA5/FRT expression vector containing your gene of interest (GOI) is cotransfected with pOG44 into the Flp-In™ Host Cell Line.

3. The Flp recombinase expressed from pOG44 catalyzes a homologous recombination event between the FRT sites in the host cells and the pcDNA5/FRT expression vector.

4. Integration of the expression construct allows transcription of the gene of interest (GOI) and confers hygromycin resistance and Zeocin™ sensitivity to the cells.

Figure 5. Mechanism of the Flp™-in system. Images and text from the Flp-in manual provided by Invitrogen (2008).
2.6 Bacterial work. All bacterial work was performed in XL1-Blue bacteria. The pGL3-basic and the pcDNA5/FRT vectors were first amplified by transformation into the bacteria which after selection on agar-amp plates were cultivated over night. The plasmids were prepared using Hispeed plasmid maxi kit from Qiagen. Then the far module was inserted and the vector was transformed and re-prepared. Next the distal module and the proximal promoter were treated as above. In the meantime the \textit{PXR}-gene was inserted into the pcDNA5/FRT vector and treated as above. Finally the whole construct of FDP-Luc was inserted behind \textit{PXR} and the final construct was amplified in bacteria as above. 

2.6.1 Transformation.
DNA was added to 50 μL of competent XL1-blue bacteria. The bacteria were kept on ice for 10-30 min. The samples were then lowered into a 42°C water bath for 40 seconds and put on ice for an additional 5 minutes. 1 mL of pre-heated (37°C) antibiotic free LB-medium was added to the bacteria and then incubated for one hour at 37°C on a shaking board. The bacteria were centrifuged down and the supernatant discarded. The pellet was re-suspended in 100 μL LB and plated on an agar-ampicillin plate.

2.7 Transient transfection.
Cells were cultivated in 24 well plates until 90% confluency was reached. Each well was transfected, with pGL3-FD/FDP alone or together with PXR-pCMV6/XL4, according to the protocol for Lipofectamine 2000 provided by Invitrogen. The cells were then left over night. On the next day, the cells from three wells were trypsinized and counted. From each well aliquots of 60,000 cells were seeded to every third well on a 96 well plate. Thereby making all triplicates as equal as possible. (Figure 6)

![Figure 6: Seeding of transfected cells.](image)

Each well was filled with DMEM to a total of 200 μL. The cells were allowed to settle for five hours. After that, 100 μL was removed and replaced with fresh medium containing different inducers and controls. After 17 hours the medium was removed and the cells were lysed by the addition of 50 μL of a cell lysis buffer containing NP40, in order to expose the luciferase.
For optimization purposes the cells were transfected with various PXR amounts ranging from 0.01 µg to 0.75 µg per well using a 24 well plate. For all other plasmids 0.25 µg were used.

2.8 Stable transfection.
The PXR-FDP-Luc-pcDNA5/FRT plasmid was transfected (see section 2.7) into HEK 293 cells already containing pFRT/LacZeo in the genome. Selection of transfected cells was performed by hygromycin treatment. Colonies were grown in wells and treated with various reference molecules.

2.9 Luciferase readings.
Luciferase measurements were conducted at both Karolinska Institute and Medivir AB, hence two different methods. In the first method used at Karolinska, co-transfection with a renilla luciferase vector was conducted in order to get normalization between the different transfections.
Renilla luciferase in comparison to the firefly luciferase uses another substrate for its reaction. Firefly luciferase uses Luciferin as substrate and needs ATP to process it. While renilla luciferase only needs its substrate, coelenterazine without ATP. This reaction emits a blue light at 480 nm while the firefly luciferase reaction emits at 550-570 nm. Comparing the intensity of the renilla luciferase between transfections gives a fairly normalized response free from differences in cell amounts and degree of transfection.
This, of course, is only the case if the same amounts of renilla luciferase is used for all experiments.
For the experiments lacking the renilla-vector we used BCA protein concentration assay kit, from Pierce, Rockford USA, in order to normalize the activity.
After the cell lysis (see section 2.7) 10 µL of each sample was transferred to a new 96 well plate for later protein concentration measurements. Luciferine was added in 100 µL aliquots to each well and the plate was inserted into a luminometer. ATP (100 µL) was injected and the luminescence measured.

2.10 BCA protein concentration assay.
In order to get a normalized response from the Luciferase readings corrected for differences in cell amounts, the total protein concentration in each well was measured.
Cell lysate (10 µL) was mixed with a BCA reagent that turns catalytic upon binding to proteins. A substrate was added according to the manufacturer’s protocol and absorption was measured at 562 nm.
A standard curve was made from a number of known concentrations of BCA. The protein concentration of each sample could then be determined.
2.11 Western Blot.
The PXR gene was found to contain a rather large untranslated region (UTR) (www.ensembl.org 2008). It has been found that PXR is indeed regulated by microRNA. (Takagi, Nakajima et al. 2008) This could be a problem if there are microRNAs produced in the HEK cells that have affinity for this region. By transfecting HEK 293 cells with the PXR–vector alone and performing a Western blot the level of PXR expressed was detected.

Three cultures of HEK 293 were prepared in 100 mm dishes. At 70 % confluence, they were transfected with 0.5 µg and 1 µg PXR leaving one dish untransfected as a control. The next day the cells were scraped and centrifuged. The pellet was re-suspended in 100 mL RIPA lysis buffer, and the lysate was re-centrifuged to remove cell debris.

The protein concentration of the supernatant was determined in a Bradford assay and 50 µg protein per culture was prepared for SDS-gel separation. The proteins were boiled together with SDS loading buffer to denature protein and make it possible to separate the proteins by size. After separation, the proteins were transferred onto a blotting membrane. The membrane was put in a bath containing a primary PXR-antibody (from rabbit) and left over night at 4°C. The membrane was then washed with TBST with milk protein added to block unspecific binding. After this, the secondary antibody labeled with a horse radish peroxidase (HRP) with affinity for rabbit IgG, was added to the bath. After one hour, the membrane was washed with TBST, and then SuperSignal West Pico Chemiluminescent substrate, from Pierce, was added to the membrane and the resulting chemiluminescence was capture on film.

2.12 Sequencing.
To establish the sequence of the plasmids they were sent to MWG Biotech AG, Germany. The pGL3-FD and pGL3-FDP vector were analyzed. Primers used for the sequencing were as follows: Far-sense, Far-antisense, Distal-sense and Distal-antisense (see section 2.3).

Two primers proposed in the technical manual for pGL3-basic provided by Promega were also used. These primer-sites are situated on either side of the multiple cloning site. They are called RVprimer3, clockwise over the MCS, and GLprimer2, counterclockwise. Since the insertion of the proximal promoter is debated, its primers were discarded and the GLprimer2 would hopefully show what actually was situated directly ahead of the Luciferase gene.

2.12.1 Alignment.
The sequence obtained was compared with available sequence information. Comparing sequences manually is time consuming and not very accurate. A better idea is to use one of the many bioinformatic tools/programs designed for this purpose. The web based bioinformatic program called MAFFT v6.240 was used. (http://align.genome.jp/mafft/) The default settings were not adjusted.

Obtained sequences were compared both to each other as well as to the hypothetical sequence, restriction sites included. It was also aligned to 13 000 bases 3´ of the CYP3A4 gene.
2.13 Calculations.

2.13.1 Fold induction.
Calculations for fold induction have been performed in Excel in the following way. The activity of each sample was normalized for the total protein concentration and the triplicates were averaged. The result for the vector transfected without PXR was subtracted from the other results. Then the obtained numbers for the vectors co-transfected with PXR and treated with inducers were divided by the result obtained for vector co-transfected with PXR but not treated with inducer. These calculations give a Δ Fold induction for the luciferase readings with the value of one for the non-induced Vector co-transfected with PXR.

2.13.2 Renilla co-transfection.
The calculations for the luciferase readings performed at KI (Figure 10 & Figure 11) which included renilla luciferase were performed automatically by the program used by the machine. The program simply normalized the readouts to the intensity of the renilla luciferase, e.g. a low renilla response awards a higher readout from the firefly response and vice versa.

3 Results.

3.1 Cloning.
Cloning proved to be a bigger obstacle than anticipated. The far module was cloned out in the first experiment with a Mg^{2+} concentration of 2 mM and an annealing temperature of 56°C. The distal module was not as easy as the far module. Several different annealing temperatures and Mg^{2+}-concentrations were tried. Finally, when the primers were checked they did not match the ones used by Goodwin et al. (Goodwin, Hodgson et al. 1999). A new pair of primers were ordered and with the new primers the distal module was cloned out at 2 mM Mg^{2+} concentration and at 55°C annealing temperature (Figure 7). The proximal module was also cloned out at an annealing temperature of 55°C. Here all three Mg^{2+} concentrations gave fragments.
Figure 7. PCR isolation of the proximal (left half) and distal (right half) modules.

All three modules were re-amplified to get a sufficient yield. (Figure 8)

Figure 8. Re-amplification of the three modules, the 1 kb+ ladder used as standard.

The far module and the pGL3-basic were digested with the restriction enzymes designated for the far module. The digested vector and module were then run on a low-melting agarose gel. Controls showed that the digestion was successful. The module and vector was cut from the gel and purified with the Qiaquick® gel purification kit. They were then ligated with T4-ligase and transformed into bacteria and plated on agar-amp plates. The ligated vector and control were allowed to grow over night, but not a single colony was detectable the next day. Various conditions, such as different ratios between vector and modules, different ligation times and short UV exposure time, were used for ligation of the other modules. The results were always the same, no colonies.
The digested vectors and modules were purified with the Qiaquick® PCR purification kit instead. The ligated vectors grew this time, but so did the controls. Therefore the colonies were screened to see whether fragments had been inserted. For the far and distal module this was quite straightforward and those modules were inserted and verified, by digestion, within two weeks. The ligation of the proximal module proved to be more persistent. When it was inserted, control experiments indicated that something was wrong. Two colonies from the colony screening were in fact positive for the Proximal-module (Figure 9).

Figure 9. Colony screening for the proximal module. Two out of 24 colonies were positive, from the top left corner, nr 3 and 7.

Both colonies were cultivated and the vectors were extracted with a Qiagen Hi-speed maxiprep kit.
3.2 Control experiments.

Both vectors were transfected into HEK 293 cells, either alone or together with PXR, and induced with rifampicin. The vector containing only the Far and Distal modules (pGL3-FD) was also checked for activity. The first pGL3-FDP vector had almost no activity; it was less than that of the control of pGL3-basic (Figure 10).

![Graph](image)

**Figure 10.** Rifampicin (10 µM) induction of pGL3-FDP1 and FD co-transfected with PXR (0.5 µg of each vector was used per well in a 12 well plate, 0.1 µg was used for renilla). pGL3-basic with and without PXR and an untransfected well was included as controls.

The second pGL3-FDP gave a very high luciferase activity when transfected without PXR but the activity decreased when transfected together with PXR (Figure 11). The pGL3-FD vector consistently showed an increase in activity when co-transfected with PXR and a higher activity than that from the pGL3-basic control but this increase was not statistically significant (Figure 10 & Figure 11). The second pGL3-FDP vector was chosen for further experiments.
Induced by rifampicin

![Graph showing induction of pGL3-FDP2 and FD co-transfected with PXR](image)

Figure 11. Rifampicin induction of pGL3-FDP2 and FD co-transfected with PXR (0.5 µg of each vector was used per well in a 12 well plate, 0.1 µg was used for renilla). pGL3-basic with and without PXR and an untransfected well were used as controls. Also FDP2 (1 µg) without rifampicin (-RIF) were included for comparison reasons.

A PCR experiment was performed to see whether all modules in the second pGL3-FDP vector were inserted. The distal module could not be detected in this experiment (Figure 12).

![PCR experiment result](image)

Figure 12. PCR screening of the pGL3-FDP and the pGL3-FD vector to see if the modules were inserted and whether the size of the FDP-Luc fragment was correct. * indicates that pGL3-FDP was used as template. Notice that the distal module is missing and that the FD-Luc is close to the same size as FDP-Luc.
A digestion of the pGL3-FDP vector with the restriction enzymes designed for the three modules was also performed. The distal module appeared to be only 400 base pairs big, compared to the expected 600 base pairs. There were also some debris bands in the other restriction reactions, although the predicted bands were present (Figure 13).

![Image of restriction digestion](image.png)

**Figure 13.** Restriction digestion of pGL3-FDP with enzymes corresponding to the different modules. The distal module seems to have a size of 400 base pairs.

### 3.3 Sequencing.

Both the pGL3-FD and FDP vectors were sent for sequencing. The sequence of the pGL3-FD vector met the predicted one and the modules had the correct orientation. Some single nucleotides differed from the template (~3 mutations out of ~1900 possible). These were not considered to be of importance since the actual recognition sites were not affected. For the pGL3-FDP vector, the sequence showed that approximately 200 bases at the 3’ end of the ~600 bases long distal module had been removed, although the proximal module had been inserted correctly.

### 3.4 PXR optimization.

Two experiments were performed to optimize the PXR concentration. A control without PEST was included to check whether it affected induction by rifampicin. The highest fold induction was achieved with 0.1 µg PXR per well (in a 24 well plate). PEST did cause induction but it was decided to be irrelevant, since it seemed to give the same response whether treated with rifampicin or not (Figure 14 & Figure 15).
Figure 14. PXR optimization with pGL3-FD, with and without PEST, and rifampicin.

Figure 15. PXR optimization with pGL3-FDP, with and without PEST, and rifampicin.
Interestingly the very high induction seen for pGL3-FDP with 0.1 µg PXR was not observed in any earlier or later experiments (Figure 15). Data for 0.01, 0.05, 0.2, 0.5, 0.7 and 1 µg PXR are not shown.

### 3.5 Induction experiments.

A final transient transfection experiment was conducted in order to test the system, both with the FD and the FDP vector. A wide range of chemicals, both known inducers and known non inducers, were added to the medium after transfection. The luminescence response varied between triplicates. Even when normalizing for total protein concentration, the results varied a lot. It was thus difficult to draw any conclusions from the results of this experiment.

However, for the pGL3-FD vector we could see a general trend that induction did occur (Figure 16), e.g. the chemicals known induction potential seemed to agree with the data collected (not shown).

\[
\frac{(\text{FD}_{\text{PXR inducer}})-\text{FD}}{(\text{FD}_{\text{PXR}})-\text{FD}}
\]

**Figure 16.** pGL3-FD $\Delta$ fold induction, compared to FD-PXR which is set to 1.

pGL3-FDP transfected together with PXR consistently showed a slightly higher activity than FDP transfected alone when no inducers were present. FDP without PXR, induced with rifampicin, gave higher response than FDP without both PXR and rifampicin. Finally we could see a tendency that FDP together with PXR and induced by rifampicin emitted more luminescence than non-induced FDP with PXR (Figure 17). However, one should remember that the variance between the wells was so great that these results were not significant.
Figure 17. Average activity from pGL3-FDP triplicates.

### 3.6 Western blot.

A western blot experiment was also performed to see whether PXR was inhibited by microRNA. The idea was that if PXR could be detected after transfection, it should not be a necessary to make any further investigations into this area. PXR was shown to be present after transfection in a concentration dependent manner. (Figure 18) Some faint bands could possibly be seen even for the untransfected control.

![Western blot](image)

**Figure 18. Western blot.** Three 100 mm dishes were either transfected with 1 µg of PXR, 0.5 µg of PXR or not transfected at all. After cell lysis, 50 µg of protein from each dish was loaded on the gel. From the left; 1 µg, 0.5 µg and untransfected.

### 4 Discussion.

Isolation of fragments from genomic DNA is nowadays routinely performed. However, that does not mean that it always works. Several reactions and a new pair of primers were needed before all three fragments could be isolated. There are many variables to consider. The Mg$^{2+}$ concentration in the reaction buffer is important, as well as the composition of the primer. These are variables to consider during primer design. Yet, when they come to use, they may still not work. The general idea today is to try it again and again until it works, making small adjustments and perhaps designing new primers.
When the proximal module was introduced approximately 200 base pairs were lost from the 3´ side of the distal module. Presumably a point mutation had been introduced during PCR and created a recognition site for the NheI restriction enzyme. The same could also happen if the genomic DNA used in these experiments had a CYP3A4 sequence which differed from the one registered in the Ensembl (2008) database. Another possibility is star activity, which is a term used for restriction enzymes when they cut other sequences than expected.

The increased basal expression for the FDP vector was also observed by Liu et al. (Liu, Song et al. 2008), but they did not describe the decrease found here when PXR and inducers were added. Liu et al. described cis-elements flanking the CYP3A4 enhancer elements and to these cis-elements a protein called hepatocyte nuclear factor-4α (HNF-4α) will bind and enhance transcription. Another study (Tirona, Lee et al. 2003) has shown that the most important cis-element is the one in the distal module ranging from -7783 to -7771, that is 5´ of the PXR enhancer element. Is it possible that the ~200 3´ bases somehow can disturb the HNF-4α activation and with its loss giving HNF-4α a margin to act? The activation of PXR could then explain the inhibition of Luciferase activity by competing with HNF-4α for binding to the DNA strand. This theory however needs some further investigation and seems unlikely since Tirona et al. showed that HNF-4α is crucial for PXR mediated induction. Most studies performed on CYP3A4 induction show a 2-4 fold activation. Whereas Tirona et al. get a 104 fold activation in HepG2 cells when PXR and HNF-4α are co-transfected and the cells are treated with rifampicin. Liu et al. have, in contrast to this study, used a distal module ranging from -7836 to -6038 base pairs (Liu, Song et al. 2008) while in this study a module described by Goodwin et al. (Goodwin, Hodgson et al. 1999), which goes only to -7208, has been used.

Liu et al. noticed a difference, a slightly higher basal expression in their results compared to the results of Goodwin et al, although that difference is not in the proximity of the results produced in this study.

HEK 293 cells were used for these experiments since they were readily available from Invitrogen as pre-made Flp™-in cells, e.g. the first vector of the Flp™-in system had already been introduced and the cell line had been tested for viability. Since CYP3A4 is primarily a hepatic enzyme, using a kidney derived cell line can be a problem. CYP3A4 regulation is a whole lot more complex than just PXR mediated induction. There is cross-talk between PXR and other xeno-sensors, for example CAR, and there are many other mediators of which many probably have not been studied so far. By moving the CYP3A4 enhancer elements to an environment not natural to the system, the fact that many crucial factors in the vicinity could be lacking becomes a major predicament to this study. As previously mentioned, Tirona et al. (Tirona, Lee et al. 2003) have shown that cell lines lacking HNF-4α give much lower induction response and although HNF-4α is found in kidney (Unigene 2008), the HEK cells are so altered that it is possible that they lack HNF-4α, although that remains to be investigated.

Worth noticing from the optimization induction experiments was that lower PXR concentrations gave the highest induction and at high PXR concentrations the luciferase
response was lower than that from cells not transfected with PXR. What this implies is hard to deduce. The PXR might be toxic at high concentrations, since there was a loss of cell adhesion. Whether this was due to PXR or something else, could not be determined at the time.

We also proposed that an excess of PXR could lead to homodimerisation of PXR, (Noble, Carnahan et al. 2006) instead of dimerisation with RXR, and that this might somehow inhibit the transcription of Luciferase, yet this seems to be a far fetched theory seeing that Noble et al. showed that disruption of PXR homodimerisation lowers CYP3A4 induction by rifampicin (Figure 19). Liu et al. also showed that PXR did not bind to the distal and proximal modules in the absence of RXR, although the far module seemed to allow for such interactions. (Liu, Song et al. 2008)

Another interesting idea is that an experiment with PXR null mice has shown a four fold increase in basal expression of CYP3A11. (Staudinger, Goodwin et al. 2001) They proposed that PXR without a ligand functions as a repressor by binding to a silencing mediator of retinoid and thyroid hormone receptors (SMRT) and inhibits its binding to DNA and thereby inhibits basal expression. However in another study, Xie et al. showed that this was not the case for CYP3A4 (Xie, Darwick et al. 2000). However, Johnson et al. disputed whether those data could be the result of naturally occurring differences between mouse lines (Johnson, Li et al. 2006).

There are different hypotheses in the literature for the interactions between SMRT and PXR but we can at least hypothesize that if PXR is expressed in high concentrations it is possible that the inhibitory effect of ligand free PXR is increased.

To finalize the discussion, we propose that the most likely reason for variability in signal between wells, and the most probable reason to the unsignificant results, is varying
transfection efficiency. Although some trends were visible, the lack of internal controls during the last luciferase experiments caused such significant differences between wells in the triplicates that the results between different treatments could not be determined as statistically significant. Repeating the experiment several times could, however, have reduced the standard deviations.

5 Future perspectives.
For future work in this project some ideas are proposed to be taken into consideration. First to the cloning steps, instead of adding restriction sites to every fragment it is possible to add a sequence that overlaps onto the other fragments thereby merging the fragments together in a PCR reaction, before insertion into the vector. This would probably save a lot of time and effort. (Jonsson 2008)
Another idea concerns the problem with normalization of the Luciferase activity. For transient transfections it would probably be best to co-transfect with *Renilla* Luciferase as this will give both the transfection efficiency and the number of cells. It would also be possible to perform a quantitative mRNA (RT-PCR) analysis of PXR, RXR or some constitutive protein, or perhaps all three. This experiment could also be performed to make sure whether PXR and/or RXR are expressed constitutively in our batch of cells. For a stable cell line it is recommended that you compare activity to the concentration of some constitutive protein, this could also be done with quantitative mRNA analysis, but we would like to propose another way, which would be to incorporate the *Renilla luciferase* gene into the vector under stringent control of, for example, a CMV promoter. That would make the experiments fairly easy with no need for extra controls.
As Tirona et al. have shown that the induction of CYP3A4 is much more efficient in the presence of HNF-4α, (Tirona, Lee et al. 2003) it would be preferable to also incorporate that gene into the construct, unless it is constitutively expressed in the cell line chosen. One other idea that could be implemented without too much work is to test the present versions of vectors in liver derived cell lines, such as HepG2 or Huh7 cells. If these cells give a response, widely different from the HEK 293 cells, it would be a good idea to start making a Flp™-in cell line from these liver derived cells instead.

6 Conclusions.
Although the original objective of this study was not met, a long step has been taken along the way. The far, distal and proximal modules were successfully isolated from genomic DNA. At least two of them have so far been correctly inserted and we have seen some interesting results. In the final experiment, with the pGL3-FDP vector, the luciferase signal was successfully induced by rifampicin. It seems that there is still a lot of work to do but under the right conditions and with a little luck, the final assay should be there, looming, not in the far or distal but in the proximal future.
7 Acknowledgements.

There are a lot of people to whom I would like to express my gratitude, because without you this thesis would not have been a reality today. First of all I would like to thank Ylva Terelius, my head supervisor, for giving me the chance to work with this interesting project, for always keeping the door open, for finding me all that reading material, for taking me under your wing, for interesting conversations, for all help and so many other things. Thank you.

Magnus Ingelman-Sundberg for letting me come and work in his group, for taking an interest in my work and always giving valuable comments on it. My thanks also to Nalle, my helpful teacher, who gave me my first real insight into scientific research.

Alvin Gomez, without you my experiments would probably have taken twice the time and the double effort or more. He helped me with exactly everything during my time at KI. ‘Some times science is ingenting’. Thank you Alvin, really, thank you.

Mikael Edlund, again a person who always lends a hand, both during experiments or if I just had a question regarding anything.

Anders Eneroth, for showing me the rat and ordering things for me. I also wish to thank Lasse Nyberg who many times has come in to my office, always with an encouraging word on his lips.

My thanks also go out to everyone in the MIS group at KI, there was not a single one of you who didn’t help me. Amanda, Angelica, Anna, Begüm, Etienne, Inger, Isa, Jana, Jessica, Kristian, Linn, Louise, Margareta, Marika, Mike, Monica, Rasmus, Sarah, Souren, Susanne, Ylva, and Åsa, thank you.

All the people at Medivir AB, you have been so kind to me and so many of you have helped me in my thesis work, if I mention you by name I am afraid I would forget someone. You know who you are. Thank you! And a special thanks to the Bandy gang, those Wednesdays helped me clear my mind.

Pascal, Calle, Peter, Erik, Anders, John and Thomas, tack.

To my mother, the foundation on which we all rest on. I doubt that any other mother can love as much as mine.

Pappa som alltid fått oss att blicka vidare, ut över horisonten. Utan er två, ingenting. Jag älskar er båda.

My wonderful sister, where would one have been if I had not tried to be more like you. Almost last. But, as you well should know by now, not least, my companion, my friend and the love of my life. Thanks for your support and love during this, during what’s been and during what’s to come.

I love you Sara.

All other people who has wandered through my life and think you should be under this header, now you are.

THANKS!
8 References.
http://drnelson.utmem.edu/cytochromep450.html (visited may 2008)
APPENDICES.

APPENDIX A Digestion protocol.
3 µL of PCR product was mixed with 1 µL of each restriction enzyme and 1 µL of restriction buffer. If needed, 1 µL of BSA was added and finally water to a total volume of 10 µL (Table 1). The restriction reaction was allowed to proceed over night at 37°C. Restriction buffers were chosen to be the best possible match from the point of view of both enzymes. The vector was digested by the same enzymes. As controls, two vectors was partially digested by one of the enzymes and one vector were left undigested but still exposed to buffer. BSA was used, in the reaction buffer, for the Distal and Far modules since KpnI and NheI needed it for efficient digestion.

Table 1. Digestion protocol.

<table>
<thead>
<tr>
<th>Digestion</th>
<th>PCR product (µL)</th>
<th>Vector (µL)</th>
<th>Control 1 (µL)</th>
<th>Control 2 (µL)</th>
<th>Undigested (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme 1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Enzyme 2</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Buffer</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>DNA</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>(BSA)</td>
<td>(1)</td>
<td>(1)</td>
<td>(1)</td>
<td>(1)</td>
<td>(1)</td>
</tr>
<tr>
<td>Water</td>
<td>4/(3)</td>
<td>4/(3)</td>
<td>6/(5)</td>
<td>6/(5)</td>
<td>7/(6)</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

APPENDIX B Ligation protocol.
After separation on an agarose gel, fragments were mixed with the cleaved vector at a ratio of 3:1. T4 ligase and ligase-buffer were added and the reaction was allowed to proceed over night at RT (Table 2). Controls consisted of a vector ligated without the fragments. After transformation into E. coli, selection was performed on agar-amp plates. Ligation success was checked by comparing the number of colonies on both plates.

Table 2. Ligation protocol.

<table>
<thead>
<tr>
<th>Ligation</th>
<th>Ligation reaction (µL)</th>
<th>Control (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X Ligation buffer</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Insert DNA</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>pGL3-basic</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>T4 ligase</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Water</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>
APPENDIX C PCR protocol.
For all PCR reactions, except the colony screening, we used the Elongase® enzyme mix from Invitrogen. We used the following protocols. (Table 3 & Table 3. PCR reaction mix protocol for Elongase®.Table 4)

Table 3. PCR reaction mix protocol for Elongase®.

<table>
<thead>
<tr>
<th>PCR reaction</th>
<th>Volumes for genomic target (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM dNTP mix</td>
<td>1</td>
</tr>
<tr>
<td>10 µM Forward primer</td>
<td>1</td>
</tr>
<tr>
<td>10 µM Reverse primer</td>
<td>1</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1</td>
</tr>
<tr>
<td>5X Buffer A</td>
<td>Mixing buffer A and B in different ratios, to a total volume of 10 µL, gives Mg(^{2+}) concentrations between 1 and 2 mM</td>
</tr>
<tr>
<td>5X Buffer B</td>
<td>1</td>
</tr>
<tr>
<td>Elongase Enzyme Mix</td>
<td>1</td>
</tr>
<tr>
<td>Water</td>
<td>35</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>50</strong></td>
</tr>
</tbody>
</table>

10 µL Buffer A gives a Mg\(^{2+}\) concentration of 1 mM and 10 µL of B gives a 2 mM concentration. 1, 1.5 and 2 mM were used.
The temperatures and times were derived from the technical manual for Elongase® enzyme mix from Invitrogen (Table 4). 35 cycles were performed and the pre-amplification denaturation time was three minutes. After the 35 cycles an additional 7 minutes of extension at 68°C was performed to make sure all DNA fragments were complete.

Table 4. PCR temperature and cycling protocol. (Adapted from manual for Elongase® enzyme mix, Invitrogen.)

For primers with a T\(_{\text{m}}\) below 68°C (three-step cycling):

1. Pre-amplification denaturation: 94°C for 30 s, 1 cycle
2. Thermal cycling: Denaturation: 94°C for 30 s
   Annealing: 55-65°C for 30 s
   Extension: 68°C for 45-60 s per kb of target

30-35 cycles
For the colony screenings Taq-polymerase from Advanced Biotechnologies was used. Temperatures and cycling protocol was performed as mentioned above (Table 4), except for the first denaturation step which was performed at 99°C for five minutes and the extension temperature which was 72°C for the Taq polymerase. Mixing conditions are found in the table below (Table 5).

<table>
<thead>
<tr>
<th>Table 5. PCR reaction mix protocol for colony screening with Taq-polymerase.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taq-PCR (50 µL)</td>
</tr>
<tr>
<td>30 µL bacterial water</td>
</tr>
<tr>
<td>1 µL Taq-polymerase</td>
</tr>
<tr>
<td>1 µL dNTP</td>
</tr>
</tbody>
</table>

Bacterial water was obtained by suspending a single E. coli colony in water and boiling it for 5 minutes.

**APPENDIX D** Reporter gene assay.

Figure 20. Schematic image showing the basics of PXR mediated CYP3A4 induction. PXR expression is constant. Inducer binds to PXR which in turn dimerises with RXR. Together they bind to the three modules, far, distal and proximal (only one module shown in image). The transcription of the luciferase gene is enhanced. (Willson and Kliewer, 2002)