
# Regulation of rat *CYP2B1* gene expression:

# The role of nuclear receptors

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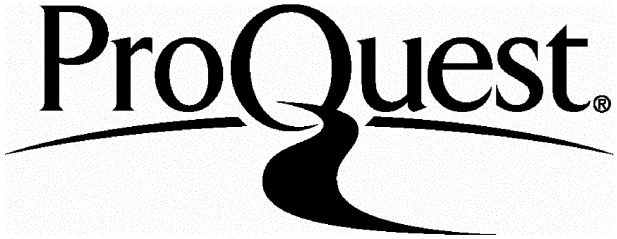

# Abstract

Cytochrome P450 (CYP) plays a central role in the metabolism of endogenous and exogenous compounds. This thesis aims to investigate the molecular mechanism which underlies CYP2B1 induction in response to xenobiotics, particularly phenobarbital (PB), by characterisation of the phenobarbital-responsive element (PBRE) of the *CYP2B1* gene and identification of nuclear regulatory proteins, which bind to the PBRE. *In vivo* transfection studies by biolistic particle delivery, showed that the 5'-upstream region between -2301 to -2142 (159 bp) conferred PB responsiveness on a reporter gene. Within this region, there are two nuclear receptor binding sites; NR1 and NR2, flanking a nuclear factor-1 binding site. Gel mobility shift assays showed the heterodimerisation of constitutive androstane receptor-β (CAR-β) and retinoid X receptor α (RXRα) onto the NR1 site, but not the NR2 site. Co-transfection of the expression vector for CAR-β, with a *CYP2B1* PBRE-Luc reporter construct confirmed both constitutive and xenobiotic-mediated transactivation of reporter gene expression in transfected liver and primary hepatocytes. However, HepG2, Hela, and CV-1 cell lines support only constitutive activation of gene expression by CAR-β. The co-activator SRC-1 enhances both constitutive and xenobiotic-mediated transactivation of a *CYP2B1* PBRE-Luc reporter gene via CAR-β in primary hepatocytes. The SRC-1 stimulation of CAR-β transactivation is not observed when a reporter construct containing only the NR1 site was used. The regulation of the *CYP2B1* gene in response to xenobiotics is therefore mediated by a nuclear

receptor mechanism and involves the interaction between several transcription factors and co-activators. PB and pregnenolone 16α-carbonitrile (PCN) increase the expression of both *CYP2B1* and *CYP3A1* genes. PCN-induction of CYP3A1 is mediated by a pregnane X receptor (PXR). Both CAR-β and PXR were shown to bind to and transactivate via the same DNA element, either the *CYP2B1* NR1 or the *CYP3A1* PXRE, in primary hepatocytes and *in vivo*. Thus, the versatility of these promiscuous nuclear receptors in regulating of more than one CYP may enable an organism to efficiently respond to xenobiotics.

To my dear parents,

*Ruangsak & Sangwian Muangmoonchai*

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# Table of Contents

Abstract....2
Acknowledgements....5
Table of contents....7
List of figures....13
List of tables....16
Abbreviations....17
**INTRODUCTION**....20

1.1 OVERVIEW OF XENOBIOTIC METABOLISM....20
1.2 THE CYTOCHROME P450 SUPERFAMILY....23
1.3 EVOLUTION AND NOMENCLATURE OF CYTOCHROME P450....25
1.4 REGULATION OF *CYP* GENE EXPRESSION....27
1.5 TRANSCRIPTIONAL ACTIVATION OF *CYP* GENES BY DIFFERENT CLASSES OF INDUCERS....30
1.5.1 Aromatic hydrocarbon-inducible CYP1A family....30
1.5.2 The phenobarbital-inducible CYP2B family....31
1.5.3 Steroid-inducible CYP3A family....35
1.5.4 Peroxisome proliferator-inducible CYP4A family....36
1.6 ACHIEVEMENTS IN THE STUDY OF THE MOLECULAR MECHANISM UNDERLYING *CYP2B* GENE EXPRESSION BY PB....38
1.6.1 CYP induction in Bacillus megaterium as a model for mammalian *CYP* gene regulation....39
1.6.2 Proposed phenobarbital-responsive elements within the proximal promoter....40
1.6.3 Distal phenobarbital-responsive enhancer elements....42
1.7 THE NUCLEAR RECEPTOR SUPERFAMILY....46
1.7.1 Retinoid X receptor....51
1.7.2 Nuclear orphan receptors....52
1.7.3 Regulation of *CYP* gene expression by nuclear receptors....56
1.8 AIMS AND SCOPE OF THIS THESIS....58

**MATERIALS AND METHODS** ..... 60

2.1 CHEMICALS AND REAGENTS ..... 60

2.2 DNA CLONES, EXPRESSION VECTORS AND OLIGONUCLEOTIDES ..... 60

2.3 ANIMALS AND CELL LINES ..... 61

2.4 CLONING OF DNA FRAGMENTS ..... 62

2.4.1 Linearisation of the plasmid vector ..... 62

2.4.2 Dephosphorylation of linearised plasmid DNA ..... 62

2.4.3 Preparation of DNA fragments for subcloning ..... 63

2.4.4 Ligation reaction ..... 64

2.4.5 Transformation of bacteria ..... 65

2.4.6 Mini-preparation of the recombinant plasmids ..... 68

2.4.7 Checking for the right insert ..... 69

2.4.8 Preparation of glycerol stocks ..... 70

2.4.9 Maxi-preparation of the recombinant plasmids ..... 70

2.5 PURIFICATION OF DNA FROM LOW MELTING POINT AGAROSE GEL ..... 71

2.6 RNA ISOLATION ..... 72

2.7 ELECTROPHORESIS OF RNA ..... 73

2.7.1 Preparation of RNA samples ..... 74

2.7.2 Gel preparation and electrophoresis ..... 74

2.8 NORTHERN BLOTTING ..... 74

2.9 RADIOISOTOPE-LABELLING OF DNA PROBE ..... 75

2.9.1 Random priming method ..... 75

2.9.2 Fill-in reaction ..... 76

2.9.3 End-labelling reaction ..... 76

2.9.4 Purification of radiolabelled DNA probe ..... 76

2.9.5 Determination of % incorporation of radioactivity into the labelled DNA probe ..... 77

2.9.6 Determination of the specific radioactivity ($S_A$) ..... 77

2.10 HYBRIDISATION ..... 78

2.11 NUCLEAR PROTEIN EXTRACTION ..... 79

2.11.1 Treatment of animals ..... 79

2.11.2 Isolation of nuclei ..... 80
2.11.3 Nuclear protein extraction (Sierra, 1990) ..... 81
2.12 DETERMINATION OF PROTEIN CONCENTRATION BY THE METHOD OF LOWRY (LOWRY, 1951) ..... 82
2.13 GEL MOBILITY SHIFT ASSAY ..... 83
2.13.1 Supershift assay ..... 84
2.13.2 UV-crosslinking of the protein-DNA complexes ..... 84
2.14 PARTIAL PURIFICATION OF NUCLEAR PROTEINS USING PHOSPHO-CELLULOSE ION-EXCHANGE CHROMATOGRAPHY ..... 85
2.14.1 Exchanger preparation ..... 85
2.14.2 Column packing ..... 86
2.14.3 Sample loading and chromatographic elution conditions ..... 86
2.14.4 Coupling of synthesised biotinylated DNA to pre-washed magnetic beads ..... 86
2.14.5 Binding of nuclear proteins to the DNA-affinity magnetic beads ..... 87
2.15 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS ..... 87
2.16 WESTERN BLOT ANALYSIS ..... 88
2.17 IMMUNOSTAINING ..... 89
2.17.1 Blocking step ..... 89
2.17.2 Primary antibody-binding step ..... 90
2.17.3 Secondary antibody conjugation step ..... 90
2.17.4 Detection step using Enhanced chemiluminescense (ECL) ..... 90
2.17.5 Re-probing ..... 91
2.18 *IN VIVO* DNA TRANSFECTION BY BIOLISTIC PARTICLE DELIVERY - GENE GUN TECHNIQUE ..... 91
2.18.1 Preparation of DNA for *in vivo* transfection ..... 91
2.18.2 Preparation of DNA coated gold particles ..... 92
2.18.3 Preparation of the cartridges ..... 92
2.18.4 Biolistic particle delivery using the Helios gene gun ..... 93
2.19 ISOLATION AND CULTURING OF PRIMARY RAT HEPATOCYTES ..... 94

2.19.1 Isolation of primary rat hepatocytes by liver perfusion ..... 94
2.19.2 Culturing of hepatocytes ..... 97
2.20 CULTURING THE CELL LINES ..... 98
2.21 PREPARATION OF WHOLE CELL EXTRACT FROM CELL LINES ..... 98
2.22 PREPARATION OF NUCLEAR EXTRACT FROM THE CELL CULTURE ..... 99
2.23 TRANSIENT TRANSFECTION ..... 100
2.23.1 Transfection by calcium phosphate method ..... 100
2.23.2 Transfection by Effectene™ transfection reagent ..... 101
2.23.3 Transfection by TransFast™ transfection reagent ..... 102
2.24 TISSUES / CELL LYSIS ..... 102
2.24.1 Lysis of the whole liver tissues ..... 103
2.24.2 Lysis of the cultured cells ..... 103
2.25 DETERMINATION OF DUAL-LUCIFERASE ACTIVITY ..... 104
2.26 CHLORAMPHENICOL ACETYL-TRANSFERASE (CAT) ASSAY ..... 104
2.26.1 Standard curve for CAT assay ..... 105
2.27 β-GALACTOSIDASE ASSAY ..... 105
2.27.1 Standard curve for β-galactosidase assay ..... 106
2.28 TNT® COUPLED RETICULOCYTE LYSATE SYSTEM ..... 106

**RESULTS AND DISCUSSION** ..... 108

3.1 SUBCLONING OF 5'- UPSTREAM SEQUENCES OF THE RAT *CYP2B1* GENE ..... 108
3.2 *IN VIVO* TRANSFECTION USING BIOLISTIC PARTICLE DELIVERY ..... 111
3.2.1 Reporter constructs used for *in vivo* transfection ..... 113
3.2.2 The -2301/-2142 (159 bp) region of the rat *CYP2B1* gene confers PB responsiveness in vivo ..... 116
3.3 STUDIES ON PROTEIN-DNA INTERACTIONS USING THE GEL MOBILITY SHIFT ASSAY ..... 126
3.3.1 Rat liver nuclear protein extracts bind to the 159 bp element (-2301/-2142) of the *CYP2B1* 5' flanking sequence and form multiple complexes ..... 129
3.2.2 Nuclear protein binding is localised within the -2214/-2142

(72 bp) region of the 159 bp element ..... 132
3.4 THE SEARCH FOR NUCLEAR PROTEINS THAT BIND TO THE *CYP2B1* PB-RESPONSIVE ELEMENT ..... 138
3.4.1 Purification of DNA-binding proteins using magnetic DNA-affinity beads ..... 138
3.4.2 RXRα is one of the proteins that binds to the NR1 and NR2 binding sites of the *CYP2B1* PB-responsive element ..... 143
3.4.3 RXRα heterodimerises with nuclear orphan receptor CAR-β and binds to the *CYP2B1* NR1 region ..... 152
3.5.1 Preparation of cells for transfection ..... 161
3.5.2 CAR-β transactivates the *CYP2B1* PBRE-*SV40*-Luc reporter gene expression in response to xenobiotics in primary hepatocyte cultures, but not in transformed cell lines ..... 164
3.5.3 Transactivation of a *CYP2B1* $(NR1)_2$-*SV40*-Luc reporter gene expression via CAR-β upon treatment of xenobiotics ..... 175
3.5.5 Overexpressed RXRα and 9-cis retinoic acid down- regulate PB-induced *CYP2B1* PBRE-SV40-Luc activity ..... 178
3.6 CROSS-TALK BETWEEN PROMISCUOUS RECEPTORS AND CYTOCHROME P450 GENE EXPRESSION ..... 181
3.6.1 Both CAR-β and PXR1 bind to the *CYP2B1* NR1 and *CYP3A1* PXRE motifs ..... 183
3.6.2 PXR1 and CAR-β transactivate gene expression via the *CYP2B1* PBRE ..... 190
3.6.3 CAR-β and PXR1 transactivate *CYP2B1* PBRE-*SV40*-Luc reporter gene expression *in vivo* ..... 194
3.7 THE EFFECT OF THE STEROID RECEPTOR CO-ACTIVATOR-1 (SRC-1) IN THE TRANSACTIVATION OF GENE EXPRESSION BY THE NUCLEAR RECEPTORS, CAR-β AND PXR1 ..... 200
3.7.1 SRC-1 enhances the transactivation of *CYP2B1* PBRE-*SV40*-Luc reporter via CAR-β, but not via PXR1 ..... 202

3.7.2 SRC-1 does not enhance the transactivation of the *CYP2B1* $(NR1)_2$-SV40-Luc reporter ..... 207
3.7.3 SRC-1 interacts with CAR-β but not PXR1, in the transactivation of the *CYP3A1* $(PXRE)_4$-tk-CAT reporter ..... 211

**GENERAL DISCUSSION** ..... 215

**REFERENCES** ..... 224

# List of Figures

1.1 Pleiotropic effects of phenobarbital.....33
1.2 Structure and functional domains of nuclear receptors.....44

3.2.1 Circle maps of the pGL3-Promoter, pGL3-Control, pGL3-Basic and pRL-TK reporter vectors.....115
3.2.2 24 hr post-transfection is an appropriate time to monitor gene expression.....120
3.2.3 The 159 bp DNA element confers PB-responsiveness in *vivo* and the fold PB induction is optimal at 24 hr.....121
3.2.4 The *CYP2B1* minimal promoter exhibits lower basal expression than a heterologous SV40 promoter.....122
3.2.5 The 159 bp (*Bgl*II)-*SV40*-Luc and 159 bp (*BamH*I)-*SV40*-Luc confer similar PB-responsiveness, but exhibit luciferase activity to different extents.....125

3.3.1 Multiple proteins bind to the *CYP2B1* -2301/-2142 (159 bp) region...131
3.3.2 Nuclear factor 1 (NF-1) can bind within the -2241/-2142 region.....133
3.3.3 The protein binding sites within the -2301/-2142 (159 bp) region are localised within -2214/-2142 (72 bp).....135
3.3.4 Protein complexes on the *CYP2B1* 72 bp element.....137

3.4.1 Purification of DNA-binding proteins using magnetic DNA-affinity beads.....139
3.4.2 Magnetic DNA-affinity purification of proteins that bind to the NR2 sequence.....142
3.4.3 The core PB-responsive element contains two nuclear receptor

binding sites separated by a NF-1 site.....145
3.4.4 Supershift analysis confirms that retinoid X receptor α (RXRα) is one of the nuclear proteins that can bind to nuclear receptor 1 (NR1) binding site.....147
3.4.5 RXRα can bind to the nuclear receptor 2 (NR2) binding site.....149
3.4.6 RXRα can bind to both NR1 and NR2.....150
3.4.7 Determination of CAR expression in rat liver.....154
3.4.8 *In vitro* translated CAR-β and RXRα heterodimerise and bind to the NR1 site.....158
3.4.9 CAR-β and RXRα do not bind to *CYP2B1* NR2.....159

3.5.1 CAR-β supports constitutive and xenobiotic activation of reporter gene expression.....167
3.5.2 CAR-β constitutively activates expression from the *CYP2B1* PBRE-*SV40*-Luc reporter plasmid in transformed cell lines.....170
3.5.3 CAR-β protein is expressed in a functional form in HepG2 cells.....171
3.5.4 PB-treatment does not increase the abundance of protein binding to the *CYP2B1* NR1.....174
3.5.5 CAR-β transactivates reporter gene expression via the *CYP2B1* NR1 element.....177
3.5.6 Overexpressed RXRα and 9-cis retinoic acid down regulate the *CYP2B1* PBRE-*SV40*-Luc expression.....180

3.6.1 Sequence of *CYP2B1 NR1* and *CYP3A1* PXRE.....184
3.6.2 Binding of *in vitro* translated CAR-β and RXRα to *CYP2B1* NR1 and *CYP3A1* PXRE.....185
3.6.3 Binding of *in vitro* translated PXR1 and RXRα to *CYP3A1* PXRE and *CYP2B1* NR1.....186

3.6.4Competition assay for the nuclear proteins bound to NR1 and PXRE.189
3.6.5 PXR1 transactivates via the *CYP2B1* NR1 element in response to steroid treatment.....192
3.6.6 PCN and dexamethasone activate *CYP2B1* PBRE-*SV40*-Luc gene expression through PXR1.....193
3.6.7 Both CAR-β and PXR1 transactivate *CYP2B1* PBRE-*SV40*-Luc reporter gene expression *in vivo*.....196
3.6.8 PCN induces *CYP3A1* $(PXRE)_4$-*tk*-CAT expression via PXR1.....199

3.7.1 SRC-1 increases CAR-β transactivation of *CYP2B1* PBRE-*SV40*-Luc reporter gene expression.....203
3.7.2 SRC-1 does not effect PXR1-mediated transactivation of *CYP2B1* PBRE-*SV40*-Luc reporter in response to PCN and Dex.....206
3.7.3 SRC-1 does not increase CAR-β transactivation of the reporter plasmid *CYP2B1* $(NR1)_2$-*SV40*-Luc.....209
3.7.4 SRC-1 does not further enhance *CYP2B1* $(NR1)_2$-*SV40*-Luc reporter activation by PXR1.....210
3.7.5 SRC-1 has no effect on PXR1-mediated transactivation of *CYP3A1* $(PXRE)_4$-*tk*-CAT reporter in response to PCN.....213
3.7.6 SRC-1 further enhances CAR-β-mediated transactivation of *CYP3A1* $(PXRE)_4$-*tk*-CAT reporter in response to PB.....214

4.1 Model of nuclear receptors-mediated *CYP2B* gene expression in response to xenobiotics.....223

# List of Tables

1.1 Examples of drug-metabolising enzymes in phase I and phase II reactions.....22

1.2 Regulation of *CYP* gene expression.....28

1.3 Orphan nuclear receptors: their ligands/activators and physiological actions.....54

1.4 Nuclear receptor mediated CYP induction: endogenous ligands and DNA response elements.....57

# Abbreviations

| | |
|---|---|
| AhR | Aryl hydrocarbon receptor |
| AR | Androgen receptor |
| Arnt | AhR nuclear translocator protein |
| ATP | Adenosine triphosphate |
| bHLH | Basic helix-loop-helix |
| bp | Base pairs |
| cAMP | Cyclic adenosine monophosphate |
| CAR-β | Constitutively active receptor-beta |
| | Constitutive androstane receptor-beta |
| CAT | Chloramphenicol acetyltransferase |
| C/EBP | CCAAT/Enhancer binding protein |
| CMV | Cytomegalovirus |
| COUP-TF | Chicken ovalbumin upstream promoter transcription factor |
| CYP | Cytochrome P450 |
| dATP | 2'-Deoxyadenosine 5'-triphosphate |
| dCTP | 2'-Deoxycytidine 5'-triphosphate |
| dGTP | 2'-Deoxyguanosine 5'-triphosphate |
| dNTP | 2'-Deoxynucleoside 5'-triphosphate |
| dTTP | 2'-Deoxythymidine 5'-triphosphate |
| DBD | DNA-binding domain |
| Dex | Dexamethasone |
| DMSO | Dimethylsulfoxide |
| DNase | Deoxyribonuclease |
| DR | Direct repeats |
| DTT | Dithiothreitol |
| EDTA | Ethylenediaminetetra-acetic acid |

| | |
|---|---|
| ER | Everted repeats |
| GR | Glucocorticoid receptor |
| GRE | Glucocorticoid-responsive element |
| HRE | Hormone response element |
| Hsp90 | Heat-shock protein 90 |
| IPTG | Isopropyl-β-D-thiogalactopyranoside |
| IR | Inverted repeats |
| kb | Kilobase |
| LBD | Ligand-binding domain |
| Luc | Luciferase |
| LXR | Liver X receptor |
| MR | Mineralocorticoid receptor |
| NAD(P)H | Nicotinamide-adenine dinucleotide phosphate (reduced) |
| NF-1 | Nuclear factor-1 |
| NGFI-B | Nerve growth factorI-B |
| NR | Nuclear receptor |
| PAGE | Polyacrylamide gel electrophoresis |
| PB | Phenobarbital |
| PBRE | Phenobarbital responsive element |
| PBREM | Phenobarbital responsive enhancer module |
| PCN | Pregnenolone 16α-carbonitrile |
| PCX | Picrotoxin |
| PKA | Protein kinase A |
| PMSF | Phenyl methyl sulfonyl fluoride |
| PPAR | Peroxisomal proliferator-activated receptor |
| PR | Progesterone receptor |

| | |
|---|---|
| psi | Pound per square inch |
| PXR | Pregnane X receptor |
| PXRE | Pregnane X receptor response element |
| RAR | Retinoic acid receptor |
| RARE | Retinoic acid response element |
| REs | Response elements |
| RNAP | RNA polymerase II |
| RT | Room temperature |
| RXR | Retinoid X receptor |
| SDS | Sodium dodecyl sulfate |
| SRC-1 | Steroid receptor coactivator-1 |
| SV40 | Simian virus 40 |
| TAF | TBP associated factor |
| TBP | TATA binding protein |
| TCPOBOP | 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene |
| TE | Tris-EDTA |
| TFIID | Transcription factor IID |
| TK | Thymidine kinase |
| TR | Thyroid hormone receptor |
| VDR | Vitamin D receptor |
| VDRE | Vitamin D receptor response element |

# CHAPTER 1

# Introduction

## 1.1 Overview of xenobiotic metabolism

Living organisms, from prokaryotes to humans, are exposed to a large number of foreign compounds. These include natural occurring and synthetic chemicals such as drugs, environmental contaminants, chemical carcinogens, mutagens and plant metabolites. These chemicals are generally hydrophobic and have the potential to affect normal cell functions, unless they are metabolised to water-soluble forms, which can be readily eliminated from the body. The biotransformation of these xenobiotic compounds is carried out by drug-metabolising enzymes in two phases; phase I (functionalisation) and phase II (conjugation) (Williams, 1971). The enzymes associated with these two phases of detoxification are shown in Table 1.1. Cytochromes P450 are well-known as the major detoxification enzymes of phase I. These proteins introduce an oxygen atom into their substrates, producing reactive metabolites which can then be conjugated by the enzymes in phase II, such as glutathione-S-transferase, UDP-glucuronosyl-transferase, and sulfotransferase, resulting in the water-soluble products. Cytochromes P450 have a broad range of substrate specificity and can metabolise numerous structurally diverse compounds. Upon exposure of an organism to foreign compounds, many cytochromes P450 are substantially induced. This induction process is very

sensible as it results in the reduction of the concentration of the substrate, in this case, referred to as an "inducer". Moreover, the induction process allows organisms to adapt to chronic exposure to toxic compounds or pollutants (Josephy, 1997). However, in some cases, metabolism by cytochromes P450 can be harmful because they convert some substrates into strong electrophiles, which reactively bind to cellular macromolecules such as DNA and proteins. This process thus enhances chemical toxicity, mutagenicity, and carcinogenicity (Guengerich, 1988).

Table 1.1 **Examples of drug-metabolising enzymes in Phase I and phase II reactions.** (Reproduced from Nebert, 1994)

| **Phase I enzymes** |
| --- |
| Cytochrome P450 |
| Flavin-containing monooxygenase |
| Aldehyde dehydrogenase |
| Epoxide hydrolase |
| NADPH-cytochrome P450 reductase |
| Monoamine oxidase |
| Carboxylesterase |
| **Phase II enzymes** |
| UDP-glucuronosyltransferase |
| Glutathione-S-transferase |
| Acetyltransferase |
| Methyltransferase |
| β-glucuronidase |
| Sulfotransferase |

## 1.2 The cytochrome P450 superfamily

Cytochrome P450 (CYP) comprises a large superfamily of oxidative enzymes (MW~45000-55000). CYP was first discovered in pig and rat liver microsomes. By treating microsomes with sodium dithionite, a reducing agent, and gassing with carbon monoxide, a unique absorption spectrum at 450 nm was observed (Garfinkle, 1958; Klingenberg, 1958). This microsomal fraction was further characterised as containing a haem-binding protein which was named cytochrome pigment 450 or P450 (Omura and Sato, 1964). CYPs oxidise a wide variety of structurally diverse compounds by insertion of one atom of molecular oxygen into the substrate. The monooxygenase reaction carried out by CYPs is accomplished by the assistance of other components in the cytochrome P450-dependent mixed function oxygenase system (Lu and Coon, 1968). These components include NAD(P)H-cytochrome P450 reductase, cytochrome $b_5$, cytochrome $b_5$ reductase and phospholipid. Once the substrate binds to the active site of CYP, NAD(P)H donates electrons to CYP via NAD(P)H-cytochrome P450 reductase or, in some cases, via cytochrome $b_5$ and cytochrome $b_5$ reductase (Porter and Coon, 1991). This is followed by the incorporation of an oxygen atom from molecular oxygen to the substrate. The interaction between membrane-bound CYPs and NAD(P)H-cytochrome P450 reductase is facilitated by the phospholipid. The overall reaction is as follows, where RH represents the substrate.

$$RH + O_2 + NAD(P)H + H^+ \xrightarrow{\textit{Cytochrome P450}} ROH + H_2O + NAD(P)^+$$

However, CYPs do not only catalyse oxidation reactions but also *N*-, *O*-, *S*-dealkylation, deamination and *N*-oxide reduction. CYP proteins, generally, are hydrophobic and tightly associated with the intracellular membranes of the smooth endoplasmic reticulum. Some CYPs in the steroidogenic tissues are present in the mitochondrial membrane such as the two CYP11 members, CYP24, and CYP27 (Nelson *et al.*, 1996; Waterman and Simpson, 1989). Bacterial CYPs are located in a soluble cytosolic form. Amongst all microsomal CYPs, the regions that are conserved include an N-terminal hydrophobic stretch of ~ 20 amino acids for membrane anchorage; a cysteine residue for haem binding; a site that interacts with NAD(P)H: cytochrome P450 reductase for oxygen activation; and sites that can be phosphorylated for post-translational regulation of CYPs. On the other hand, regions of great structural difference between CYPs are proposed to be involved in substrate recognition and binding and in redox partner interaction (Graham-Lorence and Peterson, 1996).

There have been extensive studies on CYPs in various organisms, including bacteria, insects, plants, rodents, and human. Up to 500 CYPs have been identified in 85 eukaryotes and 20 prokaryotes (Nelson *et al.*, 1996). A number of CYPs database is expected to increase by the end of the genome-sequencing project. Individual *CYP* genes range from 50 to 60 forms are present in any mammalian species (Nebert, 1991). CYPs on the basis of amino acid similarity are divided into 74 families, 17 of which are found in mammals. Members of the families 1 to 4 are responsible for metabolism of foreign compounds, in addition to metabolism of

endogenous compounds including steroids, bile acids, fatty acids, prostaglandins, eicosanoids, and retinoids. The other 13 families (CYPs 5, 7, 8, 11, 17, 19, 21, 24, 26, 27, 39, 46, and 51) typically do not metabolise foreign chemicals. They play a major role in the biosynthetic pathway of endogenous compounds. For example, CYPs 5 and 8A are essential in thromboxane and prostacyclin biosynthesis; CYPs 11, 17, 19, and 21 catalyse reactions required for steroid hormone biosynthesis from cholesterol; CYPs 7, 8B, 24, 27, and 51 catalyse reactions required for the biosynthesis of bile acids, vitamin $D_3$ and cholesterol and CYP26 catalyses hydroxylation of retinoic acids (Nelson, 1999; Nelson *et al.*, 1996).

## 1.3 Evolution and nomenclature of cytochrome P450

The CYP ancestral gene is thought to have existed earlier than the time of prokaryote/eukaryote divergence: a time pre-dating drugs, animal-plant interaction, and the combustion of organic matter (Nelson *et al.*, 1993). Prediction of the unit evolutionary period (UEP) indicates that the time required for a 1 % change in amino acid sequences in a CYP ranges from 2-4 million years. Compared to histones, with a UEP of 400 million years, CYPs are considered to evolve rapidly. The evolutionary relationship between various CYPs, as shown by a phylogenetic tree, is determined by comparing the amino acid sequences in all species identified. The oldest CYPs are the mitochondrial cholesterol-metabolising enzymes of the CYP11 family and those that metabolise steroids, fatty acids and eicosanoids. It is believed that these proteins may have evolved

to maintain membrane integrity in early eukaryotes (Nebert and Gonzalez, 1985). During the past 400 millions year ago, the explosion of new *CYP* genes is thought to be a consequence of animal-plant warfare, as aquatic vertebrates moved onto land. Changes in dietary habits led animals to develop new CYPs to detoxify the toxins contained within land vegetation (Gonzalez and Nebert, 1990).

Because of the identification of increasing numbers of cytochromes P450 and the numerous names given for the same enzyme, a nomenclature system for CYPs was developed. This system is based on amino acid sequence similarity between these proteins. CYPs with> 40% amino acid sequence similarities are placed into the same family and those with >55% similarities are placed in the same subfamily. Each CYP gene is designated by the italic prefix *CYP* (Cytochrome P450) for all species, except "*Cyp*" for mouse and drosophila. An Arabic numeral designating a family, then a letter to specify the subfamily and another Arabic numeral to indicate the individual gene within the subfamily i.e. *CYP1A1*, *CYP2B1*, *CYP2H1* and etc. The cDNAs, mRNAs, and enzymes in all species include all capital letters, and without italics or hyphens (Nelson *et al.*, 1996).

## 1.4 Regulation of *CYP* gene expression

Regulation of *CYP* gene expression is complex. It is subject to developmental-, gender-, tissue- and strain-specific regulation. Moreover, hormones, physiological stress, and exposure to many xenobiotics also modulate the expression of *CYP* genes (Atchison and Adesnik, 1986; Gonzalez, 1989; Guengerich *et al.*, 1987). Constitutive expression of many *CYP* genes in rat, for example, is dependent on gender, age, strain, growth hormone, starvation and high blood pressure (reviewed in Soucek and Gut, 1992). The expression of *CYP* genes is primarily regulated at the level of transcription. However, certain *CYP* genes are regulated at the level of mRNA stability, post-translational modification and protein stabilisation, other than transcription (Eliasson *et al.*, 1990; Song *et al.*, 1987). The mechanism and regulation of some *CYP* genes is described in Table 1.2. CYPs are present in greatest abundance in the liver, where they account for nearly 20% of the proteins in the endoplasmic reticulum and 2-3% of the total cellular protein. However, lower amounts of specific isoforms are also found in extra-hepatic tissues such as the gastrointestinal tract, lung, kidney, olfactory epithelia, skin, and the central nervous system (Gonzalez and Lee, 1996).

Table 1.2 **Regulation of *CYP* gene expression**
(Reproduced from Soucek and Gut, 1992)

| *CYP* | Mechanism and regulation of *CYP* gene expression[a] |
|---|---|
| *1A1* | *Inducible* (all tissues) -MC, TCDD, ACLR, ISF, BNF: Transcriptional activation/mRNA stabilisation |
| *1A2* | *Constitutive* (liver) : Transcriptional activation/mRNA stabilisation<br>*Inducible* (all tissues) -MC, TCDD, ACLR, BNF, ISF : Transcriptional activation/mRNA stabilisation |
| *2A1* | *Constitutive* (liver)-age, sex, growth hormone, diabetes, hypertension : activation<br>*Inducible* -MC, PB, ACLR, BNF, PCN, ISF : Transcriptional activation |
| *2A2* | *Constitutive* (liver)-age, sex, growth hormone : Transcriptional activation |
| *2A3* | *Inducible* (lung) -MC |
| *2B1* | *Constitutive* (lung, testis)-age, strain, colony, growth hormone : Transcriptional activation<br>*Inducible* -PB, AE, AC, ISF, PCN, starvation : Transcriptional activation/mRNA stabilisation |
| *2B2* | *Constitutive* (liver)-age, growth hormone<br>*Inducible* -PB, ACLR, ISF, PCN, DEX, starvation : Transcriptional activation/mRNA stabilisation |
| *2B3* | *Constitutive* (liver) |
| *2B12* | *Constitutive* (liver, lung, kidney, testis)<br>*Inducible* -PB : Transcriptional activation |
| *2C6* | *Constitutive* (liver, brain)-age : Transcriptional activation<br>*Inducible* -PB |
| *2C7* | *Constitutive* -age, sex, diabetes : Transcriptional activation<br>*Inducible* –PB, AE |
| *2C11* | *Constitutive* (liver) –age, sex, growth hormone, diabetes, hypertension : Transcriptional activation |
| *2C12* | *Constitutive* (liver) –age, sex, growth hormone, diabetes : Transcriptional activation |
| *2D1* | *Constitutive* (liver, kidney)-age, sex, strain : Transcriptional activation<br>*Inducible* –MC, PB : Transcriptional activation |
| *2E1* | *Constitutive (liver, kidney)*-age, growth hormone: Transcriptional activation ; diabetes, starvation : Transcriptional activation/mRNA stabilisation<br>*Inducible* (liver, lung)-ISN, AE, AC, B : protein stabilisation |

## Table 1.2 **Regulation of *CYP* gene expression (continued)**

| *CYP* | Mechanism and regulation of gene expression[a] |
|---|---|
| *3A1* | *Inducible* –PCN, DEX, PB, hypertension : Transcriptional activation/mRNA stabilisation ; TAO : protein stabilisation |
| *3A2* | *Constitutive* (liver, intestine)-age, sex, growth hormone, diabetes, starvation : Transcriptional activation |
| *4A1-3* | *Constitutive* (liver, kidney) : Transcriptional activation<br>*Inducible* – clofibrate, starvation : Transcriptional activation |
| *4B1* | *Constitutive* (lung) : Transcriptional activation |

[a]Abbreviations used : AC = Acetone, ACLR = Aroclor 1254, AE = Ethanol, , B = Benzene, BNF = β-Naphthoflavone, DEX = Dexamethasone, ISF = Isosafrole, ISN = Isoniazide, MC = 3-Methylcholanthrene, PB = Phenobarbital, PCN = Pregnenolone-16α-carbonitrile, TAO = Triacetyloleandomycin, TCDD = Tetrachlorodibenzo-p-dioxin
*Physiological factors, which affect CYP gene expression*: age, sex, diabetes, growth hormone, hypertension, and starvation
*Mechanism of CYP gene expression*: Transcriptional activation, mRNA/protein stabilisation

## 1.5 Transcriptional activation of *CYP* genes by different classes of inducers

As mentioned previously, CYP family members 1-4 are mainly responsible for xenobiotic metabolism. These proteins are highly induced by four major groups of structurally dissimilar compounds and classified accordingly. Only substantially induced-*CYP* genes are described.

### 1.5.1 Aromatic hydrocarbon-inducible CYP1A family

The CYP1A family contains two members; CYP1A1 and CYP1A2. Potent inducers of CYP1A1 are polycyclic aromatic hydrocarbons including benzo(a)pyrenes (natural combustion products), heterocyclic amines (dietary constituents) and dioxins (manufacturing by-products) (Johnson and Muller-Eberhard, 1977; Sakaki *et al.*, 1984), whereas CYP1A2 potent inducers are aromatic and heterocyclic amines (Goldstein *et al.*, 1984; Johnson *et al.*, 1980). These inducers have in common a unique planar and aromatic structure. CYP1A1 is not detectable in rat liver, whereas CYP1A2 is constitutively expressed. The mechanism of CYP1A induction is the most understood with respect to *CYP* gene regulation by xenobiotics. It is mediated via a specific receptor, termed the aryl hydrocarbon (Ah) receptor (Hankinson, 1995). The Ah receptor belongs to a basic helix-loop-helix/PAS (bHLH/PAS) transcription factor family, a subclass of bHLH proteins that contain a region of homology with the proteins *P*er (a circadian rhythm factor in *Drosophila*), *A*rnt (the AhR nuclear translocator

protein), and Sim (a *Drosophila* neurogenic factor). Within an Ah receptor molecule, the basic region binds to DNA, the helix-loop-helix region forms a dimerisation surface for a partner, Arnt, and the PAS domain possesses ligand and Hsp90 binding activity (Whitelaw *et al.*, 1994). The mechanism of CYP1A induction involves dissociation of the Ah receptor, upon binding to an aromatic hydrocarbon compound, from cytosolic heat shock proteins, Hsp90 (Phelan *et al.*, 1998). Hsp90 is believed to mask a nuclear localisation signal in the receptor. Subsequently, a heterodimeric complex is formed between the activated Ah receptor and the Arnt. The heterodimer complexes are then able to bind to specific xenobiotic response elements of the *CYP1A1* and other Ah-responsive flanking sequences, leading to enhanced transcription (reviewed in Hankinson, 1993). The finding that the Sp1 transcription factor, which binds to BTE (basic transcription element) located next to the TATA box of *CYP1A1*, can bind to and interact synergistically with AhR and Arnt, suggests that such protein-protein interaction may disrupt chromatin structure and allow the assembly of additional transcription factors on the *CYP1A1* promoter (Kobayashi *et al.*, 1996).

### 1.5.2 The phenobarbital-inducible CYP2B family

The pleiotropic effects of PB, an anti-epileptic drug, have been observed in several cellular processes including the induction of several members of phase I and phase II drug-metabolising enzymes (Figure 1.1) (Waxman and Azaroff, 1992); (reviewed in Honkakoski and Negishi, 1998). Using

differential display mRNA analysis, 29 cDNAs in chick embryo have been shown to be modulated, either positively or negatively, after PB treatment *in ovo* for 48 hr (Frueh *et al.*, 1997). It was suggested that PB may exert the effects via multiple mechanisms according to different doses and time course for PB induction as well as the extent of induction level among these detoxification enzymes (Kocarek *et al.*, 1990). Moreover, many hydrophobic compounds structurally unrelated to PB such as 2-allyl-2-isopropylacetamide,1,1,1-trichloro-2,2-bis (*p*-chlorophenyl) ethane (DDT), chlordane, isosafrole, 5,5-diphenyl-hydantoin, dieldrin, and some polychlorinated biphenyls, also induce a similar set of *CYP* genes as PB. They are referred to as 'PB-like inducers'.

Rat CYP2B1 and CYP2B2 have been extensively studied because they are the CYPs most highly inducible by PB. Our research interest is in the genes encoding these proteins and so a more detailed emphasis is given to these CYPs. CYP2B1 and CYP2B2 have similar and overlapping substrate specificities. They catalyse benzphetamine N-demethylation, hexobarbital 3-hydroxylation, testosterone 16α-hydroxylation and androstenedione 16α- and 16β-hydroxylation (Guengerich *et al.*, 1982; Waxman *et al.*, 1983; Wood *et al.*, 1983). Androstenedione 16β-hydroxylase is a specific marker for CYP2B1 activity (Kedzie *et al.*, 1991). CYP2B1 exhibits a 2-10 fold higher activity towards most substrates compared to CYP2B2, except for 2-hydroxylation of estradiol-17β, which CYP2B2 has greater activity (Waxman and Walsh, 1982).

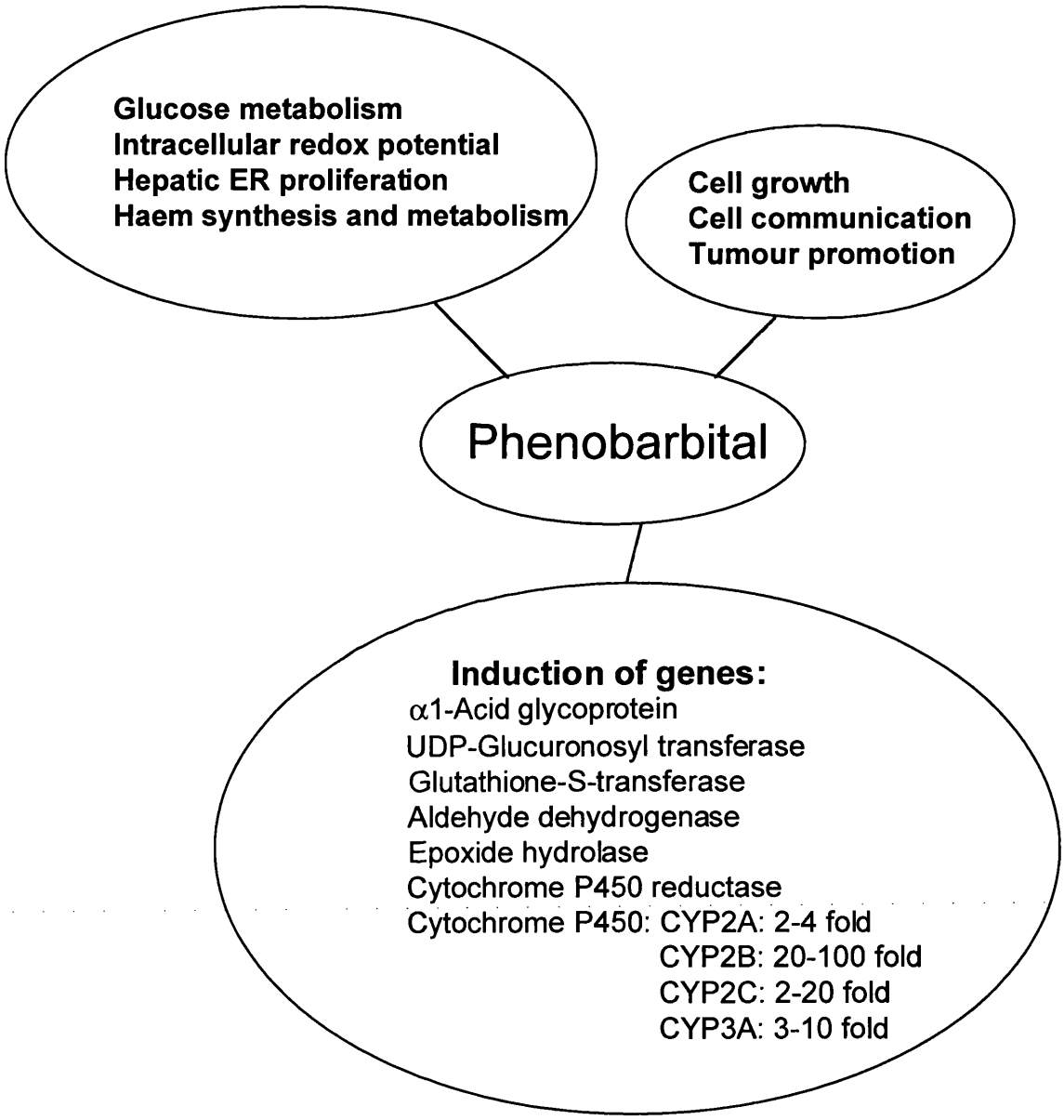



Figure 1.1 **Pleiotropic effects of phenobarbital.**
(Reproduced from Honkakoski and Negishi, 1998)

The *CYP2B1* and *CYP2B2* genes are closely linked on rat chromosome 1 (Rampersaud and Walz, 1987). Both genes have nine exons and eight introns and their overall lengths are 23 kb and 14 kb, respectively. *CYP2B1* has at least six alleles, whereas *CYP2B2* has two alleles or more (Rampersaud and Walz, 1983). *CYP2B2* has a similar nucleotide sequence to *CYP2B1.* There are only 40 base pair substitutions in the coding region. This results in 14 out of 491 amino acid substitutions in the final peptide product. Thus the two proteins exhibit 97% amino acid sequence similarity. These substitutions are primarily distributed in exons 6-9 (Suwa *et al.*, 1985), which result in a variable region towards the carboxyl terminus of the protein. In addition, there is a major difference between *CYP2B1* and *CYP2B2* in their first intron, which is approximately 12 kb long in *CYP2B1*, but only 3.2 kb in length in *CYP2B2.* The transcription initiation site for both *CYP2B* genes is putatively ~30 bp upstream from the ATG translation initiation site with a modified TATA box, CATAAAA, ~20 bp further upstream. At about -255 bp there is a repeating alternate purine/pyrimidine sequence, $(CA)_5$ in *CYP2B1* and $(CA)_{19}$ in *CYP2B2*, which has the potential to form a regulatory Z-helical structure (Suwa *et al.*, 1985). In addition to their similarities, CYP2B1 and CYP2B2 show marked differences in their expression patterns. CYP2B1 mRNA is barely detectable in the liver, whereas CYP2B2 is constitutively expressed. Both *CYP2B1* and *CYP2B2* are transcriptionally activated within 30-60 min of treatment with PB, and reach a peak (~23 fold increase) at 6 hr and decline to ~14 fold at 24 hr (Atchison and Adesnik, 1983; Hardwick *et al.*, 1983b). PB induces CYP2B1 mRNA to 4-5 fold higher than CYP2B2

mRNA (Omiecinski *et al.*, 1985), but both are induced to the same final amount (Akrawi *et al.*, 1993). In extra-hepatic tissues, CYP2B1 is constitutively expressed in lung, testis (Christou *et al.*, 1987) and small intestine (Traber *et al.*, 1990), while CYP2B2 is absent from these tissues regardless of PB treatment (Omiecinski, 1986). However, CYP2B1 is not PB-inducible in lung and testis, but it is PB-inducible in the small intestine (Elia, 1996; Traber *et al.*, 1990).

### 1.5.3 Steroid-inducible CYP3A family

The observation that pregnenolone 16α-carbonitrile (PCN) induces hepatic CYP content, other detoxification enzymes and acts to proliferate endoplasmic reticulum, led to the identification of a protein designated PCN1 (Elshourbagy and Guzelian, 1980). It is now known as a CYP3A family member. CYP3A hydroxylates steroid hormones such as corticosteroids, progesterone, and androgen and detoxifies two-thirds of xenobiotics (Nebert and Gonzalez, 1987). In humans, CYP3A4 is the most abundant CYP enzyme and accounts for 30% and 50 % of the total CYP content in the liver and intestine, respectively. Moreover, many pharmaceutical drugs, at least 50%, are metabolised by CYP3A4. Therefore, CYP3A metabolism has a major impact on drug-drug interactions, especially in humans. In rat, the *CYP3A1* gene is transcriptionally inactive in the uninduced state. Upon PCN treatment, it is up regulated. On the other hand, CYP3A2 is constitutively expressed and is PCN-inducible (Hardwick *et al.*, 1983a). Interestingly, both of these

genes are induced by PB and PB-like inducers (Schuetz *et al.*, 1986). Studies on CYP3A1 and CYP3A23, which is related to CYP3A1, has shown that induction by the antiglucocorticoid; PCN, and the glucocorticoid; dexamethasone is not mediated via a classical glucocorticoid receptor pathway (Huss *et al.*, 1996; Quattrochi *et al.*, 1995). The activation of CYP3A by these inducers has now been shown to be mediated through a novel orphan receptor, PXR (pregnane X receptor) that binds, as a dimer with RXR, to a DR-3 glucocorticoid and PCN-responsive element (Kliewer *et al.*, 1998; Quattrochi *et al.*, 1998; Schuetz *et al.*, 1998). Lehman and colleagues also demonstrated the activation of hPXR that regulate human *CYP3A4* gene expression by many drugs and steroids (Lehmann *et al.*, 1998). The CYP3A response to steroids is primarily regulated at the transcriptional level. However, administration of triacetyloleandomycin (TAO), a macrolide antibiotic, induces CYP3A by reducing the rate of CYP3A degradation (Watkins *et al.*, 1986). Thus, regulation of CYP3A expression can be at the post-transcriptional level.

### 1.5.4 Peroxisome proliferator-inducible CYP4A family

CYP4 is one of the oldest P450 families and is thought to have evolved along with the cholesterol metabolising enzymes to maintain membrane integrity in early eukaryotes. CYP4A genes encode CYP enzymes that catalyse terminal omega (ω) -carbon hydroxylation and, to a lesser extent, the ω-1 hydroxylation of saturated and unsaturated fatty acids (Sharma *et*

*al.*, 1989) as well as in the ω-hydroxylation of various prostaglandins (Matsubara *et al.*, 1987). Among the 13 subfamilies of CYP4A, rat CYP4A1, CYP4A2 and CYP4A3 are the most extensively studied. These proteins are constitutively expressed in male rat liver and kidney and substantially induced by peroxisomal proliferators including clofibrate, a hypolipidemic drug (Kimura *et al.*, 1989). CYP4A represent only 1-4% of the total CYP protein content in the liver of an untreated animal. However, it is increased up to 16-30% of the total CYP content following clofibrate treatment. The activation pathway controlling induction of CYP4A is mediated by a peroxisome proliferator-activated receptor (PPAR) mechanism and is now well-characterised (Aldrige *et al.*, 1995; Mangelsdorf and Evans, 1995; Sher *et al.*, 1993).

## 1.6 Achievements in the study of the molecular mechanism underlying *CYP2B* gene expression by PB

The mechanism of PB-induced *CYP* gene expression has been under intense investigation in many species, from bacteria to human. Progress has been hampered by the inability to identify a PB receptor. Unlike the unique planar and aromatic structure of CYP1A inducers, PB and PB-like inducers share little common structural similarity, except for their hydrophobic nature. Therefore, it was proposed that these inducers may bind to a receptor with an elastic fit (Okey, 1990). A photoaffinity probe, such as a modified PB, *p*-azido-PB, was proposed for use in tracing a putative PB receptor. However, the induction of CYP2B1/2 by *p*-azido-PB is low compared to the parent compound (Shinohara *et al.*, 1997). To date, a receptor for PB has not been identified. In fact, much of the work in this area has been devoted to the identification of PB-responsive elements in the promoters of the *CYP2B* genes. Initially, functional studies were based on *in vitro* transcription systems. Subsequent advances in primary hepatocyte cell culture systems led to the identification of a PB-responsive element in several *CYP2B* genes. Moreover, the technology of DNA transfection into live animals and cell cultures as well as transgenic mice experiments is now providing key evidence for the mechanism(s), which underlie *CYP* gene regulation by phenobarbital.

### 1.6.1 CYP induction in *Bacillus megaterium* as a model for mammalian *CYP* gene regulation

CYP102 and CYP106 induction by barbiturates in *Bacillus megaterium* has been proposed as a model system (Liang *et al.*, 1995). De-repression of these genes by barbiturates is mediated by removal of a repressor protein, BM3R1, which binds to a palindromic operator region and the Barbie box sequence of both CYP102 and CYP106 genes (Liang and Fulco, 1995). Upon PB treatment, the binding of repressor to these sites is inhibited by direct interaction with positive regulatory factors and by competition for binding sites. The Barbie box sequence is also present in many mammalian *CYP* genes including rat *CYP2B1*, *CYP2B2*, *CYP3A2* and rabbit *CYP2C1* (Liang *et al.*, 1995). It was therefore thought that the conserved Barbie box element might play a role in PB induction. Fulco and colleagues have shown greater abundance in binding of liver nuclear extracts from PB-treated rats to the Barbie box-like sequence at -89/-73 of the *CYP2B1* gene promoter. The addition of PB to liver nuclear extracts from untreated rats, increased binding to the Barbie box (He and Fulco, 1991). Similar results were observed with the Barbie box containing sequence of other rat *CYP2B* genes (Upadhya *et al.*, 1992). In contrast, gel shift assays performed in our laboratory did not show any binding of liver nuclear extract from either untreated or PB-treated animals to the Barbie box-like sequence of *CYP2B2*, which is identical to that of *CYP2B1* (Shephard *et al.*, 1994). Moreover, experiments performed by several other

laboratories also failed to demonstrate protein binding to the Barbie box (Luc *et al.*, 1996; Park and Kemper, 1996; Ramsden *et al.*, 1993; Sommer *et al.*, 1996). Mutation or deletion of the Barbie box has no effect on PB-responsive transcription of *CYP2B1* or *Cyp2b10* reporter constructs (Honkakoski *et al.*, 1996; Park *et al.*, 1996). In the mouse PB-regulated *Cyp2b10* gene, the Barbie box is disrupted by a 42-bp DNA insertion (Honkakoski *et al.*, 1996). Recent evidence has been obtained against the Barbie box as a key element in barbiturate-mediated induction of CYP106 (Shaw *et al.*, 1998).

### 1.6.2 Proposed phenobarbital-responsive elements within the proximal promoter

A number of experiments support the role of regulatory elements within the proximal promoter in the PB induction of CYP2B. Cell-free transcription of the *CYP2B2* proximal DNA fragment from -179 to +181 was increased with liver nuclei from PB-treated rats (Rangarajan and Padmanaban, 1989). The same DNA fragment was shown to confer PB responsiveness in an *in vivo* transcription assay using an asialoglycoprotein-DNA complex liver targeting system (Prabhu *et al.*, 1995). Within the region -179 to +181, the same protein (26-28 kDa) has been shown to bind to both a negative element at -160/-127 (Ram *et al.*, 1995) and positive element at -98/-69 (Upadhya *et al.*, 1992), depending on its phosphorylation status. It was therefore proposed that in the absence of PB, this protein binds to a

negative cis-element in a dephosphorylated form, whereas in the presence of PB, the protein is phosphorylated and binds preferentially to the positive cis-element thus attracting other transcription factors and so enhancing *CYP2B2* transcription (Prabhu *et al.*, 1995). Work in our laboratory has shown two DNA sequences that bind nuclear proteins enriched or activated in the liver upon PB treatment. These sequences are located at -183/-199 and -31/-72. In addition, DNase I footprinting analysis revealed three protein binding sites; -117/-142, -48/-66, and -10/-35 (Shephard *et al.*, 1994). Other laboratories have demonstrated similar protein-binding sites in the *CYP2B* proximal promoter as follows: -116/-129, -45/-64, and -8/-36 (Luc *et al.*, 1996); -119/-138, and -45/-64 (Park and Kemper, 1996); -116/-143 and -47/-61 (Sommer *et al.*, 1996). The sequence from -120 to -140 resembles a NF-1 binding site, whereas -10 to -35 is likely to be a binding site for the TATA binding protein. The region within -45 to -65 has been proved to be a functional CCAAT/ enhancer binding protein (C/EBP) binding site (Luc *et al.*, 1996; Park and Kemper, 1996). Deletion or mutation of this C/EBP binding site dramatically decreased promoter activity of rat *CYP2B1* and mouse *CYP2b10* gene reporter constructs in transient transfection assays. But mutation of the C/EBP site has no affect on PB-induced reporter gene activity. This evidence strongly suggests that the C/EBP protein may not be involved in PB-induction, but instead contributes to *CYP2B* basal promoter activity (Honkakoski *et al.*, 1996; Park *et al.*, 1996).

### 1.6.3 Distal phenobarbital-responsive enhancer elements

In contrast to the evidence implicating proximal promoter sequences as PB-responsive elements, there are increasing numbers of studies that support the role of 5'-distal elements to the promoter in mediating PB induction. Studies in chicken embryo hepatocytes provided the first evidence that the region upstream from -5.9 kb to -1.1 kb of the *CYP2H* gene conferred PB responsiveness (Hahn *et al.*, 1991). Subsequently, transgenic mice containing either 19 kb or 800 bp of the 5' flanking region of *CYP2B2* were examined. Transgenic mice with the 800-bp *CYP2B2* transgene displayed a high basal transcription in the liver. Treatment with phenobarbital did not further increase transcription in these animals. In addition, these animals expressed a high level of CYP2B mRNA in kidney. In contrast, mice harbouring the 19 kb transgene of the *CYP2B2* 5' flanking region expressed CYP2B mRNA only in liver. Moreover, this transgene conferred PB-responsiveness indicating that the *CYP2B2* proximal promoter region is not adequate to support PB-induction. Instead, distal upstream elements between -800 bp and -19 kb are required to achieve PB-responsiveness and to restrict *CYP2B2* expression to the liver (Ramsden *et al.*, 1993). Attempts to specify the upstream sequence, which plays a role in the PB response, have continued. However, the lack of a cell culture system that maintains *CYP2B* expression and retains PB-responsiveness has made progress difficult in identifying functional PB-responsive elements. But now, several cell culture conditions for primary hepatocytes have been established that use an extracellular matrix

basement membrane i.e. collagen or laminin or co-culture with liver epithelial cells (Brown *et al.*, 1995; Lerche *et al.*, 1997; Lindblad *et al.*, 1991). By transfection of DNA constructs containing different lengths of the *CYP2B2* promoter into primary rat hepatocytes, Trottier *et al.* identified a region between -2155 and -2318 (163 bp) of the *CYP2B2* gene, which conferred PB responsiveness to a reporter gene. They called this sequence the phenobarbital responsive element (PBRE). The PBRE was active in both orientations, upstream and downstream of the heterologous promoter. Hence, it has the properties of a transcriptional enhancer (Trottier *et al.*, 1995). This finding was confirmed in the rat liver using *in situ* transient transfection that a reporter gene containing one or three copies of a 163 bp PBRE under the control of *CYP2C1* and *CYP2B2* promoter responded to PB treatment (Park *et al.*, 1996). The 5'-flanking sequence of the mouse *Cyp2b10* gene (-2397/-1850) was shown to confer PB responsiveness on a reporter gene linked to either the proximal *Cyp2b10* promoter or the SV40 heterologous promoter. This region has 91 % sequence similarity to the rat *CYP2B2* 163 bp fragment. The 177 bp was studied in more detail. A 132-bp fragment was found to confer PB-responsiveness on a reporter gene. Six *in vitro* DNase I footprints were detected when this fragment was incubated with nuclear protein extracts, of these only three were strongly protected. Equal binding was observed with liver nuclear extracts from both untreated and PB-treated mice (Honkakoski and Negishi, 1997). Subsequently, Negishi and his group defined a 51-bp element within the 132-bp sequence, which confers responsiveness not only to PB, but also to 16 structurally unrelated PB-

type inducers on a reporter gene. They named this element the phenobarbital-responsive enhancer module (PBREM). Within the 51-bp element, they identified a 33-bp core element that contains two nuclear receptor (NR)-binding half sites; NR1, NR2 and an NF-1 site (Honkakoski *et al.*, 1998a). The above experimental evidence supports the role of the distal region and the PBRE in CYP2B induction *in vivo*. Importantly, DNase I footprinting *in vivo* shows a ~20 bp protected region within the PBRE in untreated animals, which is increased to ~60 bp upon PB treatment (Kim and Kemper, 1997). This suggests that PB treatment alters the composition or structure of pre-existing protein complexes in native chromatin. Very recently, work from the same group demonstrates protein binding in native chromatin and the nucleosomal structure of the PBRE and proximal promoter in liver and kidney, where CYP2B1/2 are expressed and not expressed, respectively. Although, the *in vitro* DNase I footprinting detected the same pattern from both liver and kidney nuclear extracts, protein binding to the PBRE and proximal promoter was detected in the liver, but not kidney native chromatin. Moreover, Southern analysis of micrococcal nuclease-digested chromatin from untreated rats showed hypersensitive regions in the proximal promoter and PBRE in the liver, but not in the kidney. The hypersensitivity in both regions was increased upon PB treatment. These data suggest that, in the liver, PB treatment alter protein binding to the PBRE and substantially alter chromatin structure in both the PBRE and proximal promoter. On the other hand, in the kidney, the closed chromatin structure of the *CYP2B1/2* gene probably prevents access of regulatory factors (Kim *et al.*, 2000). The evidence that PB-

induced *CYP* gene expression does not require *de novo* protein synthesis (Sidhu and Omiecinski, 1998) also supports the effect of PB treatment on pre-bound proteins on the PBRE.

## 1.7 The nuclear receptor superfamily

Small lipophilic molecules, either hormones or metabolites, as well as xenobiotics serve as signalling molecules that modulate the expression of responsive genes. The action of these molecules is mediated through the binding to specific nuclear receptors, which bind directly to DNA response element in the gene promoter. These proteins play a vital role in morphogenesis, cell growth, homeostasis, and differentiation, by converting extracellular signals into transcriptional responses (Beato *et al.*, 1995; Evans and Hollenberg, 1988; Mangelsdorf and Evans, 1995). More than 150 nuclear receptors have been identified in species as diverse as flies and humans. Most nuclear receptors exist as isoforms, which are encoded by different genes (Lazar, 1993; Zelent *et al.*, 1989). Figure 1.2 shows that the nuclear receptors are composed of six distinct domains that are capable of functioning independently (Laudet *et al.*, 1992; Schwabe, 1996). The highly conserved cysteine-rich DNA binding domain (DBD) comprises 66 amino acids containing two zinc fingers, which are essential for targeting the receptor to a specific DNA sequence (Evans and Hollenberg, 1988). The hinge region, located immediately C-terminal to the DBD, contributes to the DNA binding and specificity of the receptor by acting as a molecular ruler to determine the partner subunit interaction (Rastinejad *et al.*, 1995). The C-terminal ligand binding domain (LBD), which encompasses approximately 250 amino acids, is conserved for multiple functions such as ligand binding, receptor dimerisation, nuclear translocation, transactivation (AF-2) (Carson-Jurica *et al.*, 1990) and

transcriptional suppression (Baniahmad *et al.*, 1992). Within the N-terminal domain, there is another transactivation region, called AF-1. In contrast to AF-2, transactivation through the AF-1 region is constitutive and ligand-independent.

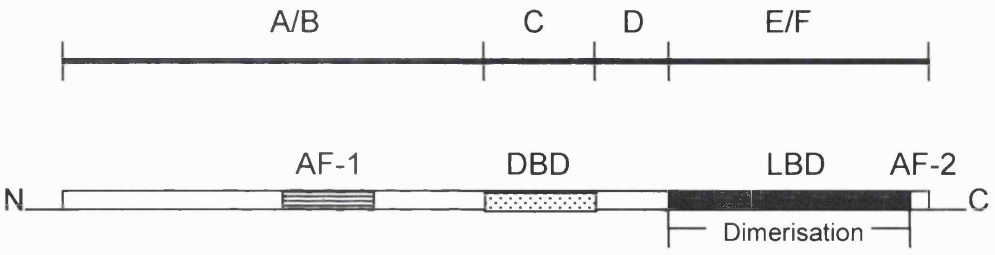



Figure 1.2 **Structure and functional domains of nuclear hormone receptors.** Typical nuclear receptors consist of six domains; A/B, C, D, E/F. A/B, F = modulating regions, C = DNA binding domain (DBD), D = hinge region, E = Ligand binding domain (LBD), AF = Activation function (Reproduced from Mangelsdorf, 1995)

The nuclear receptors can be classified according to the ligand to which they bind: *(i) The steroid hormone receptors* include those, which bind to glucocorticoids, progestins, mineralocorticoids, androgens, and estrogens; *(ii) The non-steroid hormone receptors* represent those that bind to non-steroid lipophilic molecules such as thyroid hormone, vitamin D3, retinoids, and prostaglandin; *(iii) Orphan receptors* comprise most members of the nuclear receptor superfamily that bind unknown ligands (Mangelsdorf and Evans, 1995). The non-steroid hormone receptors, including orphan receptors, differ from steroid hormone receptors in some aspects. In the absence of ligand, non-steroid hormone receptors do not bind to heat shock proteins and always reside in the nucleus (Dalman *et al.*, 1990). In contrast, steroid hormone receptors are sequestered in the cytoplasm in association with heat shock proteins (Mangelsdorf and Evans, 1995). Symmetric repeats of DNA response elements (REs) with a 3 bp-spacer determine the binding of steroid hormone receptors as homodimers. The non-steroid hormone receptors including orphan receptors, however, bind as heterodimers with a common partner, retinoid X receptor (RXR) to asymmetric DNA REs, either direct repeats (DRs), everted repeats (ERs), or inverted repeats (IRs) of two degenerate -AGGTCA- core sites with a spacer of 1 to 5 nucleotides. The number and composition of the nucleotide spacer determines the specificity of the nuclear receptor assembly on DNA direct repeats (Glass, 1994; Mangelsdorf and Evans, 1995). For example, heterodimers of RXR with PPAR, VDR, $T_3R$ and RAR bind to DNA direct repeats with a 1, 3, 4, or 5 bp spacer, respectively (Kliewer *et al.*, 1992; Umesono *et al.*, 1991). Upon

ligand binding, the receptors transform to the transcriptionally active state. In other words, a ligand-induced conformational change of the ligand-binding domain occurs so that the protein is now able to interact with co-activators that serve as mediators in the interaction with the basal transcription factors. These co-activators normally possess intrinsic histone acetyltransferase activity to remodel the chromatin structure in such a way that allows the assembly of the pre-initiation complex at the core promoter. Thus, the expression of responsive genes is initiated (Tjian and Maniatis, 1994).

Recently, a nomenclature for the nuclear receptor superfamily has been established. This is based on the nomenclature system devised for the cytochrome P450 superfamily (Nebert *et al.*, 1987). Members of the same family share at least 80-90% similarity in the DNA-binding domain and at least 40-60% in the ligand-binding domain. As an example, NR1A1, NR2B1, NR1I3 represent the trivial names of TRα, RXRα and CARα, respectively (Nuclear receptors nomenclature committee, 1999). However, the use of trivial names is preferred for this thesis.

### 1.7.1 Retinoid X receptor

Retinoid X receptors (RXRs) were first identified as orphan receptors, which bind no known ligand (Mangelsdorf *et al.*, 1990). Subsequently, 9-cis retinoic acid was recognised as ligand for RXRs (Mangelsdorf and Evans, 1995). Three RXRs isoforms, α, β and γ,are present in mammals (MW~50000). The expression of RXRβ is widespread. RXRα is found in the liver, kidney, and skin, whereas RXRγ is expressed in muscle and heart (Mangelsdorf and Evans, 1995). Efficient binding of several nuclear receptors to their REs requires an accessory factor which proved to be RXR (Glass, 1994; Kliewer *et al.*, 1992; Zhang *et al.*, 1992). Within the DR-3, DR-4 and DR-5 motifs, which RXR heterodimers bind to, RXR has been shown to occupy the 5' half-site and the nuclear receptor partner occupies the 3' half-site of REs (Perlmann *et al.*, 1993). In contrast, RXR is able to bind as either a homodimer or as a heterodimer with RAR on a DR-1 motif, such that the polarity of the RAR/RXR heterodimers is reversed (Kurokawa *et al.*, 1994). Moreover, RXR can play a role in transcriptional silencing or activation depending on its partner. For instance, RXR heterodimers with VDR, TR or RAR suppress responsiveness to 9-cis retinoic acid (Forman *et al.*, 1995b). But when RXR is heterodimerised with PPAR, NGFI-B, LXRα or FXR, transcription is increased in response to the cognate ligand (Kliewer *et al.*, 1992)(reviewed in Minucci and Ozato, 1996). The interaction of RXR with the TATA box binding protein (TBP) and $TAF_{II}110$ in the TFIID complex suggest that RXR can play a role in transcription initiation (Schulman *et al.*, 1995).

## 1.7.2 Nuclear orphan receptors

Structurally related proteins that belong to the nuclear receptor/steroid receptor superfamily, but have an unidentified ligand are classified as orphan nuclear receptors. Recently, the discovery of ligands for many orphan receptors has revealed novel nuclear hormone signalling pathways (Forman *et al.*, 1997; Forman *et al.*, 1995a; Kliewer *et al.*, 1998). Examples of orphan nuclear receptors and their ligands are shown in Table 1.3. The approach to seek for these unknown ligands has defined a new era - reverse endocrinology. The orphan receptors have been used to search for their ligands, instead of using purified hormones to identify their receptors. Basically, the ligand-binding domain (LBD) of the orphan receptor was fused to the DNA-binding domain (DBD) of the yeast transcription factor GAL4. This construct was co-transfected with a reporter vector containing the GAL4 DNA binding site. The transfected cell line was then treated with a series of natural and synthetic compounds. The responsiveness to a specific ligand was shown by high activity of the reporter protein (Kliewer *et al.*, 1998). This assay provides a screening tool for the nature of ligand, although it cannot distinguish between an indirect activator and a *bona fide* ligand. Therefore, a ligand-binding assay is essentially required to determine if the compound acts as a true ligand. Recently, ligands for five nuclear orphan receptors: the peroxisome proliferator-activated receptor (PPARs), the constitutive androstane

receptor-β (CAR-β), the liver X receptors (LXRs), the pregnane X receptor (PXR), and the farnesoid X receptor (FXR) have been identified (Forman *et al.*, 1997; Forman *et al.*, 1998; Kliewer *et al.*, 1998; Krey *et al.*, 1997; Lehmann *et al.*, 1997; Parks *et al.*, 1999).

Table 1.3 **Orphan nuclear receptors: their ligands/activators and physiological actions** (Reproduced from Forman, 1998)

| Receptor | Ligand / Activator | Major physiological actions |
|---|---|---|
| DAX | (lack conserved DBD) | Adrenal & gonadal development |
| SHP | (lack conserved DBD) | ? |
| GR | Glucocorticoid | Gluconeogenesis, anti-inflammatory |
| MR | Mineralcorticoids | Urinary sodium reabsorption |
| AR | Androgens | Male sexual maturation, increase muscle mass |
| SF-1α/β | ? | Steroid biosynthesis, Adrenal,gonadal development |
| PR | Progestins | Endometrial glandular secretion |
| T3Rα<br>T3Rβ | Thyroid hormone<br>Thyroid hormone | Growth, development & metabolism |
| RARα<br>RARβ<br>RARγ | All-trans retinoic acid<br>All-trans retinoic acid<br>All-trans retinoic acid | Development & morphogenesis |
| CARα | ? | ? |
| VDR | 1,25–dihydroxyvitamin $D_3$ | Bone mineral homeostsis |
| LXRα<br>LXRβ | Oxysterols<br>Oxysterols | ?<br>? |
| EcR | Ecdysteroids | larval-to-adult metamorphosis (insect) |
| FXR | Farnesoids | ? |
| PPARα | Fatty acids, fibrates | Fatty acid catabolism, peroxisome proliferation |
| PPARδ | Fatty acids | ? |
| PPARγ | 15-deoxy-$\Delta^{12,14}$-PGJ2, thiazolidinediones | Adipogenesis, insulin sensitization |

| | | |
|---|---|---|
| RORα | ? | Cerebellar development |
| RORβ | ? | ? |
| RORγ | ? | ? |
| NGF1Bβ | ? | ? |
| NGF1Bγ | ? | ? |
| NGF1Bα | ? | Apoptosis, negative selection of thymocytes |
| ERα | Estrogen | Female sexual maturation, |
| ERβ | Estrogen | Bone density |
| HNF-4α | ? | Glucose-stimulated insulin secretion |
| HNF-4β | ? | ? |
| RXRα | 9-cis retinoic acid, phytanic acid | Common dimerisation partner |
| RXRγ | 9-cis retinoic acid, phytanic acid | Common dimerisation partner |
| RXRβ | 9-cis retinoic acid, phytanic acid | Common dimerisation partner |
| COUPα | ? | ? |
| COUPγ | ? | ? |
| COUPβ | ? | ? |
| TLX | ? | ? |

(Table 1.3 continued)

? = Unidentified ligands/activators and/or physiological actions

### 1.7.3 Regulation of *CYP* gene expression by nuclear receptors

Nuclear receptors are generally known as ligand-modulated transcription factors. The steroid and non-steroid ligands that bind nuclear receptors are often associated with increases in particular subsets of CYPs and other drug-metabolising enzymes. It was proposed that drug-metabolising enzymes might regulate the steady state levels of these ligands that serve as signalling molecules in a variety of cellular processes (Nebert, 1991). The fundamental role of CYPs in metabolism of many endogenous compounds supports this proposal. Beyond this, the relationship between nuclear receptors, their ligands, and CYPs, is demonstrated by the finding that *CYP* gene expression, in response to both endogenous and exogenous compounds, is regulated by the nuclear receptors. For example, the transcriptional activation of members of the CYP families 2, 3, and 4 involves the nuclear orphan receptors CAR PXR and PPAR, respectively. The endogenous ligands, which can act either as agonists or antagonists and the respective DNA response elements of these nuclear receptors, are shown in Table 1.4. The exogenous compounds have also been shown to activate *CYP* genes via direct binding to these nuclear receptors. Very recently, many of the compounds that induce *CYP3A* gene expression have been shown to bind directly to human PXR by using a novel scintillation proximity binding assay (Jones *et al.*, 2000).

Table 1.4 **Nuclear receptor mediated CYP induction: endogenous ligands and DNA response element** (Reproduced from Waxman, 1999)

| Inducers | Responsive CYPs | Nuclear receptor | Endogenous ligands | DNA response element |
|---|---|---|---|---|
| Phenobarbital | 2B1, 2B2 | CAR | androstanol, androstenol | DR-4 |
| Dexamethasone | 3A1,3A2, 3A23 | PXR | Pregnenolone, corticosterone | DR-3, ER-6 |
| Fibrate drugs | 4A1, 4A2, 4A3 | PPARα | linoleic acid, arachidonic acid | DR-1 |

## 1.8 Aims and scope of this thesis

This thesis aim is to characterise the molecular mechanism(s) underlying the regulation of rat *CYP2B1* gene expression by xenobiotics, particularly phenobarbital. The 5'-upstream region of rat *CYP2B1* gene localised at -2301/-2142 is the focus of detailed investigation. To study the role of this DNA element (called the phenobarbital responsive element, PBRE) in *CYP2B1* gene activation *in vivo*, I used the biolistic particle delivery method of delivering DNA into the liver of live animals. As this element caused PB responsiveness of a reporter gene, I then used the gel mobility shift assay to identify the nuclear proteins that interact with this DNA element. The region where the proteins bind to was localised by competition assays. I have also made attempts to characterise and purify the nuclear regulatory proteins that bind within the PBRE by using magnetic DNA-affinity beads. During the course of this study, the nuclear receptor, CAR-β was identified as a regulatory factor that binds to the phenobarbital responsive enhancer module (PBREM) and activates the mouse *Cyp2b10* expression (Honkakoski *et al.*, 1998b). So the transactivation of the *CYP2B1* PBRE by CAR-β was examined in a rat model by co-transfection of the expression vector for CAR-β along with a PBRE-containing reporter plasmid in animals that were treated with PB. Because *CYP2B1* is up regulated by structurally dissimilar compounds such as picrotoxin, dexamethasone, and pregnenolone 16α-carbonitrile, I then investigated whether these compounds activate *CYP2B1* transcription via the same DNA element. Transactivation via the PBRE, in response to these xenobiotics, by CAR-β

was assessed *in vivo*, in primary hepatocytes and cell lines. PB and PCN increase the expression of both *CYP2B1* and *CYP3A1*, and PCN-activation of *CYP3A1* is mediated via PXR. A gel mobility shift assay showed that two different nuclear receptors, CAR-β and PXR1, bind to the same DNA element. The effect of xenobiotics on both CAR-β and PXR1-mediated activation of the *CYP2B1* PBRE and *CYP3A1* PXRE reporter plasmids was therefore investigated. The effect of the steroid receptor coactivator (SRC-1) on nuclear receptor transactivation was also examined. This study provides evidence, *in vivo* and in primary hepatocytes, of direct activation of CAR-β in response to PB, which is further enhanced by SRC-1. In addition, the finding that different nuclear receptors are capable of binding to and mediating gene activation via the same DNA element suggest that animals have evolved promiscuous receptors that regulate CYP induction in response to xenobiotic exposure.

# CHAPTER 2

# Materials and methods

## 2.1 Chemicals and reagents

All chemicals were either analytical reagents (AR) grade or tissue culture grade. They were purchased from Sigma Chemical Co., UK and BDH, UK. Restriction enzymes were purchased from Amersham Pharmacia Biotech, UK and New England Biolab, UK. Supershift antibodies were purchased from Santa Cruz Biotechnology Inc., UK. William's E medium and Dulbecco's Modified Minimal Essential Medium (DMEM) were purchased from GibcoBRL, UK. Matrigel® basement membrane matrix was purchased from Becton Dickinson Labware, Bedford, MA. Transfection kits were purchased from Promega, UK (TransFast® and Tfx™-20), QIAGEN Ltd., UK (Effectene®), and Amersham Pharmacia Biotech, UK (CellPhect®). Enhanced chemiluminescence kit was purchased from Amersham Pharmacia Biotech, UK.

## 2.2 DNA clones, expression vectors and oligonucleotides

A clone of the *CYP2B1* gene 5' flanking sequence, from -3878 to +27, in pBLCAT3 was a generous gift from Dr. Peter Shaw. Expression vectors of mouse constitutive androstane receptor-β (CAR-β), human constitutive

androstane receptor (hCAR), were kindly provided by Dr. David Moore and mouse retinoid X receptor α (mRXRα) was provided by Prof. Pierre Chambon. The expression vector for steroid receptor coactivator (SRC-1) was a generous gift from Dr. Malcolm Parker. Expression vectors of mouse pregnane X receptor1 (PXR1) and human pregnane X receptor (hPXR), DNA constructs of four copies of the *CYP3A1* promoter sequence, *CYP3A1* $(PXRE)_4$-*tk*-CAT, were kindly provided by Dr. Steven Kliewer. Consensus oligonucleotides were purchased from Santa Cruz Biotechnology Inc., UK. All other oligonucleotides were synthesised by Amersham Pharmacia Biotech, UK or Oswel® laboratory, UK.

## 2.3 Animals and cell lines

Male Sprague-Dawley rats, used for *in vivo* studies, preparation of primary hepatocyte cultures and nuclear protein extraction, were supplied by UCL biological services. Human Caucasian hepatocyte carcinoma (ECACC No: 85011430)-HepG2 cells; Human Negroid cervix epitheloid carcinoma (ECACC No: 93021013)-Hela cells; and Monkey African Green kidney fibroblast (ECACC No: 87032605)-CV-1 cells; used for transfection studies, were obtained from the European collection of cell cultures (ECACC).

## 2.4 Cloning of DNA Fragments

### 2.4.1 Linearisation of the plasmid vector

**Materials:**

**Plasmid vectors:** *pUC 19, pGL3-Basic, pGL3-Promoter*

**10x One-Phor-All buffer (Pharmacia®):** *100 mM Tris-acetate (pH 7.5), 100 mM magnesium acetate, and 500 mM potassium acetate. This was used with the restriction enzymes from Pharmacia® according to the manufacturer's instruction.*

**Method:**

The closed circular plasmid vector was digested with the appropriate restriction enzyme(s) to produce linear plasmid with ends compatible with those of the insert DNA. The reaction contained 1/10 volume of 10x One-Phor-All buffer, vector DNA and 1 unit of restriction enzyme(s) per microgram of DNA (not more than 1/10 of total volume) and distilled water to make up the required volume. The mixture was incubated at 37°C for 3 hr.

### 2.4.2 Dephosphorylation of linearised plasmid DNA

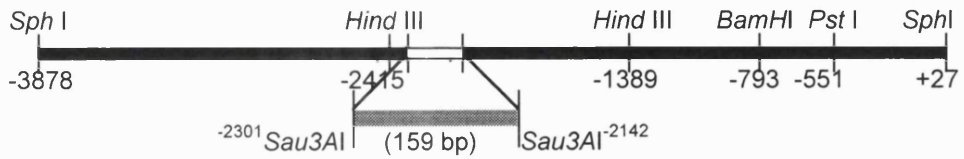
Linearised plasmid DNA was extracted with phenol (liquefied washed in Tris buffer):chloroform (1:1,v/v) and precipitated from the aqueous phase with two volumes of absolute ethanol for 15 min at -70°C. DNA was recovered by centrifugation at 12,000 g for 10 min at 4°C. DNA was redissolved in 90 µl of 10mM Tris.Cl (pH8.3), and then 10 µl of 10x One-Phor-All buffer and 1 µl of calf intestinal alkaline phosphatase; CIP (1000

U/ml) were added. The mixture was incubated at 37°C for 30 min. At the end of incubation period, SDS and EDTA (pH 8.0) were added to a final concentration of 0.5% and 5 mM, respectively. To inactivate CIP, the mixture was heated at 75°C for 10 min. After cooling the reaction to RT, it was extracted once with an equal volume of phenol and once with equal volume of phenol:chloroform (1:1,v/v). The plasmid DNA was precipitated at -70°C for 15 min by adding two volumes of ethanol. Plasmid DNA was recovered by centrifugation at 12,000 g for 10 min at 4°C. The pellet was washed with 70% ethanol, redissolved in distilled water and stored at -20°C.

## 2.4.3 Preparation of DNA fragments for subcloning

### *2.4.3.1 CYP2B1 gene*

The +27 to -3878 region of the *CYP2B1* gene has previously been cloned into the plasmid vector, pBLCAT3, at the *Sph*I site. This recombinant plasmid was digested with the appropriate enzymes to produce smaller fragments according to the restriction map of *CYP2B1* gene as follows;




The fragments to be cloned were separated by electrophoresis through a 0.8% low melting agarose gel. The fragments were excised from the gel and recovered by using a Spin-X 0.2 mm cellulose acetate

centrifuge filter unit (Costar®). The concentration of DNA was determined either by measuring the absorbance at 260 nm or by electrophoresing 1 µl of DNA on a 0.8% agarose gel and estimating roughly, by eye, by comparison with a known amount of DNA.

#### *2.4.3.2 Synthetic oligonucleotides*

Each oligonucleotide strand was phosphorylated by 1 unit of polynucleotide kinase per nanomole of DNA, in a final concentration of 1 mM ATP, at 37 °C for 45 min. Equal molar amounts of two phosphorylated complementary strands were mixed together and heated at 70-80°C, for 10 min. This mixture was allowed to cool down at RT.

### 2.4.4 Ligation reaction

#### *2.4.4.1 Sticky ends*

Both the plasmid DNA and DNA fragment were incubated at 50°C for 5 min and then chilled on ice immediately before ligation. The reaction mixture (20 µl) contained dephosphorylated linearised plasmid DNA and the DNA fragment of interest in a molar ratio of 1 to 5, 4 µl of 5 mM ATP, 2 µl of 10x One-Phor-All buffer and 1 µl of $T_4$ DNA ligase (5,500 Weiss unit/ml). This was incubated at 16°C overnight. After the ligation reaction was completed, the mixture was incubated at 65°C for 10 min.

#### *2.4.4.2 Blunt ends*

The condition of ligation is similar to that described in section 2.4.4.1, except that the reaction mixture contained 0.5 mM ATP, instead of 1 mM, and a very high concentration of either DNA ligase or blunt-ended termini was employed.

### 2.4.5 Transformation of bacteria

**Materials:**

**Ampicillin:** *A stock solution of ampicillin (50 mg/ml) was prepared using sterile distilled water and stored at -20°C. This was added to autoclaved medium to give a final concentration of 100 µg/ml*

**Luria-Bertani (LB) medium:** *Capsules of LB medium were placed in distilled water to give a solution of 10 g bacto-tryptone, 5 g bacto-yeast extract and 10 g NaCl per litre. The solution was autoclaved and stored at RT.*

**Luria-Bertani (LB) Agar:** *Capsules of LB agar were placed in distilled water to give a solution of 10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl and 15 g bacto-agar per litre. The solution was autoclaved and cooled to at least 55°C before adding selective antibiotics.*

**Transformation buffer:** *10 mM potassium acetate, 45 mM $MnCl_2$ .$4H_2O$, 10 mM $CaCl_2$, 100 mM KCl, 3 mM hexaminecobalt chloride and 10% glycerol. The solution was filter sterilised using a 0.45-µm nitrocellulose filter, and stored at 4°C.*

**SOB medium:** *Capsules of SOB medium were placed in distilled water to give a solution of 20 g bacto-tryptone, 5g of bacto-yeast extract, 0.58 g*

*NaCl and 20 mM $MgSO_4$ per litre. The solution was autoclaved and stored at RT.*

**Bacterial strain:** *E. Coli strain DH5α; deo R, end A1, gyr A96, hsd R17 [rk-mk+], rec A1, sup E44, thi 1,*(lacZYA-arg FV169), f 80d lac Z *M15,F-,I- (D.Hanahan,1983): host strain for pUC19*

*: E. Coli strain JM109, e14$^-$(McrA$^-$), recA1, endA1, gyrA96, thi-1,hsdR17(rk$^-$mk7), supE44, RelA1(lac-proAB )[F$'$traD36, proAB, lacI$^q$ ZM15: host strain for pGL3-Basic and pGL3-Promoter*

**Method:**

***2.4.5.1 Freshly prepared competent E. Coli***

*E. coli* was cultured overnight in 10 ml LB-medium, without ampicillin, at 37°C. The absorbance at 550 nm of the culture was measured using LB-medium as a blank. The culture was grown to log phase (an optical density (O.D.) of 0.6-0.8). The culture was transferred to a sterilin® universal tube and incubated on ice for 10 min. After centrifugation at 700g for 10 min, the supernatant was discarded and the pellet was resuspended in 8-ml ice-cold transformation buffer and incubated on ice for a further 10 min. The mixture was centrifuged at 700g for 10 min. After removing the supernatant, 2 ml of transformation buffer was added to the bacterial pellet. This was thoroughly re-suspended. 200 µl of this 'competent' culture was aliquot into a 1.5-ml Eppendorf® tube, ready for transformation.

### *2.4.5.2 Preparation of frozen competent E. Coli*

*E. Coli* from glycerol stock were inoculated into 1 ml SOB medium at 37°C and grown for 1 hr. The culture was poured into 100 ml SOB medium and incubated at 37°C for 2-3 hr. The culture was transferred to a 50 ml-tube and kept on ice for 10 min. The bacterial pellet was obtained by centrifugation at 4,000 g for 10 min at 4°C. The bacterial pellet was resuspended in 20-ml ice-cold transformation buffer and kept on ice for 10 min. Then, it was centrifuged at 4,000 g for 10 min at 4°C. The supernatant was drained off and the pellet was resuspended in 4-ml ice-cold transformation buffer. 140 µl of DMSO was added and the resuspended cells were left on ice for 15 min. Then, another 140 µl of DMSO was added. Aliquots of 200 µl of the competent cells were dispensed into pre-chilled Eppendorf® tubes and quickly immersed into liquid $N_2$. The frozen competent cells were thawed on ice 10 min before they were ready for transformation.

### *2.4.5.3 Transformation of bacteria using $CaCl_2$ method*

Either 2 µl or 4 µl, of ligated DNA was introduced into 200 µl of the competent *E. Coli.* For each transformation, two control tubes were included: one contained only bacterial competent cells, the other contained a typical plasmid (~0.25 µg), without any insert DNA, mixed with the competent bacteria. In most cases, the dephosphorylated linearised plasmid was also included to test for the efficiency of dephosphorylation and linearisation of the plasmid. Tubes were incubated on ice for 30 min. After that, the mixture was heat shocked at 42°C for 90 sec and quickly

chilled on ice. One ml of SOB medium was added and the mixture was incubated at 37°C for 1 hr with vigorous shaking. Either 200 µl or 400 µl of this culture was spread on LB-plates using a bent glass rod. The colonies would be observed after 24 hr. In the case of the plasmids that contain β-galactosidase gene, such as pUC19, the LB agar plates were first spread with 10 µl of isopropyl-β-D-thiogalactopyranoside (IPTG) and 50 µl of 5-bromo - 4 - chloro - 3 - indolyl - β - D - thiogalactopyranoside (X-gal) before inoculation of bacteria. After incubating in a 37°C incubator, overnight, the transformed *E. Coli*, containing the recombinant plasmid, were observed as white colonies, whereas the cells containing non-recombinant plasmids were blue.

## 2.4.6 Mini-preparation of the recombinant plasmids

**Materials:**

**Solution I:** *50 mM glucose, 10 mM EDTA (pH 8.0), 25 mM Tris-Cl (pH 8.0).*

**Solution II:** *0.2 M NaOH, 1%(w/v) SDS. The solution was freshly prepared before use.*

**Solution III:** *60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid, and 28.5 ml of sterile distilled water.*

**RNase A (DNase-free):** *A stock of pancreatic RNase A was prepared at a concentration of 10 mg/ml in 10 mM Tris (pH 7.5), 15 mM NaCl. The solution was heated at 100 ° C for 15 min and allowed to cool slowly to RT. It was stored in aliquots at -20°C.*

**Method:**

Isolated white colonies were selected and inoculated into 5-ml LB-medium containing ampicillin. Cultures were grown at 37°C, overnight, with vigorous shaking. The culture (2 ml) was poured into a 2.2 ml Eppendorf® tube and centrifuged for 30 sec in an Eppendorf® centrifuge. After removing the supernatant, 100 µl of solution I was added to the pellet and the cells were dispersed by vortexing. Then, 200 µl of freshly prepared solution II was added and the contents of the tube mixed by inversion several times. After incubation for 5 min on ice, 150 µl of solution III was added and the mixture was mixed gently by inversion. The sample was kept on ice for 5 min, then it was centrifuged for 10 min. The supernatant was poured into an Eppendorf® microcentrifuge tube containing an equal volume of phenol:chloroform. The mixture was vortexed and centrifuged for 3 min. The aqueous phase was transferred to a new tube containing two volumes of absolute ethanol. The mixture was vortexed and left on ice for 5 min. The DNA pellet was obtained by centrifugation for 10 min and then washed once with 80% ethanol. The pellet was left to dry at RT for 5-10 min and then resuspended in 20 µl of distilled water.

## 2.4.7 Checking for the right insert

Recombinant plasmid DNA, isolated by the mini-preparation method, was cut with the appropriate restriction enzyme(s) and treated with RNase A at 37°C for 2-3 hr and subjected to electrophoresis through a 0.8-2% agarose gel, as appropriate, using 1xTBE buffer and 120 V for 45 min. The insert

was observed under an UV lamp and compared to a 1-kb ladder, a standard molecular weight marker (GibcoBRL®).

### 2.4.8 Preparation of glycerol stocks

A single bacterial colony containing a correct recombinant plasmid was inoculated into 10 ml LB-medium containing the appropriate antibiotic and incubated at 37°C overnight, with vigorous shaking. An equal volume of sterile 30% glycerol, in LB-medium, was added to the overnight culture to give a final concentration of 15% glycerol. Alternatively, the overnight culture was mixed with autoclaved glycerol (100%) (1:4) to give a final concentration of 20% glycerol.

### 2.4.9 Maxi-preparation of the recombinant plasmids

Bacteria from glycerol stock were inoculated into 10 ml LB-medium, containing ampicillin (50 μg/ml), and grown at 37°C for 8 hr with vigorous shaking. The culture was then poured into 200 ml LB-medium containing ampicillin (50 μg/ml) and incubated overnight at 37°C with vigorous shaking. The culture was centrifuged at 6,000g for 15 min in a Sorvall™ GSA centrifuge. After removing the supernatant, the bacterial pellet was resuspended by vortexing in 10 ml buffer P1. Then, 10 ml of buffer P2 was added and the sample gently mixed by inversion. This was incubated at RT for 5 min. Then, 10 ml of pre-chilled buffer P3 was introduced and the contents gently mixed immediately. The tube was placed on ice for 20 min. After centrifugation at 30,000g for 30 min at 4°C, the supernatant was

filtered through a cloth mesh. The QIAGEN-Tip 500 was equilibrated by applying 10 ml of buffer QBT and allowing the column to empty by gravity flow. The filtered supernatant was then introduced to the QIAGEN-Tip500 column. The column was washed twice with 30 ml of buffer QC. Plasmid DNA was eluted by adding 15 ml of buffer QF. DNA was precipitated with a 0.7 volume of isopropanol (~10.5 ml). The DNA pellet was recovered by centrifugation at 15000 g for 30 min at 4°C. It was washed with 70% ethanol and allowed to air dry for 5 min, and then resuspended in 100-200 µl distilled water.

## 2.5 Purification of DNA from low melting point agarose gel

**Materials:**

**50x TAE buffer:** *40 mM Tris.acetate, 2 mM EDTA (pH 8.0). This was prepared by dissolving 242 g Trisma base in distilled water and adding 100 ml 0.5 M EDTA (pH 8.0) and 57.1 ml glacial acetic acid. The solution was made up to 1 litre, autoclaved, and stored at RT.*

**Running buffer:** *50x TAE buffer was diluted 1:50 to make 1x TAE.*

**Loading buffer:** *0.2% bromophenol blue, 30% glycerol in distilled water. The solution was filter sterilised using a 0.45 µm nitrocellulose filter and stored at -20°C in aliquots.*

**Method:**

Digested DNA fragments were separated by electrophoresis through a 0.8% low melting point agarose gel in 1xTAE buffer. Under UV light, the DNA band of interest was excised using a sterile scalpel blade (the minimum volume of gel was excised). The gel was placed in a Spin-X®

tube, 0.2 mm cellulose acetate centrifuge filter unit (Costar™). The gel was frozen by one of the following methods; (1) at -70°C for 15 min (2) in dry ice for 2-3 min (3) in liquid nitrogen for 2-3 min. After that, it was centrifuged for 10 min. The steps of freezing and centrifugation were repeated three times. Then 100 µl of TE buffer was added to the top section of the Spin-X® tube and the tube was centrifuged for another 10 min. The top section was removed and 0.2 volume of 3 M sodium acetate (pH 5.2) and 2 volume of absolute ethanol were added to the contents of the tube. The DNA was precipitated at -70°C. The DNA pellet was recovered by centrifugation at 4°C for 20 min. It was dried under vacuum and dissolved in 10-20 µl distilled water and stored at -20°C.

## 2.6 RNA isolation

The cells were lysed by vortexing in the Ultraspec™ RNA reagent [Biotecx laboratories] (1 ml/5-10 x $10^6$ cells). In case of the liver tissue, Ultraspec™ RNA reagent was added directly to the frozen tissue at ~ 1 ml/ g of tissue. The tissue was homogenised using a hand-held glass pestle. Following homogenisation, the homogenate was kept at 4°C for 5 min to permit complete dissociation of nucleoprotein complexes. After that, 200 µl of chloroform per 1 ml of Ultraspec™ RNA reagent was added and the mixture was vortexed vigorously for 15 sec and then kept on ice at 4°C for 5 min. The homogenate was centrifuged at 12,000 g for 15 min at 4°C. It formed two phases: the lower phase (organic phase), the interphase containing DNA and proteins, and the upper phase (aqueous phase)

containing RNA. The latter phase was carefully transferred to a new Eppendorf® tube. An equal volume of isopropanol was then added and the samples were stored at 4°C for 10 min. The sample was centrifuged at 12,000 g for 10 min at 4°C. A white pellet was obtained at the bottom of the tube. After removing the supernatant, the RNA pellet was washed twice using 75% ethanol and then centrifuged at 7,500 g for 5 min at 4°C. The pellet was dried under vacuum for 2-3 min. It is important not to let the pellet dry completely, as it will greatly decrease its solubility. Finally, the RNA pellet was dissolved in 50-100 µl distilled water and kept at -20°C.

## 2.7 Electrophoresis of RNA

**Materials:**

**10x MOPS/EDTA buffer:** *0.2 M MOPS [3-(N-morpholino) propane - sulfonic acid], 50 mM sodium acetate, 10 mM EDTA adjusted to pH 7.0 and autoclaved*

**Electrophoresis sample buffer:** *(freshly prepared or stored at -20 °C in small aliquots); 0.75 ml deionized formamide, 0.15 ml 10x MOPS, 0.24 ml formaldehyde, 0.1 ml deionized RNase-free water, 0.1 ml glycerol, 0.08 ml (w/v) bromophenol blue*

**Electrophoresis buffer:** *1x MOPS/EDTA buffer*

**Other solutions required:** *37% formaldehyde, 10x SSC, 1.0 mg/ml ethidium bromide in deionized RNase-free water*

### 2.7.1 Preparation of RNA samples

The RNA samples (~ 1 μg) were adjusted to a volume of 5 μl with distilled water. After adding 25 μl of electrophoresis sample buffer, the mixture was heated at 65°C for 15 min. Then, 1 μl of ethidium bromide was added into each sample and they were mixed thoroughly.

### 2.7.2 Gel preparation and electrophoresis

1 g Agarose, 10 ml 10x MOPS/EDTA buffer (pH 7.0) and 87 ml distilled water were mixed and heated until the agarose was dissolved. It was left to cool down to 50°C. In the fume hood, 5.1 ml of 37% formaldehyde was introduced into the agarose solution. This was poured into a gel tray. The gel was allowed to set for 1 h. Prior to loading the samples, the wells were flushed by pipetting in and out with electrophoresis buffer. After that, the RNA samples were loaded and the gel was subjected to electrophoresis at 30 V until the bromophenol blue had migrated ~10 cm into the gel.

## 2.8 Northern blotting

**Materials:**

**20x SSPE:** *175.3 g NaCl, 27.6 g $NaH_2PO_4.H_2O$ in 1 litre, the pH was adjusted to 7.4 with 10 M NaOH*

**Method:**

The nylon membrane and several pieces of 3MM Whatman® papers were cut at the same size as the gel. They were pre-wet in distilled water and left soaking in 20x SSPE buffer before use. The blotting apparatus was set up

using the pre-soaked Whatman® paper as a bridge and placing this on a raised glass plate in a 20x SSPE-containing chamber. The RNA gel was placed upside down on the Whatman® paper-bridge. The pre-soaked nylon membrane was placed on the gel, followed by several pieces of wet Whatman® papers. By using a 10 ml-pipette and rolling this on the gel and nylon membrane, any bubbles were removed. Then, a stack of dry Whatman® or blotting papers was put on top of the membrane, together with a ~500 g of weight. The transfer was carried out overnight. The nylon membrane was UV-crosslinked for 1 min before hybridisation proceeded. The membrane can be kept dry until use.

## 2.9 Radioisotope-labelling of DNA probe

**Materials:**

**Ultrapure dNTP set:** *100 mM dATP, 100 mM dGTP, 100 mM dTTP and 100 mM dCTP solution (pH 7.5); diluted to 10 mM solution with sterile distilled water*

**Easytide™ Radioactive isotope (From NEN DuPont, Germany):**

*$\alpha$-$^{32}$P-dCTP, 10.0 mCi/ml (3000 Ci/mmol)*

*$\gamma$-$^{32}$P-ATP, 10.0 mCi/ml (3000 Ci/mmol)*

### 2.9.1 Random priming method

Two mixtures, in Eppendorf® tubes, were prepared as follows:

Tube I contained 100 ng of the probe, 1.5 µl random primers (75 ng/ml) and 59.1 µl distilled water.

Tube II contained 9 µl of 20 mM DTT, 2.4 ml of 5 mM dNTPs, 9 µl random priming buffer and 60 µCi of $\alpha$-$^{32}$P-dCTP

Tube I was boiled for 5 min and immediately chilled on ice. The contents were mixed with those of tube II followed by the addition 1 µl of Klenow fragment DNA polymerase I (6,435 unit/ml). The reaction mixture was incubated at 37°C for 3 hr.

### 2.9.2 Fill-in reaction

DNA with a 5'-overhang was labelled by the fill-in reaction. The reaction mixture contained 300 ng DNA, 1x One-Phor-All buffer, 10 mM of each dATP, dGTP, dTTP and 20 µCi of $\alpha$-$^{32}$P-dCTP, 6.43 unit of Klenow fragment, DNA polymerase I, and distilled water to make up a final volume of 20 µl. The tube was incubated at 37°C for 30 min.

### 2.9.3 End-labelling reaction

The end-labelling reaction was set up in a reaction mixture of 1X One-Phor-All buffer, 100-300 ng DNA, 20 µCi of $\gamma$-$^{32}$P-ATP, and 9.5 units of $T_4$ polynucleotide kinase and sterile distilled water to make up a final volume of 20 µl. The mixture was incubated at 37°C for 30 min.

### 2.9.4 Purification of radiolabelled DNA probe

Unincorporated nucleotides were removed using a ChromaSpin +TE column (Clontech®). This was prepared by mixing its contents thoroughly and centrifuging it at 700g for 5 min to remove the equilibration buffer. The

probe solution was applied to the column followed by centrifugation at 700g for 5 min. The DNA probe solution was obtained in the eluate.

## 2.9.5 Determination of % incorporation of radioactivity into the labelled DNA probe

1 µl of the labelling reaction mixture before separation of unincorporated nucleotides was spotted onto a DE81 ion exchange membrane. The DNA probe solution after passing through a ChromaSpin +TE column was spot 1 µl onto the second DE81 ion exchange membrane.

The membranes were dried under infrared light, and then placed in scintillation fluid (Aquasol®-LSC cocktail, DuPont) - containing vials and counted by a scintillation spectrophotometer.

$$\% \text{ Incorporation (P)} = \frac{\text{after column (cpm)} \times 100}{\text{before column (cpm)}}$$

## 2.9.6 Determination of the specific radioactivity ($S_A$)

$$S_A = \frac{(\mu Ci)(2.2 \times 10^9)(P)}{M_l + [(1.3 \times 10^3)(P)(\mu Ci/S_a)}$$

Where $S_A$ is the specific activity in disintegrations per minute per microgram (dpm/µg), µCi is the amount of radiolabeled nucleotide in microcuries in the reaction mixture, P is the proportion of radiolabeled nucleotide incorporated into the probe DNA, $M_l$ is the mass of input of the

DNA template in nanograms (ng) and $S_a$ is the specific activity of radiolabeled nucleotide in curies per millimole (Ci/mmol).

## 2.10 Hybridisation

**Materials:**

**Prehybridisation solution:** *5x SSPE, 50% deionized formamide, 0.1-0.5% SDS, 100 mg/ml denatured salmon sperm DNA, 5x Denhardt's reagent*

**Hybridisation solution:** *5x SSPE, 50% deionized formamide, 0.2% SDS, 200 mg/ml denatured salmon sperm DNA, 5x Denhardt's reagent, 10% dextran sulphate*

**Method:**

The membrane was first wet with 20xSSC and then placed in a plastic hybridisation bag with 10-ml pre-hybridisation buffer. The membrane was incubated at 42°C for 2-4 hr. The denatured probe solution was prepared by mixing 10-ml hybridisation buffer with the $^{32}$P-labelled DNA probe. This was boiled for 5 min and chilled immediately on ice. The pre-hybridisation solution was removed from the hybridisation bag and the denatured probe, in hybridisation solution, was added, and incubation was continued at 42°C, overnight.

**Washing conditions**

**Materials:**

**20x SSPE:** *175.3 g NaCl, 27.6 g $NaH_2PO_4.H_2O$ in 1 litre, the pH was adjusted to 7.4 with 10 M NaOH*

**Method:**

To achieve the greatest intensity of signal and the least background from non-specific binding of the probe, it is beneficial to optimise the time and temperature of washing as follows;

The membrane was washed once with 2xSSPE, 1%SDS at RT for 15 min, followed by two washes with 1xSSPE, 1%SDS at RT for 15 min, finally with two washes in 0.1xSSPE, 1%SDS at 50°C for 15 min.

The membrane was wrapped in Saran wrap and exposed to X-ray film, in an intensifying cassette, overnight at -70°C. Subsequent exposure times were adjusted to increase or decrease the signal intensity. Exposed film was developed using a film-developing machine.

## 2.11 Nuclear protein extraction

### 2.11.1 Treatment of animals

Male Sprague-Dawley rats (230-250 g) were fed on Harlen Teklad TRM9607 standard rat and mouse pellets. Freshly prepared 0.1% sodium phenobarbital in tap water was given to animals for 4 days. On the fifth day, they were injected intra-peritoneally with sodium phenobarbital at the dose of 100 mg/kg in 0.9% NaCl. Control and PB-treated animals were starved overnight, but given tap water. They were sacrified by cervical dislocation. The liver was removed, weighed, and kept on ice for as short a time as possible. For some experiments, the rats were injected with phenobarbital intra-peritoneally at the dose of 100 mg/kg for 6 hr before being sacrificed.

## 2.11.2 Isolation of nuclei

**Materials:**

**1 M HEPES (pH 7.6):** *23.83 g HEPES in 100 ml distilled water, adjusted to pH 7.6 with NaOH*

**0.25 M PMSF:** *PMSF was dissolved in isopropanol to a final concentration of 0.25 M. It was aliquot and stored at -20°C. Before use, the solution was warmed up to 37°C to dissolve any solid present.*

**Homogenisation buffer:** *10 mM HEPES (pH 7.6), 15 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2 M sucrose, 10% glycerol. This was stored at -20°C. Just before use, it was thawed and made to 0.5 mM DTT, 0.5 mM PMSF, 1 % trasylol (aprotinin), 0.1 mg/ml antipain, 0.1 mg/ml chymostatin, and 0.1 mg/ml leupeptin. The solution was kept cold until use.*

**Nuclear lysis buffer:** *10 mM HEPES (pH 7.6), 100 mM KCl, 0.1 mM EDTA, 10% glycerol, 3 mM $MgCl_2$. Just before use, this was made to 1 mM DTT, 0.1 mM PMSF, and 1% trasylol (aprotinin). It was kept at 4°C.*

**Dialysis buffer:** *25 mM HEPES (pH 7.6), 0.1 mM EDTA, 40 mM KCl, 10% glycerol, 1 mM DTT*

**4 M $(NH_4)_2$ $SO_4$:** *52.85 g $(NH_4)_2$ $SO_4$ in 100 ml distilled water, adjusted pH to 7.9 with 10 M NaOH*

**Method:**

The whole procedure was carried out at 4°C, according to Sierra (1990). Rat livers were chopped as finely as possible into small pieces using scissors. 10-ml homogenisation buffer was used per gram of tissue. About one-third of the homogenisation buffer was added and the tissue

homogenised using a teflon tipped pestle and a Citenco electric overhead homogeniser (8 stroke). Then, the remaining homogenisation buffer was mixed with the homogenate. 10 ml of homogenisation buffer was placed in a Beckman® ultracentrifuge tubes to make a cushion. The homogenate was layered on top. Samples were centrifuged at 104,000g for 1 hr in Beckman L8 ultracentrifuge using a SW28 rotor. The solid disc at the top of the tube was removed using a sterile spatula. The supernatant was stored in aliquots at -70°C for further investigation. The nuclear pellet was at the bottom of the tubes. The side of the tubes were washed with sterile distilled water, being careful not to wet the pellet, and wiped with a tissue paper. The nuclei were kept cold at all times by laying the tubes on ice, and in a downward position so that excess solution flow down from the pellet. At this step, the nuclear pellet can be kept frozen until needed for further nuclear protein extraction.

### 2.11.3 Nuclear protein extraction (Sierra, 1990)

The nuclear pellet was suspended in 5-ml nuclear lysis buffer, either immediately after nuclei isolation, or after being frozen. The absorbance at 260 nm of the mixture was measured by dissolving 20 µl in 980 µl of 0.5% SDS (1:50) with vigorous shaking. 0.5% SDS was used as the blank. The mixture was adjusted using nuclear lysis buffer to achieve a concentration of 0.5 mg/ml and 1/10 of total volume of 4 M $(NH_4)_2SO_4$ was added. After mixing on a Spiramix® for 30 min at 4°C, the sample was centrifuged at 126,000g at 4°C for 1 hr. The supernatant was immediately poured into tubes containing 0.3 g solid $(NH_4)_2SO_4$ per ml of supernatant (otherwise

the pellet would start swelling). Samples were mixed on a Spiramix® for 45 min at 4°C and then centrifuged at 126,000g at 4°C for 20 min. The white pellet obtained was resuspended in dialysis buffer at a concentration of 10 mg/ml, based on the initial DNA concentration. The protein solution was transferred to dialysis tubing and dialysed at 4°C, against 100 volumes of dialysis buffer, with two changes of buffer at 2 hr intervals. Nuclear protein was divided into 100 µl aliquots, frozen on dry ice, and stored at -70°C.

## 2.12 Determination of protein concentration by the method of Lowry (Lowry, 1951)

**Materials:**

**Reagent A:** *(freshly prepared) (i) 2% potassium sodium tartate, (ii) 1% copper sulphate, (iii) 2% sodium carbonate in 0.1 N sodium hydroxide. Reagent (i) and (ii) were mixed first and then reagent (iii) was added in a ratio of 1:1:100(v/v/v), respectively.*

**Reagent B:** *Folin Ciocalteau reagent was diluted 1:1.5(v/v) with water.*

**Method:**

The sample (~1-2 µl) and sequential dilutions of standard bovine serum albumin were made up to 200 µl with distilled water. A tube of 200 µl distilled water was included as a blank. 1 ml of reagent A was added to each 200 µl of the samples, standard and the blank. The mixture was incubated at RT for 20 min. Then, 100 µl of reagent B was added and the samples was mixed thoroughly immediately. The reaction mixture was left at RT for 45 min before measuring the absorbance at 700 nm against the blank.

**Standard curve:**

A stock solution of bovine serum albumin (1.23 μg/μl) was prepared and used at sequential 2-fold volume to achieve concentrations as follows: 2.46, 4.92, 7.38, 9.84, 12.30, and 14.76 μg.

## 2.13 Gel mobility shift assay

**Materials:**

**4% polyacrylamide gel:** *0.5x TBE, 30% polyacrylamide, 0.1% TEMED, 10% ammonium persulfate*

**6x Gel shift loading buffer:** *0.25% bromophenol blue, 0.25% Xylene cyanol FF, 30% glycerol*

**5x Rosette buffer:** *60 mM HEPES (pH 7.9), 20 mM Tris-HCl (pH 7.9), 300 mM KCl, 75 mM NaCl, 25 mM $MgCl_2$, 25 mM DTT, 0.5 mM EDTA and 62.5% glycerol*

**Running buffer:** *0.5x TBE buffer*

**Method:**

A 20 μl reaction mixture contained 4 μl 5x Rosette buffer, 3-5 μg nuclear protein (1 μg/μl), 2.5-5 μg poly dI.dC (5 μg/μl) and 4 x $10^4$ cpm $^{32}$P- labelled probe. The 5x Rosette buffer, 7.5 mM NaCl, and poly dI.dC were prepared as a master mix and divided into each tube. Then, nuclear proteins and labelled probe were added. The reaction mixture was incubated on ice for 30 min. If competitor DNA was used, it was added to the master mix before a labelled probe and nuclear proteins were added. A control tube contained everything, but nuclear proteins, to test if the labelled probe produced any

non-specific bands. To test whether the binding of DNA and protein is specific, proteinase K was included. This sample contained all reaction components, but at the end of reaction, 1 µl proteinase K (5 µg/µl) was introduced and the sample incubated for a further 37°C for 15 min. 2 µl 10x gel shift loading buffer was added to each tube. Samples were electrophoreses through a 4% polyacrylamide gel in 0.5 x TBE at 140 V. The gel was dried using a gel-dryer machine at 80°C and then exposed to X-ray film overnight.

### 2.13.1 Supershift assay

The protein-DNA reaction was carried out as in section 2.13. After incubation for 20 min, the antibody was added to the reaction. The binding reaction was continued for another 20 min.

### 2.13.2 UV-crosslinking of the protein-DNA complexes

The protein-DNA reaction was carried out as in section 2.13. After incubation for 20 min, the reaction mixture was UV-crosslinked for 20 min at 20,000µJoule using a UV-crosslinking machine (Stratagene®).

## 2.14 Partial purification of nuclear proteins using phosphocellulose ion-exchange chromatography

**Materials:**

**0.5 M phosphate buffer pH 7.0:** *0.0027 M potassium chloride, 0.137 M sodium chloride (pH 7.4)*

**Buffer A:** *25 mM HEPES (pH 7.6), 0.1 mM EDTA, 40 mM KCl, 10% glycerol, 1 mM DTT*

**Buffer B:** *25 mM HEPES (pH 7.6), 0.1 mM EDTA, 1 M KCl, 10% glycerol, 1 mM DTT*

### 2.14.1 Exchanger preparation

**Method:**

The cellulose phosphate was mixed with 25 volumes of 0.5 N NaOH and stirred for 5 min. The supernatant was decanted off and the medium was washed with distilled water through the Whatman® 3MM filter paper in a funnel, until the pH of the filtrate was 11.0 or below. The cellulose phosphate was then stirred in 25 volumes of 0.5 N HCl for 5 min and washed with distilled water through the Whatman® 3MM filter paper, in a funnel, until the pH of the filtrate was above 3.0. It is important not to exceed the 5 min contact times in order to avoid excessive hydrolysis and swelling changes. The cellulose phosphate was stored in 0.5M phosphate buffer pH 7.0 at 4°C.

### 2.14.2 Column packing

The equilibrated cellulose phosphate was gently dispersed before packing into the column and then slowly poured into the column (width 1 cm x length 2 cm). It was allowed to settle for a while and buffer A was run through the column for 20 min. Now the column is ready to use.

### 2.14.3 Sample loading and chromatographic elution conditions

The buffer, used to dialyse nuclear extracts, was run through the column. The sample was loaded. The flow rate was 0.3 ml/min. By using the gradient of high salt buffer, buffer B, the proteins were separated and monitored at 280 nm in each fraction.

### 2.14.4 Coupling of synthesised biotinylated DNA to pre-washed magnetic beads

Streptavidin-coated magnetic beads (Dynabeads® M-280, Dynal) were washed twice with 50 µl of 1% BSA in PBS, twice with 1M NaCl in PBS and twice with TE buffer (pH 8.0). Synthesised biotinylated DNA was added to the washed beads and rotated at RT for 30 min. The ratio of biotinylated DNA to streptavidin-coated beads was 200 pmole DNA per milligram of beads.

### 2.14.5 Binding of nuclear proteins to the DNA-affinity magnetic beads

Nuclear protein was first mixed with non-specific DNA (poly dI.dC) in 1x Rosette buffer and added to DNA-affinity magnetic beads. They were allowed to bind to the beads at RT for 20 min. By using a Magnet, the excess and non-specific proteins remaining in the supernatant can be separated. After washing the non-specific protein binding off with 0.5x Rosette buffer (poly dI.dC), twice, the specific proteins bound to the beads were eluted with 0.5 M Rosette buffer containing 0.5-1 M KCl.

## 2.15 SDS-Polyacrylamide gel electrophoresis

**Materials:**

**Protogel:** *30% (W/V) acrylamide, 0.8 % (w/v) bisacrylamide (National Diagnostic®)*

**Buffer (pH 6.8):** *0.5 M Trizma® base and 0.4 % (W/V) SDS, adjusted pH to 6.8*

**Buffer (pH 8.8):** *1.5 M Trizma® base and 0.4 % (W/V) SDS, adjusted pH to 8.8*

**10% ammonium persulphate:** *dissolved in distilled water*

**10% Separating gel:** *In 40 ml; 13.3 ml of Protogel®, 10 ml of buffer (pH 8.8), 27.5 ml of distilled water, 563 µl of 10 % ammonium peroxodisulphate, and 30.7 µl of TEMED. The solution was mixed briefly and poured into a gel apparatus to ~ 1 cm below the comb. 0.1 % SDS was promptly layered onto the surface of the gel.*

**3% stacking gel:** *In 20 ml; 2 ml of Protogel®, 5 ml of buffer (pH 6.8), 12 ml of distilled water, 225 µl of ammonium peroxodisulphate, and 20 µl of TEMED.*

*The 0.1% SDS was removed and the stacking gel was poured on top of the separating gel.*

**Electrophoresis buffer:** *0.025 M Trizma® base, 0.192 M glycine and 10 % (w/v) SDS*

**2x protein loading buffer:** *1% (w/v) SDS, 10 mM EDTA (pH 8.0), 10 mM sodium phosphate (pH 7.0), 1% (v/v) β-mercaptoethanol, 15% (v/v)*

*glycerol, 4 mM PMSF and 0.01% (w/v) bromophenol blue. The solution was filtered sterilised through a 0.45 μm filter unit and stored at - 20°C.*

**Method:**

10-20 (or more) μg of protein sample was diluted with distilled water and mixed with an equal volume of 2X loading buffer. The mixture was boiled for 3 min. Then, 2 μl of β-mercaptoethanol was added to every 10 μl of the sample. This was centrifuged briefly to separate non-dissolved material. The samples were loaded onto the gel. It was electrophoresed at constant current of 26 mA for stacking gel, and 36 mA for separating gel.

## 2.16 Western blot analysis

**Materials:**

**Transfer buffer:** *0.025 M Tris, 0.192 M Glycine, 20%(v/v) Methanol*

**Method:**

The protein samples were resolved by 10% SDS polyacylamide gel electrophoresis. The blotting membrane and 3MM Whatman® papers were pre-soaked in transfer buffer. The western blot was accomplished by means of electroblotting. The nitrocellulose membrane was placed on top of the gel. Care was taken to ensure that no bubbles were trapped between the gel and the membrane before assembling the blotting apparatus. The gel was placed at the cathode side, and the blotting membrane was placed towards anode side. The transfer was carried out at constant current of 100 mA, overnight at RT. The current was turned up to 200 mA for 1 hr before proceeding to the immunostaining detection step.

## 2.17 Immunostaining

**Materials:**

**Blocking solution:** *5% non-fat dried milk, 10 mM Tris (pH 7.5), 1 mM NaCl, 10% Tween-20*

**Washing buffer:** *10 mM Tris (pH 7.5), 1 mM NaCl, 10% Tween-20*

**Method:**

### 2.17.1 Blocking step

The membrane was removed from the gel and incubated in a blocking solution at RT for 1 hr with gentle shaking. The membrane was washed briefly in washing buffer twice, then for 15 min and then twice for 5 min.

### 2.17.2 Primary antibody-binding step

The primary antibody was diluted 1:1000 in the blocking solution. The dilution may vary according to the manufacturer's recommendation and the result obtained. The membrane was incubated with the primary antibody for 1 hr at RT with gentle shaking. Then, the membrane was washed in washing buffer briefly twice, then for 15 min and then twice for 5 min.

### 2.17.3 Secondary antibody conjugation step

Since the primary antibody was raised in rabbit, the HRP-labelled anti-rabbit IgG was used for conjugation. The HRP-labelled anti-rabbit IgG was diluted in a blocking solution at 1:1000. The conjugation step was performed at RT for 1 hr. The membrane was washed in washing buffer twice briefly, then for 15 min and then for 5 min.

### 2.17.4 Detection step using Enhanced chemiluminescense (ECL) kit (Amersham®)

The following step was carried out in a dark room. The excess liquid was drained from the membrane before placing it on Saran wrap. An equal volume of detection reagent 1 and reagent 2 was mixed and spread over the surface of the membrane (0.125 ml/cm$^2$). The incubation time was exactly 1 min. The excess liquid was removed. Then, the membrane was placed in a plastic holder and exposed to X-ray film for 1 min (or longer, depending on the signal intensity).

### 2.17.5 Re-probing

If it is desirable to re-probe the blot with another antibody, the blot may be stripped of the primary and secondary antibodies by one of two methods:

(I) Rinse the blot with water and incubate in 0.1 M glycine (pH 2.9) at RT for 20 min. Rinse the blot with washing buffer and repeat the protocol from the blocking step.

(ii) Place the blot in 2% (w/v) SDS, 62.5 mM Tris (pH 6.8) and 100 mM β - mercaptoethanol at 50-70°C for 30 min. Rinse the blot with washing buffer and repeat the protocol from the blocking step.

## 2.18 *In vivo* DNA transfection by biolistic particle delivery - Gene gun technique

### 2.18.1 Preparation of DNA for *in vivo* transfection

The -2301/-2142 (159 bp), of the *CYP2B1* 5' flanking sequence, was subcloned into the reporter vector; pGL3-Promoter (Promega®), either at *BamH* I or *Bgl* II site. The presence and orientation of DNA insert was confirmed by restriction digestion and DNA sequencing (Oswel® laboratory). Supercoiled DNA is required. In order to normalise the variation caused by transfection and/or lysis efficiency, the second reporter, control vector, pRL-TK (Promega®), was mixed in a ratio of 1 to 10. The total volume was made up with distilled water and should not exceed 100 µl.

### 2.18.2 Preparation of DNA coated gold particles

**Materials:**

**PVP solution:** *0.05 mg/ml polyvinyl pyrrolidone (MW 360,000) in absolute ethanol*

**Method:**

25 mg of Au particles (radius 1 µm) was mixed with 100 µl of 0.05 M spermidine by vortexing briefly and then sonicated for 3-5 sec. 100 µg of DNA mixture (section 2.18.1) was added (the maximum volume of DNA was 100 µl) and the mixture was vortexed for 5 sec. Then 100 µl of 1 M $CaCl_2$ was added dropwise while the mixture was vortexed at moderate rate. After 10 min at RT, it was centrifuged at 12,000g for 5 sec and the

supernatant was removed. The pellet was washed 3 times with 1 ml absolute ethanol. Then it was resuspended in 200 µl PVP solution, transferred to a 15 ml-tube and the volume adjusted to 3.5 ml. The suspension is now ready for cartridge preparation or can be kept at -20°C until use.

### 2.18.3 Preparation of the cartridges

The Tubing Prep Station was set up and connected to the nitrogen tank as described in the manufacture's instructions (Bio-rad®.) The Gold-Coat tubing is purged with nitrogen for 15 min to make it completely dry. One end of the tubing was attached to a 10-ml syringe. The DNA-coated gold suspension was inverted several times and quickly drawn into the Gold-Coat tubing using the syringe, leaving a 2-3 cm space at the end. Immediately, the tubing was inserted into the opening on the right side of the Tubing Prep Station, pushed into the hole and the tubing support cylinder. The suspension was allowed to settle for 5 min. The tubing was detached from the syringe and then attached to another immobilised 10-ml syringe. Ethanol was removed at a rate of 0.5-1.0"/sec (this requires 30-45 sec). The syringe was detached and the tubing was immediately turned $180^0$ while in the groove. The gold was allowed to coat the inside surface of the tubing for 5 sec. The Tubing Prep Station was switched on to rotate so that the gold was allowed to smear the inside of tube for 30 sec. The valve on the flow meter was then opened to allow 0.35-0.4 LPM of nitrogen to dry the Gold-Coat tubing, while the Tubing Prep Station rotated for a further 5 min. The motor was switched off. The nitrogen flow was turned off by

closing the valve on the flow meter. The tubing was removed and cut using the Tubing Cutter® according to the manufacturer's instruction (Bio-rad®). The cartridges were kept in desiccated vials at 4°C.

### 2.18.4 Biolistic particle delivery using the Helios gene gun

The rats were anesthetised by intra-peritoneal injection of xylazine and ketamine mixture (10 and 100 mg/kg body weight, respectively). The abdominal area was shaved to remove fur. A cut about 2-cm long was made in the skin and muscle layers. A liver lobe was pulled out and positioned on a tissue paper. In order to mark where the shooting area was, the liver lobe was nicked ~2-3mm using a scissors. Before firing to the target, the gene gun was activated as described in the manufacturer's instruction. After that, the cartridges were loaded into the cartridge holder. The gene gun spacer touched the surface of target area. The safety interlock was activated and the trigger was pressed to deliver the DNA-gold particles into the liver. The liver was shot at two different areas. The abdomen was closed by suture, using braided silk and a polyamide 6 monofilament (Ethilon$^{R}$) for muscle layer and skin, respectively.

After 24 hr, the animals were sacrified. The liver slice was taken and processed to determine dual-luciferase activity.

## 2.19 Isolation and culturing of primary rat hepatocytes

### 2.19.1 Isolation of primary rat hepatocytes by liver perfusion

**Materials:**

**Sodium pentobarbital:** *60 mg/ml solution in sterile 0.9%(w/v) NaCl. Use at 60 µg/g of rat body weight.*

**Sodium heparin:** *5000 u/ml of stock solution was diluted to 200 U/ml using sterile 0.9% (w/v) NaCl.*

**EGTA:** *25 mM stock solution in 0.1 M NaOH*

**10x EBSS, without calcium and magnesium (Earle's balance salt solution; Gibco BRL):** *Prepared a fresh 1X concentration by mixing 100 ml of 10X EBSS with 30 ml of 7.5%(w/v) $NaHCO_3$ and sterile distilled water to make up to 2 L. Adjust pH to 7.5.*

**Perfusion buffer I:** *Prepare fresh, 1x EBSS containing 0.5 mM EGTA*

**Perfusion buffer II:** *1x EBSS*

**Perfusion buffer III:** *Prepare fresh, 1x EBSS containing 5 mM $CaCl_2$, 0.08 u/ml of collagenase H and a spatula of trypsin inhibitor was added just before use.*

**Dispersal buffer:** *10 mM HEPES, 142 mM NaCl and 7 mM KCl. Adjust pH to 7.5, autoclaved. 2.5%(w/v) of BSA, fraction V was added just before use.*

**L-15 Leibovitz medium with Glutamex™ and L-amino acid:** *Add 2.5% (w/v) BSA, fraction V just before use and adjust pH to 7.5*

Trypan blue: *0.1 % (w/v) in 0.9 % NaCl*

**Method:**

This method was modified from the two-step collagenase perfusion of Seglen (Seglen, 1976). Male Sprague-Dawley rat (~230-250 g) was anaesthetised by injecting 60 μg/g body weight of sodium pentobarbital intraperitoneally. The skin layer was cut open along the mid-ventral line and across the middle of the mid-ventral line. The abdominal wall was cut the same way. The sternum was clamped using 125-mm Halstead's mosquito artery clamps. The liver was flipped upward toward the diaphragm. The stomach and intestine were moved to the right side so that the portal vein and vena cava were exposed. Two upper and lower loose ligatures were tied around the portal vein ~ 10 mm apart from each other. The third loose ligature was tied around the vena cava above the vena suprarenal vein. The 150 U of heparin was injected into the inferior vena cava. The lower ligature around the portal vein was tightened by making two knots. A cut was made just above the tightened ligature and kept open by using the vein lifter. Then, a medium, pre-filled cannula, was inserted into the portal vein carefully. The cannula was kept in place by tightening the upper ligature. The lower ligature was wound around the cannula to keep it stable, followed by tightening the ligature around the vena cava. At this stage, the liver has to be removed as quickly as possible by cutting the rib cage, diaphragm and other organs nearby. The liver was placed on the platform of the perfusion unit in a laminar hood. The unit was run for a while with perfusion buffer I to make sure there were no bubbles in the system before the perfusion was begun. All three perfusion buffers were gassed with 95% air and 5% $CO_2$ and the heat exchanger kept the temperature of all three buffers at 37°C when they reached the liver. The

liver was perfused for 3 min with buffer I, 7 min with buffer II and 20 min with buffer III. When the perfusion was completed, the liver was detached from the cannula and transferred to the Petri dish containing the supplemented L-15 Leibovitz medium. The Glisson's capsule was peeled off from each liver lobe. The hepatocytes were readily released. The cell suspension was filtered through a 64 µm nylon mesh in order to separate the membranous debris. The viable cells were left to settle at the bottom for 10 min. The supernatant was removed. The cells were gently resuspended in dispersal buffer and transferred to 50 ml Falcon tubes to be centrifuged at 50 g for 2 min at 4°C. The supernatant was aspirated and the cells were resuspended in fully supplemented medium with 10% foetal bovine serum. The cell viability was determined by using the trypan blue assay. The culturing of hepatocytes was carried out only when the cell viability exceeded 75%.

## 2.19.2 Culturing of hepatocytes

**Materials:**

**William's E medium** with $NaHCO_3$ without L-glutamine and phenol red (Gibco BRL®)

**Antibiotics:** *200 U/ml penicillin, 200 µg/ml streptomycin and 2.5 µg/ml amphotericin*

**10 mg/ml Insulin (bovine pancreas):** *100 mg lyophilised powder in 10 ml sterile distilled water and 100 µl sterile glacial acetic acid.*

**8 mM Dexamethasone phosphate:** *0.1 μM in William's E medium as a working solution.*

**Fully-supplemented culture medium:** *William's E medium containing 2 mM GlutaMAX™-I (Gibco BRL), 200 U/ml penicillin, 200 μg/ml streptomycin, 2.5 μg/ml amphotericin, 1.7 μM dexamethasone and 10% foetal bovine serum.*

**Method:**

2 ml of cell suspension was dispensed onto 60 mm Permanox® plates coated with 200 μl of Matrigel (1mg/ml)(Beckton Dickinson) at ~4-5 x $10^6$ cells/plate and swirled gently so that the cell suspension covered all the surface area. The cells were incubated at 37°C for 3 hr under 5% $CO_2$. At the end of this incubation period, the cells were adhered to the plates. The culture medium was removed and the cells were washed twice with 3 ml of William's E medium. Then, 3 ml of fully supplemented medium was added to the cells and they were cultured for 24 hr at 37°C under 5% $CO_2$ prior to transfection.

## 2.20 Culturing the cell lines

Each cell line (CV-1, HepG2 or Hela) was grown at 37°C under 5% $CO_2$ on the medium according to what is recommended from ECACC. When a frozen stock was used, the cells were allowed to adhere to the tissue culture flask overnight before the washing step to remove the unattached cells. The morphology and viability of the cells was observed under the

microscope. The cells were sub-cultured and the medium was changed regularly.

## 2.21 Preparation of whole cell extract from cell lines

For the expression of nuclear hormone receptors in cell lines, 20 μg of the expression vector was transfected into HepG2 cells, grown on 165 $cm^2$ tissue culture flasks. After 24 hr, the cells were harvested, and the whole cell extract was prepared by three cycles of freeze-thawing in 0.4 mM KCl, 20 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol, and 20% (v/v) glycerol. The extract was centrifuged at 10,000 X g for 15 min at 4°C. The supernatant was collected and PMSF was added to the final concentration of 1 mM.

## 2.22 Preparation of nuclear extract from the cell culture

**Material:**

**Hypotonic buffer:** *10 mM HEPES, pH 7.9, 10 mM KCl, 0.2 mM EDTA, 0.1 mM EGTA. Before use, add 1 mM DTT, 0.5 mM PMSF, 2.5 μg/ml aprotinin, 2.5 μg /ml leupeptin and 1.0 μg/ml pepstatin*

**High salt buffer:** *20 mM HEPES, pH 7.9, 0.4 M NaCl, 1mM EDTA, 0.02 % (v/v) NP-40. Before use, add 1 mM DTT, 0.5 mM PMSF, 2.5 μg/ml aprotinin, 2.5 μg /ml leupeptin and 1.0 μg/ml pepstatin*

**Method:**

The cells were washed twice with cold 1x PBS, then they were detached using the cell scraper. After that, the cell suspension was centrifuged at 1800 g for 5 min. The supernatant was removed and one ml of hypotonic buffer, per 2.5 x $10^7$ cells, was added to the cell pellet. This was vortexed and allowed swelling on ice for 15 min. Then, NP-40 was added to a final concentration of 0.02% (v/v). The cell mixture was passed through a no. $27^{1/2}$-gauge needle, several times, until the cells were lysed completely. The nuclear pellet was obtained by centrifugation of the cell lysate at 10,000 g for 5 min, at 4°C. The nuclei were resuspended in 500 μl of high salt buffer and rolled on a Spiralmix® for 30 min at 4°C. The nuclear protein was separated from the particulate matter by centrifugation at 10,000 g for 5 min, at 4°C. The nuclear protein was desalted and concentrated using a Microcon-10 unit (Millipore®). Briefly, 500 μl of the nuclear protein solution was added to the top section of the Microcon-10 column. This was centrifuged at 14,000 g for 50 min, at 4°C. The nuclear protein was

recovered by turning the top section upside down and centrifuged for 3 min at 1,000 g.

## 2.23 Transient transfection

### 2.23.1 Transfection by calcium phosphate method

**Materials:**

**Buffer A:** *0.5 M $CaCl_2$ in 0.1 M HEPES buffer (pH 7.0)*

**Buffer B:** *0.28 M NaCl, 0.75 mM $NaHPO_4$, 0.75 mM $Na_2$ $HPO_4$ in 0.05 M HEPES buffer (pH 7.0)*

**15% Glycerol in Isotonic HEPES:** *0.238 g of HEPES (free acid) was dissolved in 80 ml of distilled water. The pH was adjusted to 7.5 with NaOH. 18.9 g (15 ml) of glycerol and 0.877 g of NaCl was added and the total volume was adjusted to 100 ml. The solution was filter-sterilised before use.*

**Method:**

3 μg of DNA was dissolved in 120 μl of distilled water. The dissolved DNA was mixed with buffer A, vortexed briefly, and incubated at RT for 10 min. Then, 240 μl of buffer B was added and vortexed immediately for a few sec. The contents were incubated for 15 min at RT. The resulting calcium phosphate-DNA precipitate was added to the cell culture. The culture plates were rocked gently to mix the medium and the precipitate. The cells were incubated for 6 hr before they were washed twice with the medium (without any additions) to remove any remaining precipitate. Then, 1.5 ml of 15% glycerol in isotonic HEPES buffer (pH 7.5) was added and

incubated for 3 min at RT. The cells were rinsed once with medium (without any additions). Finally, 3 ml of complete medium was added and the cells were incubated in a 37°C incubator under 5% $CO_2$.

### 2.23.2 Transfection by Effectene™ transfection reagent (QIAGEN®)

**Materials:**

**Supplied in kit:** *1 mg/ml Effectene™ transfection reagent, 1 mg/ml Enhancer, and Buffer EC*

**Method:**

The procedure was carried out according to the manufacturer's handbook. Briefly, 1 μg of DNA (DNA concentration ≥ 0.1 μg/μl) was diluted in buffer EC to a total volume of 150 μl. 8 μl of Enhancer was added to DNA. The tube was vortexed briefly and incubated at RT for 2-5 min. After that, 25 μl of Effectene™ transfection reagent was added, the mixture was vortexed for 10 sec and incubated at RT for 5-10 min. The hepatocytes culture plates were washed once with William's E medium. 4 ml of fully supplemented culture medium was added to the cells. 1 ml of fully supplemented culture medium was added to the DNA complex. This was mixed by pipetting up and down twice before it was added drop-wise immediately to the cells. The cells were incubated with the DNA complex in 37°C incubator with 5% $CO_2$ for 24 hr. The cells were washed once with William's E medium. Then, the fully supplemented medium was added either in the presence or absence of 1 mM PB. The cells were cultured for

another 24 hr. At the end, the cells were lysed and assayed for dual-luciferase activity.

### 2.23.3 Transfection by TransFast™ transfection reagent

**Method:**

The procedure was carried out according to Promega manufacturer's instruction. The day before transfection, the Transfast™ reagent was resuspended with 400 µl of nuclease-free water, vortexed 10 sec. and kept at –20°C. The charge ratio between lipid and DNA was 1:1 (2:1 in some cases). This required adding of 3 µl (6 µl) of Transfast™ reagent per 1 µg of DNA. The total amount of DNA is about 5 µg. The total volume of DNA, Transfast™ reagent and medium, was 2 ml per each 60 mm culture dish. The mixture was vortexed instantly and incubated at RT for 10-15 min. The culturing medium was removed from the cells before adding the mixture of lipid-DNA complexes. The cells was incubated in a 37°C incubator with 5 % $CO_2$ for 1 hr. Then, 4 ml of fully supplemented culture medium was added to the cells. They were incubated for another 24 hr before harvesting.

## 2.24 Tissues / Cell lysis

**Material:**

**1x PBS:** *8 g of NaCl, 0.2 g of KCl, 1.44 g of $Na_2HPO_4$ and 0.24 g of K $H_2PO_4$ in 800 ml of distilled water. After the pH was adjusted to 7.4 with HCl, the volume is topped up to 1 L.*

**1x PLB:** *10x Passive lysis buffer (PLB) was diluted to 1x with distilled water*

**1x RLB:** *5x Reporter lysis buffer (RLB) was diluted to 1x with distilled water*

Method:

## 2.24.1 Lysis of the whole liver tissues

The liver slices were cut using a sharp round cutter of a diameter of 8 mm at the area of bombardment by gold particles. . Generally, this was visible as a yellowish area. The sections were frozen instantly in dry ice. The tissue was ground in a mortar and transferred to an Eppendorf® tube. 1x PLB was added, ~ 1ml/1 g, of tissue sample. The tissue was homogenised using a small hand-held pestle. The homogenates were frozen at -70°C for 15 min and then thawed out. They were spun at 14,000 g for 30 sec. The supernatant was used to determine luciferase activity.

## 2.24.2 Lysis of the cultured cells

The cultured cells were washed once with 1x TBS. Then, 400 µl of 1X PLB was added to the cells in each 60-mm culture dish. The culture dishes were shaken on the orbital shaker for at least 15 min at RT. The cells were scraped from the culture dishes and the homogenate was transferred to Eppendorf® tubes. The homogenate was subjected to a freeze-thaw cycle once before it was assayed for dual-luciferase activity. In the case of the CAT assay, the cells were washed with $Mg^{2+}$ and $Ca^{2+}$-free 1xPBS and lysed with 1x reporter lysis buffer in the same manner. However, it is not necessary to freeze-thaw the sample before assay.

## 2.25 Determination of dual-luciferase activity

Dual-luciferase activity was determined by using the Dual-luciferase™ Reporter Assay System (Promega®). Luciferase Assay Reagent II (LARII) and Stop&Gloreagent were prepared according to the manufacturer's instructions (Promega®). 20 µl of the cleared tissue or cell homogenate was added to 100 µl of LARII and mixed throughly. The tube was placed in the luminometer (Turner® Design) and the reaction initiated for the first reading, which is the Firefly luciferase activity. The reading time was set at 15 sec. The second reading, Renilla luciferase activity, was obtained by adding 100 µl of the Stop&Glo reagent to quench the signal from Firefly luciferase. Then, the Renilla luciferase was simultaneously determined. The results were expressed as normalised luciferase activity in relative light units.

## 2.26 Chloramphenicol acetyl-transferase (CAT) assay

**Materials:**

**Chloramphenicol acetyl-transferase (CAT) assay kit (Promega®):**

*supplied in kit; n-Butyryl Coenzyme A, 0.25 M Tris-HCl (pH 8.0)*

**$^{14}$C-Chloramphenicol (25 µCi/ml)** (from Amersham Pharmacia Biotech®)

**Method:**

Before the assay, the cell extract was treated at 65°C for 10 min to inactivate the endogenous deacetylase activity. 100 µl of cell extract was mixed with 6 µl $^{14}$C-chloramphenicol, 5 µl n-butyryl coenzyme A and

distilled water to the volume of 125 µl. The negative control tube contained 100 µl of 1xRLB, instead of cell extract. The standard tube contained 5 µl of diluted CAT enzyme and 95 µl of 1x RLB. The reaction mixture was incubated at 37°C for 90 min. For LSC assay, 300 µl of mixed xylene was added to the reaction mixture and vortexed for 30 sec. This was centrifuged at top speed in a microcentrifuge for 3 min. The upper phase (~250 µl) was transferred to a fresh tube containing 100 µl of 0.25 M Tris-HCl (pH 8.0). This extraction step was repeated twice to achieve high sensitivity. Then, 200 µl of the upper phase was transferred to a scintillation fluid (Aquasol®-LSC cocktail, DuPont) and the sample counted in a scintillation counter. The value of the negative control was subtracted from the value of each sample.

### 2.26.1 Standard curve for CAT assay

2 µl of CAT enzyme (10 U/µl) was diluted with 998 µl of 0.25 M Tris-HCl (pH 8.0) to obtain a concentration of 0.02 U/µl. Four serial, 2-fold dilutions, of 0.02 U/µl CAT solution were made. A 5 µl aliquot from each of these serial dilutions contain 0.05 U, 0.025 U, 0.0125 U and 0.00625 U of CAT enzyme, respectively.

## 2.27 β-galactosidase assay

**Materials:**

**β-galactosidase assay kit (Promega®):** *supplied in kit; Assay 2x buffer, 1 M sodium carbonate, 5x reporter lysis buffer*

**Method:**

100 µl of cell extract was diluted with 50 µl 1x RLB. This was mixed with 150 µl Assay 2x buffer and vortexed briefly before incubation at 37°C. The incubation period was varied from 30 min to 24 hr until a faint yellow colour was developed. The reaction was stopped by adding 500 µl of 1 M sodium carbonate. The β–galactosidase activity was determined by measuring the absorbance at 420 nm, as compared to the known enzyme standard.

### 2.27.1 Standard curve for β-galactosidase assay

10 µl of β-galactosidase enzyme (10 U/µl) was diluted with 990 µl of 1x RLB (1:100 dilution). 10 µl of 1:100 enzyme dilution was diluted with 990 µl of 1x RLB to obtain 1:10000 stock dilution. The volume of 10, 20, 30, 40, 50, 60 µl of a 1:10000 stock dilution was made up to a final volume of 150 µl with 1x RLB. These dilutions contain 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 mU of β-galactosidase enzyme, respectively.

## 2.28 TnT® Coupled Reticulocyte Lysate System

Expression vectors were transcribed and translated into proteins using TnT® coupled reticulocyte lysate system (Promega®). The reaction contained TnT® rabbit reticulocyte lysate, TnT® reaction buffer, TnT® RNA polymerase, 1 mM amino acid mixture-minus leucine, amino acid mixture-minus methionine, RNasin® ribonuclease inhibitor (40 u/µl), 1 µg DNA

template (0.5 μg/μl), and nuclease-free water to a final volume of 50 μl. The reaction mixture was incubated at 30°C for 60-90 min.

# CHAPTER 3

# Results and discussion

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## 3.1 Subcloning of 5'- upstream sequences of the rat *CYP2B1* gene

Although we have known for more than 40 years that phenobarbital (PB) induces cytochrome P450 in rat liver, the DNA sequences that control a *CYP* genes response to PB have been difficult to define. The lack of suitable cell culture systems, which maintain the expression of *CYP* genes and PB-responsiveness, led to the use of cell-free systems in an attempt to determine DNA elements that regulate *CYP* genes by PB. However, a DNA element involved in the PB response, was identified only when Trottier and colleagues transfected primary rat hepatocytes with DNA reporter constructs containing various sections of the *CYP2B2* flanking sequence (Trottier *et al.*, 1995). This element, a 163 bp-*Sau3A*I fragment, is located between -2318 to -2155 upstream of the transcription start site. Trottier's finding led to the search for PB-responsive elements in other *CYP* genes. We are very interested in the control of *CYP2B1* gene expression by PB. CYP2B1 is about 5-fold more inducible by PB than is CYP2B2. Within the 5' flanking sequence of the *CYP2B1* gene, there is a region at -2301/-2142, which is very similar in sequence to the *CYP2B2* PB-responsive element. I will call this 159 bp-*Sau3A*I fragment "the 159 bp element". To subclone this fragment for use in subsequent transfection studies, I began with a

plasmid that contains the region +27 to -3878 of the *CYP2B1* gene and its flank. This plasmid was digested with *BamH*I and *Hind*III to produce the sub-fragments, -3878/-2415 (1463 bp), -2415/-1389 (1026 bp), -1389/-793 (596 bp), and -793/+27 (820 bp). To obtain the 159 bp element, the DNA 1026 bp *BamH*I/*Hind*III fragment was restricted with *Sau3A*I. This produced three fragments of 753 bp, 159 bp, and 114 bp. Since there is no *Sau 3A*I restriction site in the pUC19 vector sequence, the 159 bp fragment was subcloned into the compatible ends of the pUC19 vector linearised with *BamH* I. This clone was subsequently prepared in large scale for studies on DNA-protein interactions using the gel mobility shift assay (see section 3.3).

When the biolistic particle mediated delivery system, so called "gene gun" technique, was introduced for *in vivo CYP2B1* gene regulation studies, cloning of the 159 bp element into a reporter vector was required. To study the role of the 159 bp element as a putative enhancer sequence, it was subcloned into the pGL-3 promoter: SV40-luciferase reporter vector (see section 3.2, Figure 3.2.1). One copy of the 159 bp element was successfully subcloned into the *Bgl* II site upstream of the SV40 promoter, generating the plasmid named "159 bp (*Bgl* II)-*SV40*-Luc". Another two clones, "159 bp (*BamH* I)-*SV40*-Luc" and "159 bp (*BamH* I x2)-*SV40*-Luc", representing one and two copies of the 159 bp element, respectively, were constructed by cloning the fragment into the *BamH* I site downstream of the firefly luciferase gene. The integrity of these clones was confirmed by appropriate restriction enzyme digestion. The DNA sequence and

orientation of the clones were confirmed by the Oswel® DNA sequencing laboratory. Unexpectedly, the 159 (*BamH*Ix2)-*SV40*-Luc reporter was shown to contain an extra 114 bp DNA fragment in between the two copies of the 159 bp element. This 114 bp fragment was thought to be a contaminant from the DNA purification step on low melting temperature agarose. This contaminant, unfortunately, was subcloned along with the 159 bp element. The three clones were used for *in vivo* transfection studies in live animals, primary hepatocytes or cell lines.

## 3.2 *In vivo* transfection using biolistic particle delivery

The assessment of the functional role of a DNA element (in this case a PB-responsive element) in an *in vivo* situation, such as in the liver of an animal offers an environment in which hepatocytes interact with other liver cell types and the extracellular matrix. A few methods have been developed to study regulation of *CYP* gene expression by PB *in vivo* such as transgenic mice (Ramsden *et al.*, 1993), hepatic targeting of DNA complexed with asialoglycoproteins (Wu and Wu, 1988), and direct injection of DNA into the liver (Malone *et al.*, 1994). The production of transgenic mice, although powerful, is time-consuming and laborious. In addition, the transgene expression is variable between different lines of transgenic mice created using the same DNA construct due to position effects of transgene DNA integration into the host chromosome (Furth *et al.*, 1991). The asialoglycoprotein-DNA complex method is rather complicated. Technically, poly (L-lysine) is coupled to asialoglycoprotein, then a reporter DNA construct is added to form a soluble asialoglycoprotein-poly (L-lysine)-DNA complex. The animals are given this asialoglycoprotein-DNA complex intravenously, as it targets to the liver by binding to asialoglycoprotein receptors in the hepatocytes. The results were analysed by slot blot hybridisation of the reporter mRNA. But the responsiveness to PB was found to be relatively low (Prabhu *et al.*, 1995). Direct injection of DNA, referred to as *in situ* transient tranfection, was achieved by using a 25-gauge, 5/8-inch needle to deliver DNA into the liver lobe. Despite the

ease and rapidity, this method requires 300-500 μg DNA to be injected in each liver due to low transfection efficiency and the use of an internal control is not possible. Moreover, the injection causes acute inflammatory infiltrates, so treatment with dexamethasone is necessary before and after the injection procedure. This is a particular concern because dexamethasone has been reported to have both stimulatory and inhibitory effects on *CYP2B* gene expression by phenobarbital (Sidhu and Omiecinski, 1995). Because of the disadvantages of these methods, we decided to use the particle-mediated gene transfer technology for *in vivo* studies on *CYP2B* gene regulation. This technology has been referred to as the "gene gun" technique, the biolistic particle delivery, the ballistic method, the particle bombardment technique and the Accell. The technique, basically, uses helium pressure to accelerate DNA-coated gold particles to high speed at adequate momentum to penetrate across the membrane barriers of the targeted cells. Then, the DNA is gradually released and maintained in a free form. Other biomolecules can also be coated onto gold particles such as RNA, proteins, peptides, and inactivated virus particles. There are two types of particle-mediated gene transfer technology; in-chamber type and hand-held type. Initially, a gunpowder discharge to impart momentum to the projectiles was used and the cells were placed in a vacuum chamber in order to minimise air impedance of the particle velocity. This is the in-chamber type of particle delivery. Later on, the hand-held type delivery method was developed, which is easy to use and can be applied to the organs of live animals. The evidences for gene transfer into internal organs using gene gun technology have been

reported in liver, pancreas, spleen, and kidney (Cheng *et al.*, 1993). This technique was first employed to transform onion cells in culture and was later introduced to use in mammalian somatic tissues, adherent and suspension cell culture, cell lines and organs in live animals. The reason for the broad application of this method to any type of cells, tissues, and organs is the way the gene is delivered. Delivery is independent of the presence of specific ligand receptors, biochemical features, or structural components on the surface of the targeted cells (Yang *et al.*, 1997). In contrast to other gene transfer methods that involve endocytosis or receptor-mediated uptake, a gene gun technique provides the instant uptake of DNA into the cells. Thus, transgene expression can be monitored within several hours after gene transfer. In order to obtain optimal gene expression, parameters such as the particle density, particle size, DNA dosage or DNA loading rate and discharge pressure should be balanced between effective transfection and minimal cell damage.

### 3.2.1 Reporter constructs used for *in vivo* transfection

The luciferase reporter gene system, pGL3 vector series (Promega®), was employed in this study. The vectors contain a cDNA encoding luciferase (*luc*+) cloned from the North American firefly (*Photinus pyralis*). The pGL3 vector series include pGL3-Promoter, pGL3-Control, and pGL3-Basic (Figure 3.2.1). The pGL3-Control, which contains the SV40 promoter and the SV40 enhancer sequences, results in strong expression of the firefly

luciferase gene. This plasmid was used as a positive control. The pGL3-Basic which contains neither promoter nor enhancer sequences was used as a negative control. The pGL3-Promoter contains an SV40 promoter upstream of the firefly luciferase gene. Insertion of a DNA element with putative enhancer activity can thus be analysed using this plasmid. The 5'-upstream region, -2301/-2142 (159 bp), of *CYP2B1* was subcloned into pGL3-Promoter either upstream, at the *Bgl* II site, or downstream, at the *BamH* I site of the firefly luciferase gene (Referred to in section 3.1). This is called the "experimental reporter vector". As an internal control, the control reporter vector, pRL-TK, was co-transfected with the experimental reporter vector so that the results could be normalised. The pRL-TK contains the herpes simplex virus thymidine kinase (HSV-TK) promoter upstream of the Renilla luciferase gene (R*luc*). All these constructs were amplified in the bacteria *E. Coli* JM 109. The purity of DNA was determined by the ratio of absorbance at 260 to 280 nm. A ratio of $\geq$ 1.8 was acceptable. The integrity of reporter vectors /reporter constructs was verified by electrophoresis on a 0.8% agarose gel. More than 90% of the DNA had to be in the supercoiled form for the DNA to be suitable for transfection, as the supercoiled DNA is transcribed more efficiently than its linear form (Weintraub *et al.*, 1986). The orientation of DNA insert was confirmed by the Oswel® DNA sequencing laboratory.

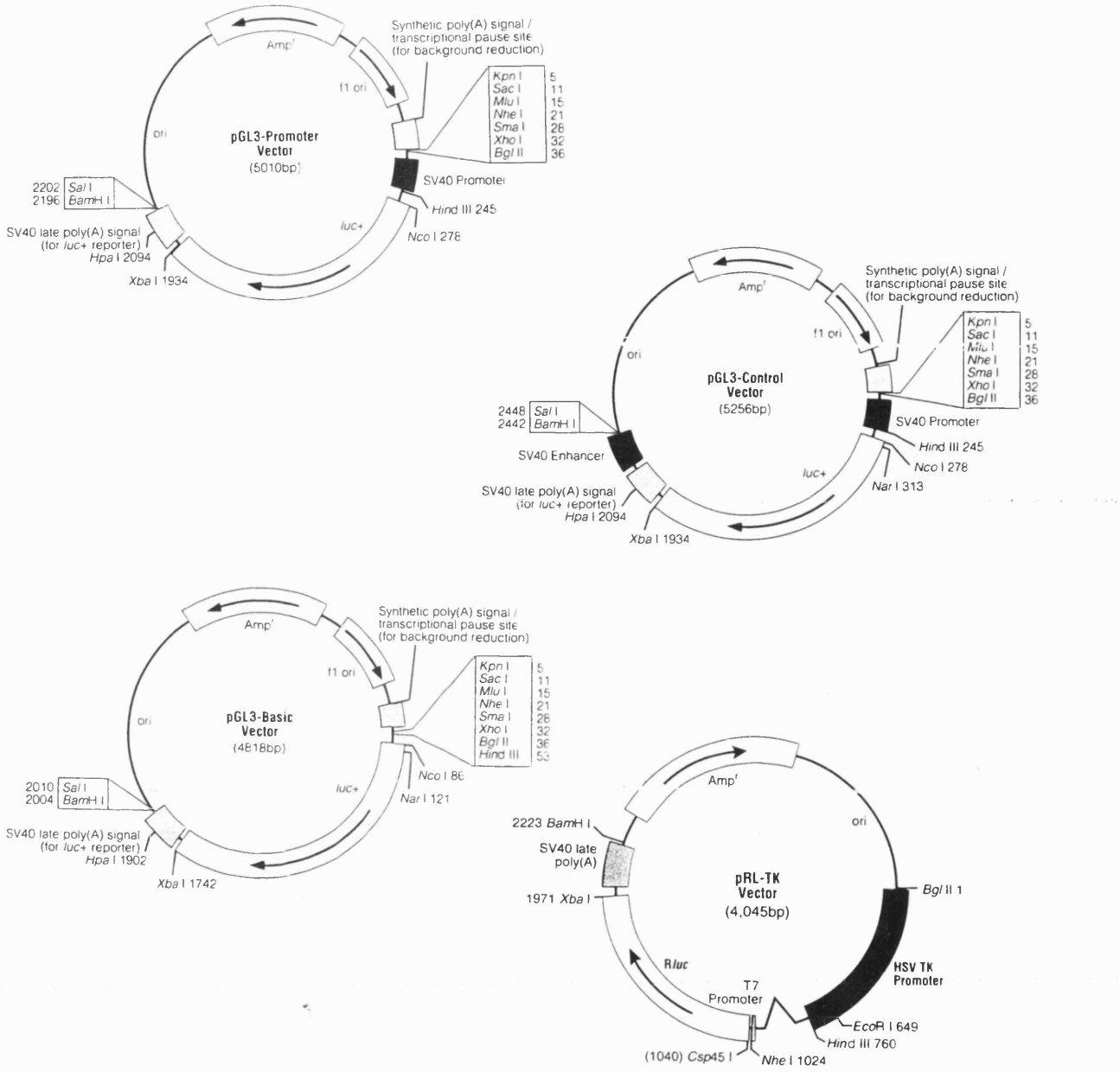



Figure 3.2.1 **Circular maps of the pGL3-Promoter, pGL-3 Control, pGL3-Basic and pRL-TK reporter vectors.** Unique restriction sites are shown. Arrows indicate the direction of transcription. Abbreviations used are as follows; Luc+ = cDNA encoding the modified firefly luciferase, Rluc = cDNA encoding renilla luciferase, Amp$^r$ = ampicillin resistance gene (Adapted from Promega's transfection guide)

### 3.2.2 The -2301/-2142 (159 bp) region of the rat *CYP2B1* gene confers PB responsiveness *in vivo*

In this study, the hand-held Helios gene gun system (Bio-Rad) was used to deliver DNA into the liver of live Sprague-Dawley rats. Initially, the He pressure used for particle delivery was optimised by varying the pressure from 150-300 psi (1 psi = 6890 Pa) and determining the luciferase activity of the pGL-3 control at 24 hr. At 250 psi, the pGL3-control expressed a high level of luciferase activity ~400-600 fold of that of the pGL3-Basic, which gave an almost undetectable value. In addition, no damage of the liver tissue was observed and the area of bombardment was noticeably yellowish from the gold particles. A pressure lower than 250 psi resulted in low luciferase activity. When the He pressure was increased up to 300 psi, haemorrhage and fissure of the liver occurred. So 250 psi of He pressure was chosen for this study. This was the same pressure as reported by Yoshida *et al* (Yoshida *et al.*, 1997). In addition, these workers found the depth of particle penetration was about 2 mm from the liver surface, and the heaviest bombardment area was about 8x8 mm. In the work described in this thesis, each tissue sample was collected by using a cutting tool of the size, ~8x8x2 mm. An area of the same size, that was not shot, was collected for the luminescence reading background.

As described in section 3.2.1, the -2301/-2142 (159 bp) DNA fragment was subcloned into the reporter vector so that the DNA element's ability to drive the expression of the reporter gene could be monitored. The transient activity level of the reporter protein is, in turn, a measure of the role of the DNA element in gene activation. Simultaneous transfection of the experimental vector with a control vector, as mentioned previously, is essential to minimise the variations caused by the differences in transfection efficiency, cell lysis efficiency, assay efficiency either within or between experiments (Promega's note). The experimental reporter vector and control vector, in a ratio 10 to 1, were co-precipitated onto the surface of gold particles in the presence of the polycation spermidine and $CaCl_2$, so that they were delivered simultaneously into the cells. In some cases, when the co-transfection of several expression vectors is required, the co-precipitation of multiple DNA constructs onto the gold particles should be adjusted properly. For example, in cryopreserved CHO cells, the effect of DNA dosage and particle number on transfection was investigated. The maximal expression was observed when 1-5 μg of DNA/mg gold particles was used. If the DNA exceeded 5 μg/mg gold particles, it caused clumping, which is not suitable for cartridge preparation (Yang *et al.*, 1997). In this study, one shot delivered approximately 2 μg of total DNA and 0.5 mg of gold particles into the cells. As the cells are transiently transfected, the DNA does not integrate into the chromosome. Many copies of the transfected gene are present, leading to high levels of expressed protein as compared to stable transfection.

After the introduction of DNA into the cells, the expression of a reporter gene generally can be analysed within 24-96 hr, depending on the DNA construct used. A 48 hr post-transfection was investigated for the luciferase level compared to a 24 hr post-transfection using the pGL3-control reporter vector. The luciferase activity of the pGL3-control reporter vector at 48 hr was much lower than at 24 hr (Figure 3.2.2). The evidences that PB rapidly induces *CYP2B* expression within 30-60 min and reaches the peak level within a few hours (Atchison and Adesnik, 1983) subsequently led us to the examination of luciferase activity of the 159 bp-*SV40*-Luc reporter and PB-responsiveness at 6 hr. Results are shown in Figure 3.2.3. The luciferase activity was high at 6 hr in untreated and PB-treated animals. However, low PB induction was observed (~ 2 fold). At 24 hr, the activity of luciferase was decreased in both untreated and PB-treated animals. Luciferase has a half-life of 3 hr, so by 24 hr the level of reporter protein declines in both untreated and PB-treated animals. But higher PB induction was obtained (~ 6 fold) because the effect of PB may reach its peak at 24 hr. Northern blot analysis was also carried out to see the effect of PB on endogenous CYP2B mRNA at 6 hr and 24 hr. The probe used was the plasmid construct p4G12 containing a 1400 bp fragment of the CYP2B2 cDNA in the pAT153 vector. This probe recognises both CYP2B1 and CYP2B2 mRNA (Phillips *et al.*, 1983). As shown in Figure 3.2.3, endogenous CYP2B1 mRNA upon PB treatment at 6 hr was increased, but was not as high as the mRNA level at 24 hr. Thus, PB induces accumulative endogenous CYP2B mRNA to a high level at 24 hr. Noticeably, the amount of the mRNA was unusually high in the liver of

the untreated animals examined 6 hr-post DNA delivery. This, in turn resulted in only 2-fold increase upon PB treatment at 6 hr. This was found to be due to the induction of CYP2B1 by the anaesthetics used in the operative procedure. However, by 24 hr the effects of the anaesthetics on CYP2B1 mRNA were not detectable. This may also explain the high basal level of reporter gene activity at 6 hr in untreated animals if the anaesthetics stimulate reporter gene expression via the 159 bp element.

The high basal expression of luciferase in the untreated animals could be due to the strong SV40 promoter, which masks the fold of PB-induction. It was therefore decided to examine PB induction using a construct containing the minimal *CYP2B1* promoter. The minimal *CYP2B1* promoter, 180 bp (-179/ +1) and the 159 bp DNA fragment were cloned into pGL3-Basic at *Hind* III/*Sma*I and *Xba*I/*Sal*I, respectively. Although the basal level of luciferase expression was much lower than found with the heterologous SV40 promoter, the PB induction at 6 hr was not increased. In addition, the absolute value of luciferase activity was low at both 6 hr and 24 hr when the minimal *CYP2B1* promoter was used (Figure 3.2.4). Subsequently, the 159 bp-*SV40*-luciferase reporter, containing a heterologous SV40 promoter was preferentially used over the construct, which contained the *CYP2B1* minimal promoter. The optimal time point to assess luciferase activity was chosen at 24 hr after transfection.

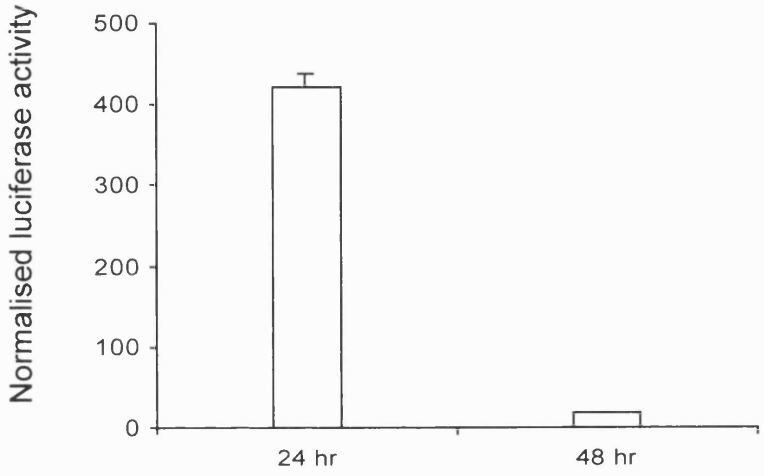



Figure 3.2.2 **24 hr post-transfection is an appropriate time to monitor gene expression.** The pGL3-Control was delivered into the liver of SD rats together with the pRL-TK plasmid by the hand-held gene gun under the He pressure of 250 psi. The liver slices were collected at 24 hr and 48 hr to assay for luciferase activity. The results are the normalised luciferase activity over the control plasmid; pRL-TK, from duplicates of three independent experiments. S.D. bars are shown.

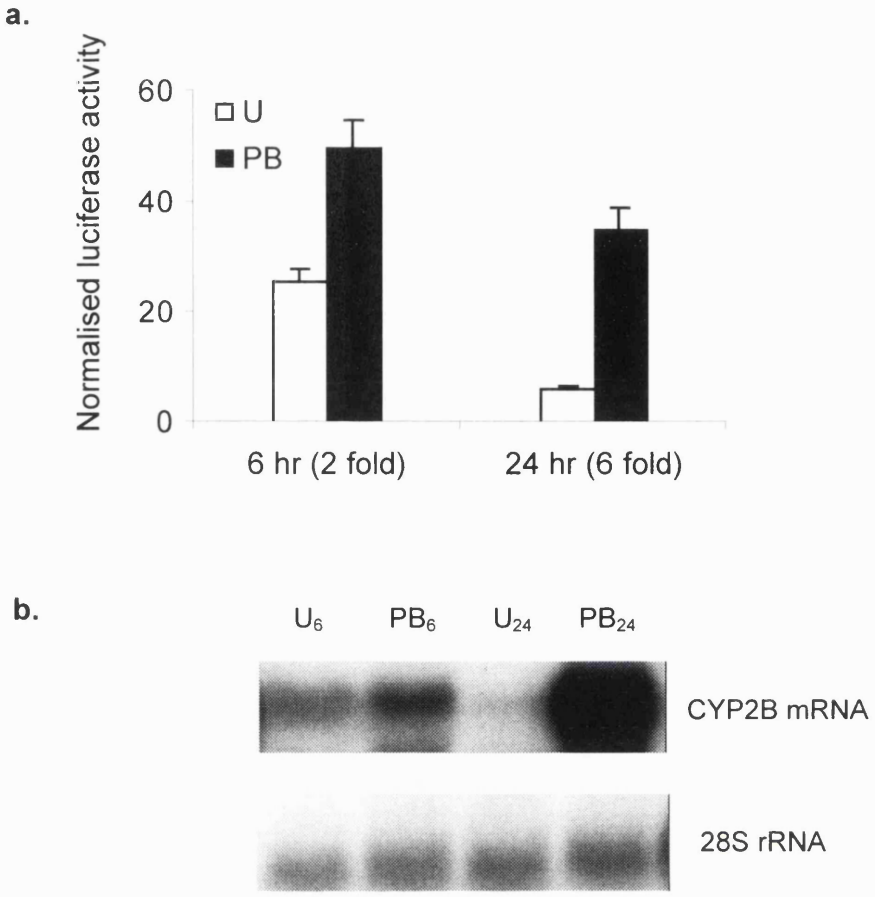



Figure 3.2.3 **The 159 bp DNA element confers PB-responsiveness *in vivo* and the fold PB induction is optimal at 24 hr.** (a) A 159 bp (*BamH* I)-*SV40*-luc reporter plasmid was co-transfected with the pRL-TK plasmid (ratio 10:1), using a hand-held gene gun, into the liver of SD rats. In the PB-treated group (PB), the animals were injected i.p. with phenobarbital (100 mg/kg body weight) shortly after the operation. The shot liver slices were taken at 6 hr and 24 hr post-transfection to assay for luciferase activity. The results represent normalised luciferase activity ± S.D., over the control plasmid; pRL-TK from duplicates of three independent experiments. (b) Northern blot analysis from the corresponding animals was carried out using a CYP2B cDNA probe and the results were normalised with a 28S rRNA oligonucleotide probe.

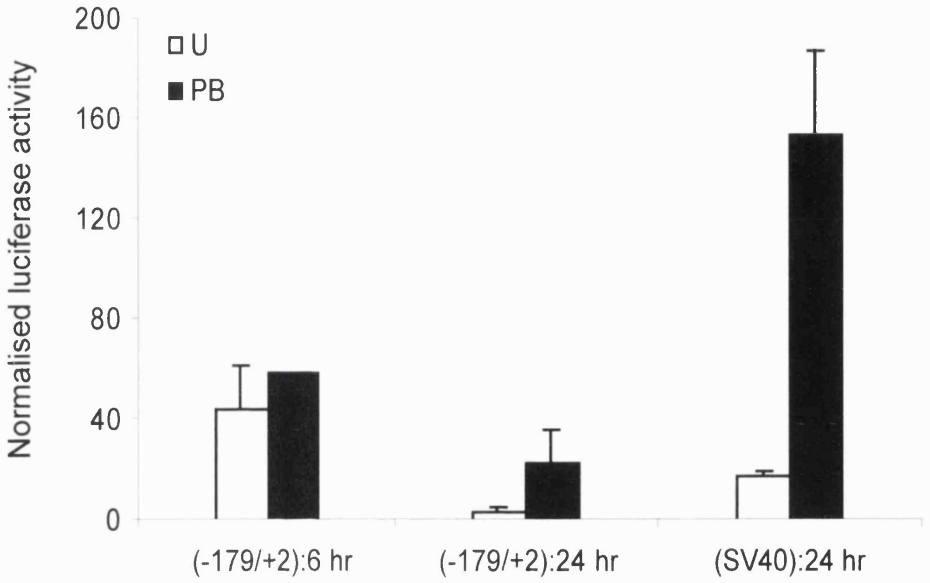



Figure 3.2.4 **The *CYP2B1* minimal promoter exhibits lower basal expression than a heterologous SV40 promoter.** The pGL3-Basic reporter construct harbouring the 159 bp element and a *CYP2B1* minimal promoter (-179/+2) was used to compare the level of a reporter expression with that driven by a SV40 promoter (159 bp (*Bam HI*)-*SV40*-Luc) using a hand-held gene gun. In the PB-treated group, the rats were injected with phenobarbital (100 mg/kg body weight) i.p. shortly after operation. The shot liver slices were assayed for luciferase activity at 6 hr and 24 hr, as indicated. The results represent the normalised luciferase activity ± S.D., over the control vector; pRL-TK.

The results represent triplicates of two independent experiments.

The luciferase system is 10-1000 fold more sensitive than the CAT assay and has a larger range of linearity (greater than four orders of magnitude). Although the luciferase activity tends to be much more variable than CAT activity because of its differential degradation during sample preparation, it offers more advantages, regarding ease, rapidity and minimal background activity.

In untreated animals, the 159 bp (-2301/-2142) *CYP2B1* DNA element, linked to the SV40 promoter, expressed basal levels of luciferase activity at about the same as that of pGL3-Promoter. A 5-8 fold induction of luciferase activity was observed when the animals were treated with phenobarbital (PB). This result shows that the 159 bp DNA element, confers PB responsiveness on the reporter gene. In addition, the 159 bp DNA element that was cloned either upstream or downstream of the SV40 promoter conferred similar PB responsiveness on the luciferase reporter gene (Figure 3.2.5). This suggests that the 159 bp element is an enhancer as it functions in an orientation independent manner. Notably, the 159 bp (*Bgl*II)-*SV40*-Luc reporter, in which the 159 bp element was cloned upstream, next to the SV40 promoter showed higher luciferase activity than the 159 bp (*BamH*I)-*SV40*-Luc reporter, in which the 159 bp was cloned downstream, far apart from the SV40 promoter. In this case, the distance between the PB-responsive element and SV40 promoter may play a role. In all subsequent experiments, the 159 bp (*Bgl*II)-*SV40*-Luc was used, and referred to as *CYP2B1* PBRE-*SV40*-Luc reporter. When the plasmid containing two copies of the 159 bp DNA element, 159 bp

(*BamH*Ix2), was used, the PB-responsiveness was not as high as expected (Figure 3.2.5). This may be due to the extra 114 bp DNA sequence inadvertently cloned in between the two copies of 159 bp element, which may negatively influence protein binding and hence gene activation.

In parallel experiments, the liver slices of non-shot areas of untreated and PB-treated animals were collected for northern blot analysis of endogenous CYP2B mRNA. The PB-treated animals showed an enormous increase in *CYP2B* mRNA, whereas in untreated animals the mRNA was almost undetectable. This confirmed the effects of PB treatment on both endogenous CYP2B and reporter gene expression.

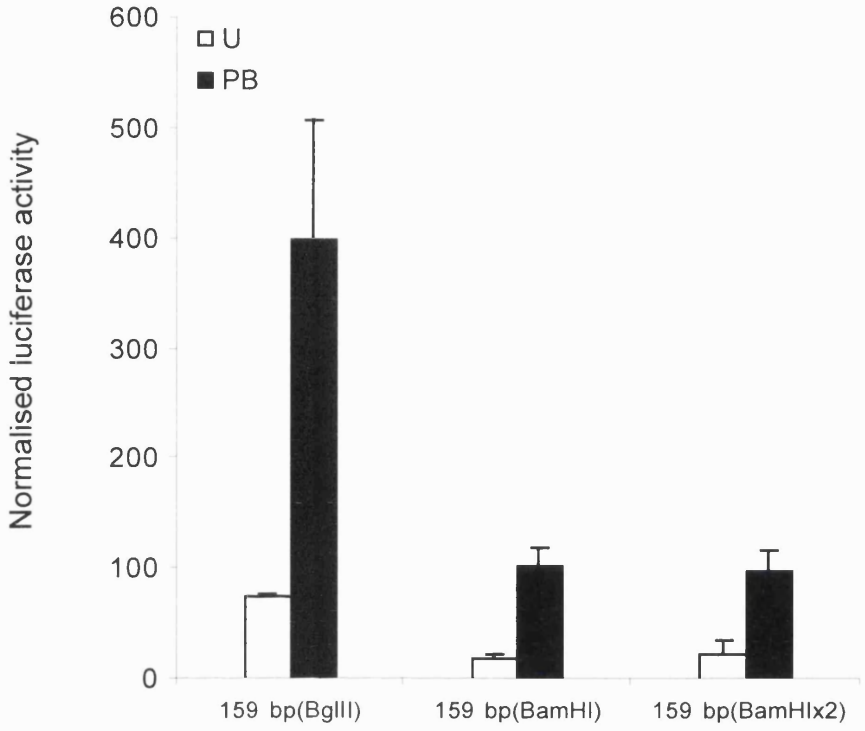



Figure 3.2.5 **The 159 bp (*Bgl* II)-*SV40*-Luc and 159 bp (*BamH* I)-*SV40*-Luc confer similar PB responsiveness, but exhibit luciferase activity to different extents.** The respective reporter constructs, 159 bp (*Bgl* II)-*SV40*-Luc, 159 bp (*BamH* I)-*SV40*-Luc, and 159 bp (*BamH* I x2)-*SV40*-Luc were delivered into the liver of live SD rats using a hand-held gene gun. In the PB-treated group, the animals were injected with phenobarbital (100 mg/kg body weight) shortly after operation. The shot liver slices were assayed for luciferase activity at 24 hr. The results represent normalised luciferase activity ± S.D. over the control vector, pRL-TK.

The results represent duplicates of three independent experiments.

## 3.3 Studies on protein-DNA interactions using the gel mobility shift assay

Protein regulatory factors interact with sequence-specific DNA elements to control the constitutive, inducible and tissue-specific gene expression in various cell types (Maniatis *et al.*, 1987; Mitchell and Tjian, 1989). The identification and characterisation of such DNA-binding regulatory factors has been greatly facilitated by the "gel mobility shift assay" or "gel retardation assay" (Fried and Crothers, 1981). This technique has also been applied to the purification of numerous transcription factors and mapping of the binding sites within target DNA sequence elements. Eventually cloning of several regulatory factors has been achieved (Singh *et al.*, 1989). The technique is based on the simple observation that the movement of a DNA molecule through a non-denaturing polyacrylamide or agarose gel is hindered when a protein molecule is bound to it. A specific protein-DNA complex is thus visualised by autoradiography migrating more slowly than does the free DNA fragment. DNA fragments with protruding ends are labelled with $\alpha$-$^{32}$P-dCTP using the Klenow fragment of DNA polymerase I, whereas blunt-ended DNA is labelled with $\gamma$-$^{32}$P-ATP using T4 polynucleotide kinase. The specific activity of the probe is measured. The DNA probe is diluted with distilled water and used at 3-4 x $10^4$ cpm/µl in each reaction. The source of proteins used included liver nuclear extracts from untreated and PB-treated rats, those from *in vitro* synthesised

protein products and nuclear and cytosolic extracts from rat primary hepatocytes and cell lines. The protein content was quantified by the method of Lowry. The integrity of proteins was verified by electrophoresis through SDS-polyacrylamide gels. Discrete multiple bands were observed (result not shown). The amount of nuclear extract used in the gel mobility shift assay was 3-10 μg in each reaction. When the *in vitro* TNT® Coupled reticulocyte lysate system was used, 1-3 μl of *in vitro* translated products was included in the reaction mix. Proteins can interact non-specifically with DNA, the addition of an excess non-specific DNA such as a synthetic copolymer; poly (dI.dC) can overcome this problem. However, the band intensity of the specific complex can be reduced if too much non-specific DNA is added. The length of DNA fragments used in the binding reaction can be varied from several hundred base pairs to short oligonucleotides. However, the results can be complex if long DNA fragments, which contain multiple protein binding sites, are used. So synthetic oligonucleotides, which span one or two protein-binding sites, are more commonly used. Competition assays, with homologous and heterologous oligonucleotides, provides information about the sequence-specificity of any DNA-protein interactions. The use of mutated oligonucleotides as competitors may identify the particular residue, which contributes to the protein binding potential. In some cases, antibodies were used to investigate the particular protein involved in a DNA-protein interaction. The antibody recognises and binds to the DNA-binding protein resulting in the supershift of the protein-DNA complexes. However, this is not true in all cases since the binding of antibody may mask the DNA-binding site and preclude the formation of

protein-DNA complexes. Lastly, a low ionic strength buffer such as 0.5 M TBE is preferred since it generates less heat, increases the speed of migration and reduces the disruption of the ionic bonds and so stabilises the complexes during their transfer from the salt-containing binding buffer into the gel.

As described in section 3.2, the 159 bp element has been shown to confer PB responsiveness *in vivo*, so the next question to be asked was which proteins bind to this DNA element and regulate *CYP2B1* gene expression in response to PB treatment. The gel mobility shift assay was introduced to investigate the pattern of protein-DNA complexes and to localise where specific proteins bind within the 159 bp DNA sequence. Competition and supershift assays were introduced to find out the identity of the proteins. Subsequently, when the DNA binding sites had been dissected, the *in vitro* synthesised proteins from an *in vitro* transcription /translation system, were required for demonstration of specific protein-DNA interactions.

### 3.3.1 Rat liver nuclear protein extracts bind to the 159 bp element (-2301/-2142) of the *CYP2B1* 5' flanking sequence and form multiple complexes

The 159 bp (-2301/-2142) element was cleaved from the vector pUC19 (2686 bp) using *Xba* I and *Ava* I, these sites lie within the pUC 19 multiple cloning site. *Sau 3A*I and *Bam H* I sites, originally used to insert the fragment, were destroyed during the cloning procedure. The fragment obtained was thus 11 bp longer than the original sequence, resulting in a 170 bp fragment. This fragment was radiolabelled with $\alpha$-$^{32}$P-dCTP and used as a probe in the gel mobility shift assay with rat liver nuclear extracts. The results are shown in Figure 3.3.1. The pattern of protein-DNA complexes obtained with liver nuclear extract from untreated or PB-treated rats are identical. Several proteins bind to the DNA probe and produce a smeared pattern, suggesting there are binding sites within this region for several proteins. No increase in abundance of protein binding is observed with nuclear extracts from PB-treated animals as compared to that from untreated animals. This may be indicative of pre-existing DNA-binding factors in the untreated condition, which *in vivo* are modified or activated in response to PB. Nevertheless, any changes in binding as a consequence of PB treatment are not detectable in this experiment. Proteinase K addition, after completion of the binding reaction, was used to verify if the complexes formed were the result of protein(s) bound to DNA. The complexes disappeared since the proteins were digested with proteinase

K. A reaction containing only a labelled DNA probe, without adding any nuclear extract, was run along side each experiment as a negative control. When a 100 molar excess of unlabelled DNA probe was included as a self-competitor, the protein-DNA complexes were inhibited, suggesting specific binding.

To see whether the phosphorylation status of the proteins might affect the protein-DNA complexes, treatment of nuclear extract with either 1 mM ATP or 10 units of alkaline phosphatase was done before adding the labelled DNA probe. There was no change in the intensity of the protein-DNA complexes (results not shown). This may indicate that phosphorylation status is not involved in this binding. Nevertheless, since several proteins bind to this DNA region, the effect of ATP or phosphatase on a particular protein might be masked. It is also possible that a kinase involved in phosphorylation events is absent from the nuclear extract. However, in primary hepatocytes treated with PB for various time intervals from 30 sec to 48 hr, no increase in cAMP level and PKA activity was observed, which indicated that the phosphorylation events are not involved in the PB induction response (Beck and Omiecinski, 1999).

| Probe | -2301/-2142 (159 bp) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Nuclear extracts | U | PB | U | PB | U | PB | U | - |
| Amount (µg) | 2.5 | | 5.0 | | | | | - |
| Self competitor | - | | | | 100 M excess | | - | |
| Proteinase K | - | | | | | | 5 µg | - |
| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |

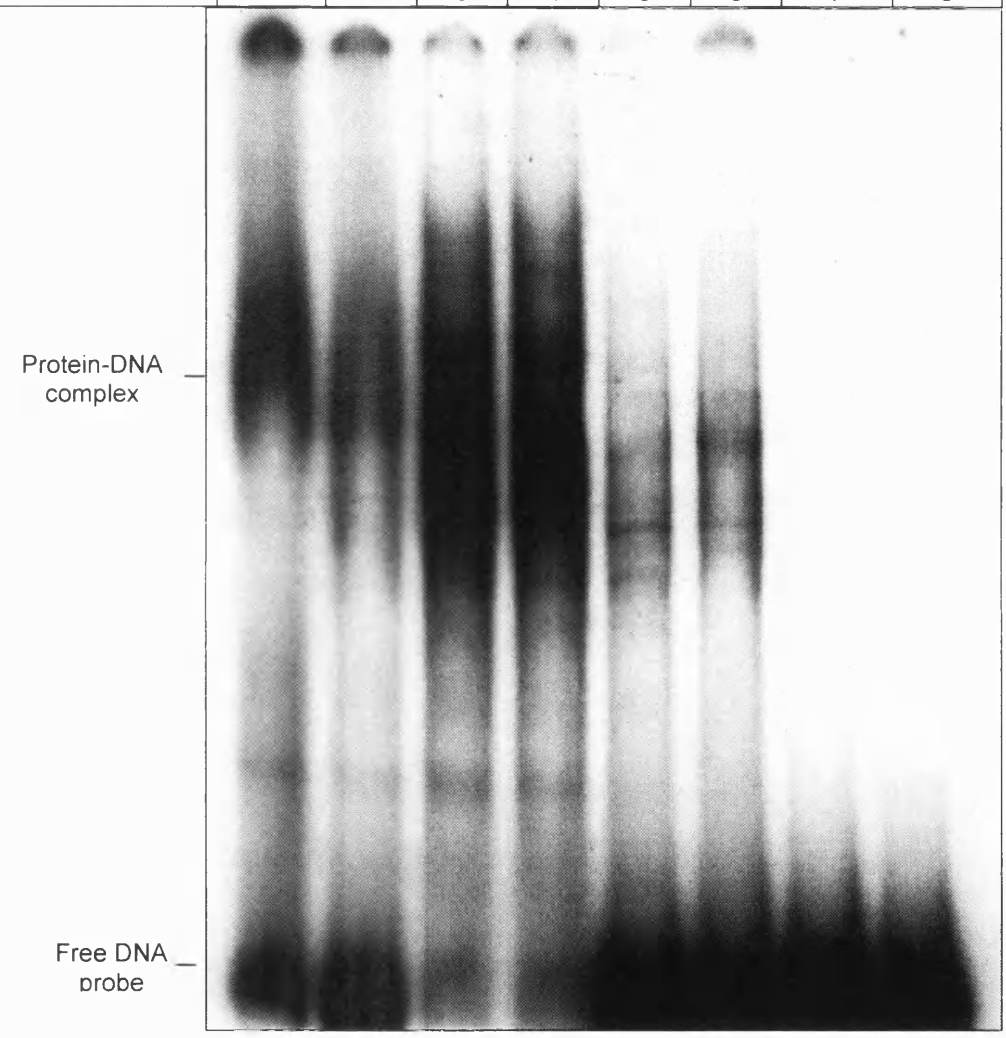
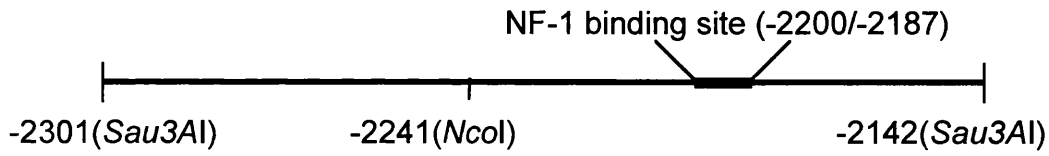



Figure 3.3.1 **Multiple proteins bind to the *CYP2B1* -2301/-2142 (159 bp) region.** Gel mobility shift assay of liver nuclear extract from untreated (U) and phenobarbital-treated (PB) rats and a radiolabelled 159 bp DNA. Lane 1 and 2 = 2.5 µg of nuclear extract, lane 3-7 = 5 µg of nuclear extract, lane 5 and 6 = 100 molar excess of unlabelled 159 bp self-competitor DNA, lane 7 = proteinase K was added after the binding reaction, lane 8 = free DNA probe

## 3.2.2 Nuclear protein binding is localised within the -2214/-2142 (72 bp) region of the 159 bp element

To specify the region within -2301 to -2142 to which proteins bind, the 159 bp element was digested with *Nco* I to produce sub-fragments as follows;




*Nco*I : -2301/2241 (60 bp) and -2241/-2142 (99 bp)

The resulting sub-fragments; -2301/-2241 (60 bp) and –2241/-2142 (99 bp), were used as competitors for the 159 bp DNA fragment in a gel mobility shift assay. The -2241/-2142 (99 bp), at 100 molar excess, is able to compete off the complexes, while the -2301/-2241 (60 bp) can not compete (Figure 3.3.2). Therefore, nuclear protein binding sites are located within the -2241/-2142 region rather than the -2301/-2241 region of the *CYP2B1* 5' flanking sequence. A potential binding site for nuclear factor 1 (NF-1) is located between -2200 to -2188. A NF-1 oligonucleotide specifying the NF-1 binding site was used as a competitor in the assay. At 100 molar excess, NF-1 is able to compete all the complexes away. This indicates that NF-1 is one of the proteins able to bind to this DNA element (Figure 3.3.2, lane 9-10).

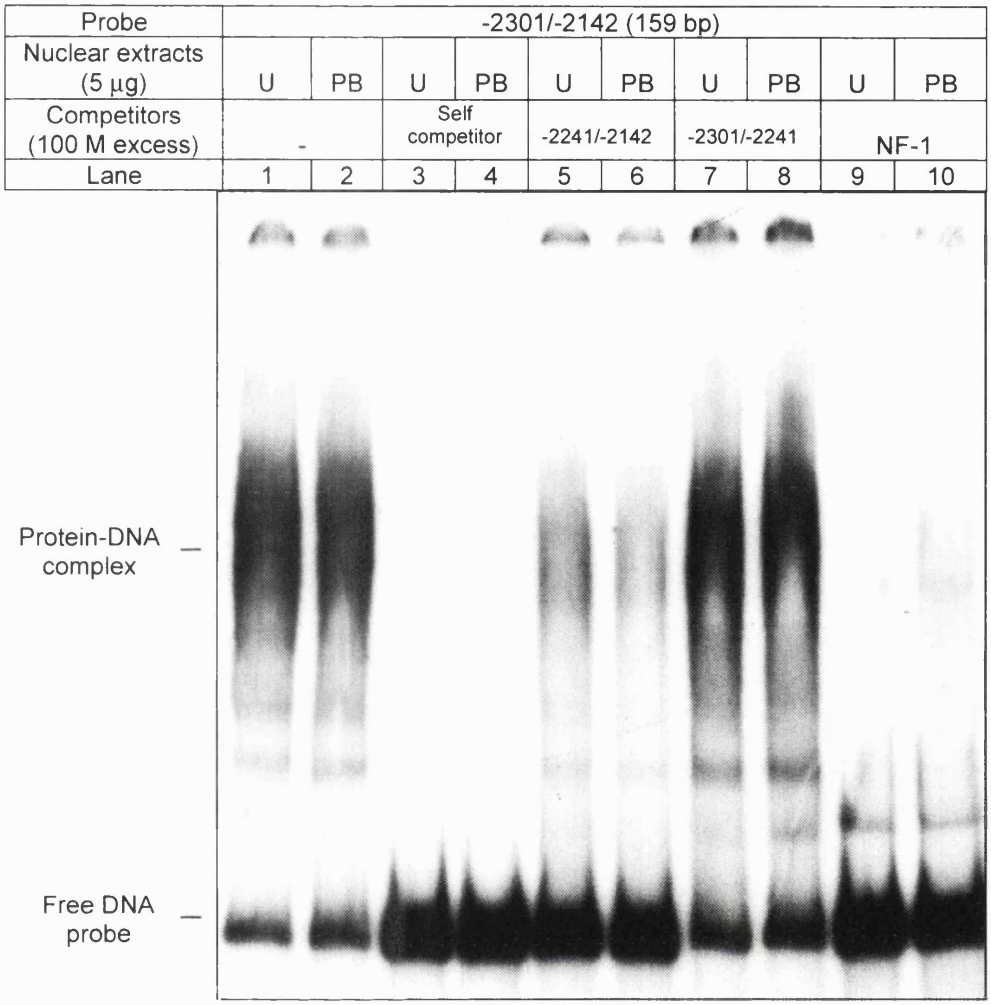
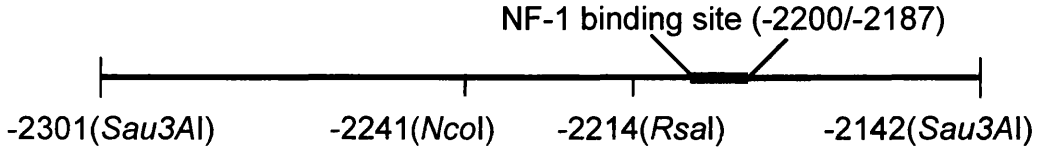



Figure 3.3.2 **Nuclear factor 1 (NF-1) can bind within the -2241/-2142 region.** A gel mobility shift assay was performed with liver nuclear extracts of untreated (U) and phenobarbital-treated (PB) rats. 100-fold molar excess of the following unlabelled DNA fragments were included as competitors; 159 bp (-2301/-2142) (lane 3 and 4), 99 bp (-2241/ -2142) (lane 5 and 6), 60 bp (-2301/ -2241) (lane 7 and 8), NF-1 consensus oligonucleotide (lane 9 and 10).

To further refine the region responsible for protein binding within the 159 bp element, the fragment was digested with *Rsa* I restriction enzyme to produce sub-fragments of 87 and 72 bp as shown below;




*Rsa*I : -2301/-2214 (87 bp) and –2214/-2142 (72 bp)

The 72 bp (-2214/-2142) fragment is able to compete the protein-DNA complexes formed by the whole 159 bp (-2301/-2142) fragment (Figure 3.3.3, lane 5-6). In contrast, when a 100 molar excess of the 87 bp (-2301/-2214) fragment was used as competitor, the protein-DNA complexes of the whole 159 bp (-2301/-2142) were not inhibited (Figure 3.3.3, lane 7-8). These results indicate that nuclear proteins bind within the -2214 to -2142 region of the 159 bp element.

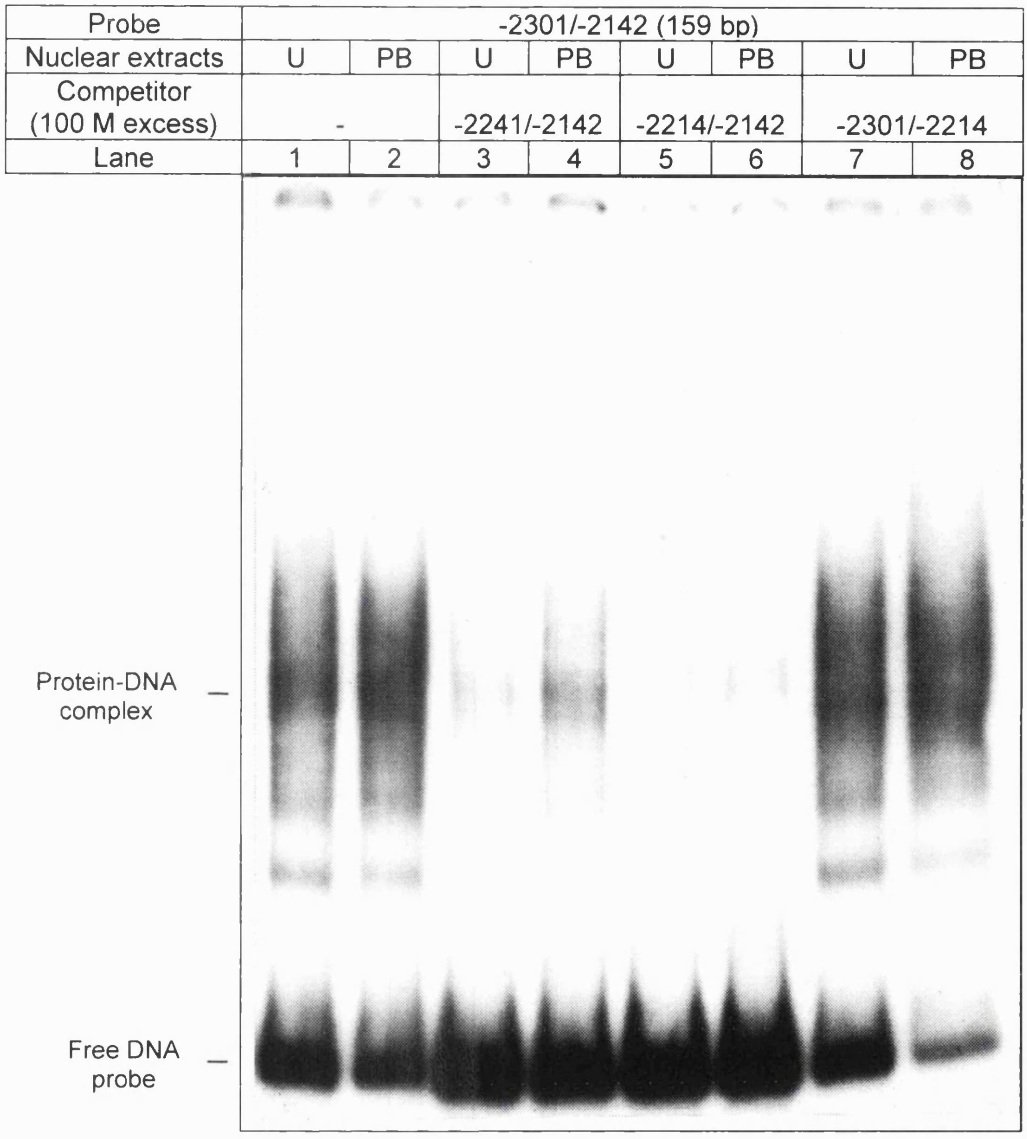



Figure 3.3.3 **The protein binding site within the -2301/-2142 (159 bp) region are localised within -2214/-2142 (72 bp).** A gel mobility shift assay was performed with liver nuclear extract from untreated (U) and phenobarbital-treated (PB) animals and the radiolabelled 159 bp DNA element. The competition assay was carried out with a 100-fold molar excess of unlabelled 99 bp (-2241/-2142) fragment (lanes 3 and 4), 100 molar excess of unlabelled 72 bp (-2214/-2142) fragment (lanes 5 and 6), 100 molar excess of unlabelled 87 bp (-2301/-2214) fragment (lanes 7 and 8).

The pattern of protein-DNA complexes observed when liver nuclear extracts from either untreated or PB-treated animals bind to the -2214/-2142 (72 bp) fragment is indistinct and smeared as is the case with the complexes formed on the 159 bp element. No increase in protein binding with nuclear extract from PB-treated rats was observed compared to extracts from untreated animal. When a 100 molar excess of unlabelled -2214/-2142 (72 bp) was included as a self-competitor, it was able to compete the complex(es) away. The same result was obtained when a 100 molar excess of the -2241/-2142 (99 bp) and -2301/-2142 (159 bp) fragment, and a NF-1 oligonucleotide, were used as competitors (Figure 3.3.4). Notably, the NF-1 oligonucleotide completely inhibited the protein-DNA complex(es) formed either on the labelled 159 bp element or 72 bp element. This might suggest that NF-1 is needed to stabilise the complexes formed by the additional, unidentified proteins. These results show that liver nuclear proteins from both untreated and PB-treated animals are able to bind to sequences within the -2214 to -2142 (72 bp) sequence of the *CYP2B1* 5' flank. The identification of other proteins that bind to this DNA sequence is described in the next section.

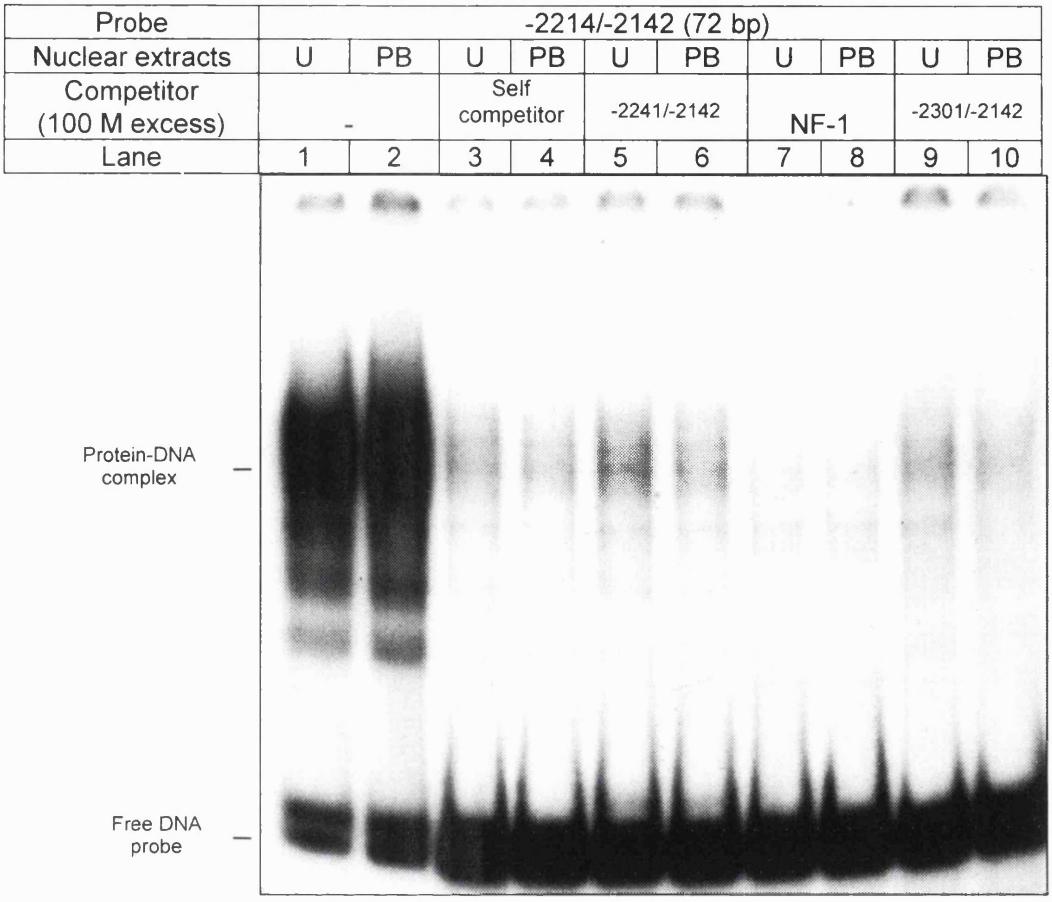



Figure 3.3.4 **Protein complexes on the *CYP2B1* 72 bp element.** A gel mobility shift assay was performed with liver nuclear extracts from untreated (U) and phenobarbital (PB)-treated animals and a radiolabelled -2214/-2142 (72 bp) DNA fragment. The competition assay was carried out with a 100 molar excess of unlabelled fragments of -2214/-2142 (lane 3 and 4), -2241/-2141 (99 bp) (lane 5 and 6), NF-1oligonucleotide (lane 7 and 8), and -2301/-2142 (159 bp) (lane 9 and 10).

## 3.4 The search for nuclear proteins that bind to the *CYP2B1* PB-responsive element

### 3.4.1 Purification of DNA-binding proteins using magnetic DNA-affinity beads

The approach that I took to search for the regulatory proteins that bind to the *CYP2B1* PB-responsive element, was to use magnetic DNA-affinity beads. I began by ligating a 159 bp sequence to a biotinylated tether DNA. The use of tether DNA, which incorporates a restriction site at its end, provides the means to analyse the bound protein using a gel mobility shift assay and following elution from the DNA, analysis by SDS-PAGE (as shown in Figure 3.4.1.). The biotin containing ligated DNA binds to streptavidin-coated beads. The nuclear extracts are allowed to bind to the DNA under conditions similar to those used in the gel shift assay. After washing off non-specific binding proteins, the purified proteins are either released from the DNA by elution or by restriction digestion to detach the bound protein-DNA complex. The proteins are eluted under the unfavourable conditions of low pH or high salt buffer.

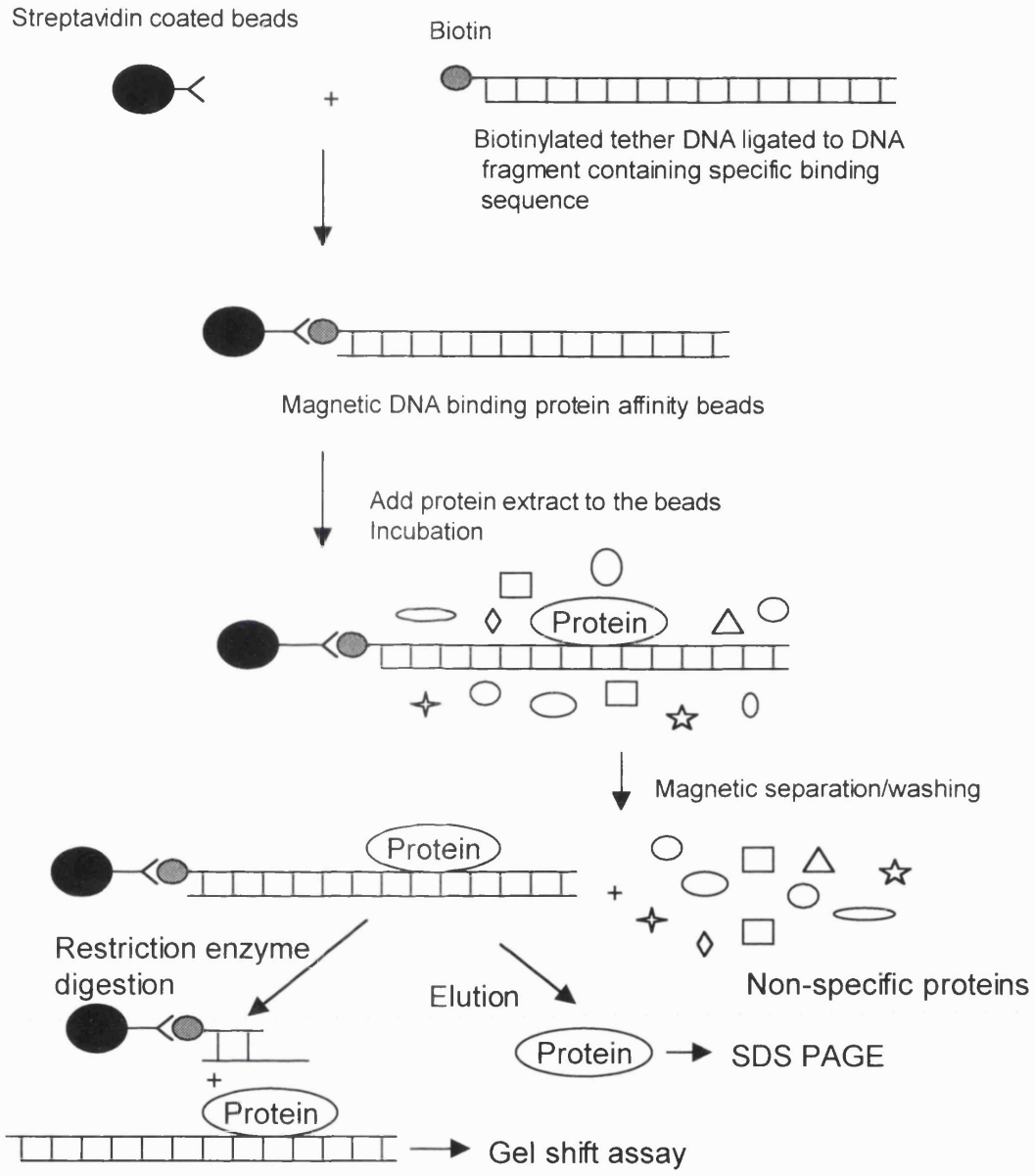


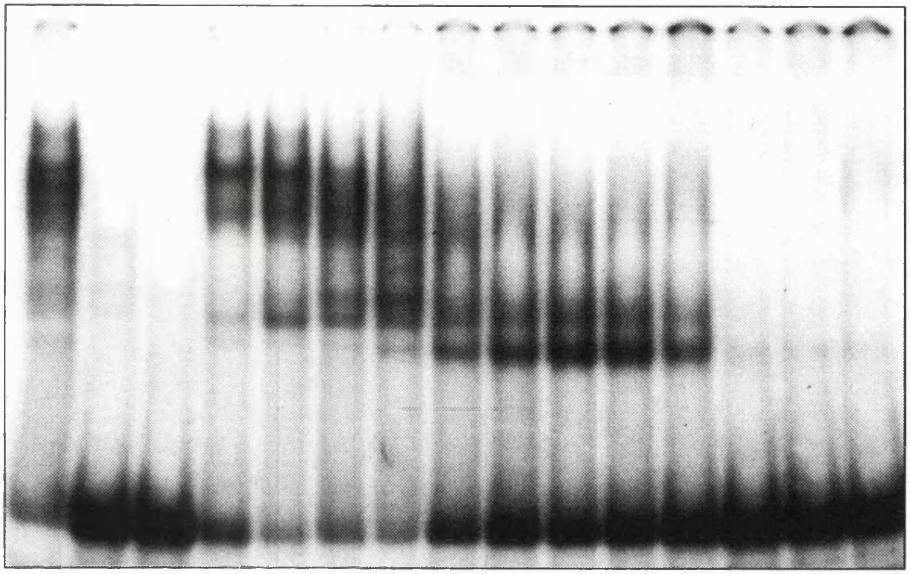

Figure 3.4.1 **Purification of DNA-binding proteins using magnetic DNA-affinity beads** (adapted from Dynal® technical handbook, Biomagnetic techniques in molecular biology).

Firstly, the 159 bp element was separated from the plasmid pUC19 using digestion with *Sal*I and *Ava*I. This resulted in a 176 bp DNA fragment. The *Sal*I site is necessary for subsequent ligation to the tether DNA because a tether DNA was designed with a *Sal*I site overhang at the end. In order to be certain that the 159 bp element had ligated to the $^{32}$P-tether DNA, the ligated product was electrophoresed along with the $^{32}$P-tether DNA on a 20% polyacrylamide gel. The binding conditions were optimised by varying the salt concentration in the binding buffer from 60, 120, 180, 240, to 300 mM KCl. Each binding buffer was mixed with the magnetic DNA affinity beads and the same amount of liver nuclear extract was added to each tube. Proteins were allowed to bind to the beads. The supernatants were then separated from the beads by placing the tube in a magnetic rack. Each supernatant was collected and tested for binding activity using a gel mobility shift assay. Supernatants from the binding buffer containing 180 mM KCl showed the least binding to the probe, which in turn, meant that most of the proteins in the liver nuclear extract had bound to the DNA-coupled beads. So the binding buffer containing 180 mM KCl was used in subsequent experiments. The elution conditions were also optimised by varying the elution buffer containing the following salt concentration of 0.5, 0.8, 1.0, 1.5, to 2.0 M NaCl. The protein content in the supernatants of the wash and elution fractions was determined by SDS-PAGE and gel mobility shift assays. Unfortunately, there was no detectable protein in any of the elution fractions.

The NF-1 binding site within the 159 bp *Sau3A*I fragment is flanked by potential nuclear receptor binding sites of the imperfect -AGGTCA- motif (Glass, 1994) organised as direct repeats with a 4 bp-spacer. The 5' element is called NR1 and the 3' element is known as NR2 (Referred to in Figure 3.4.3). Oligonucleotides for each of these nuclear receptor-binding sites were also used in attempts to affinity purify proteins. Each oligonucleotide was ligated to the tether DNA. The crude liver nuclear extract was partially purified by phosphocellulose column chromatography to enrich the proteins that bind to *CYP2B1* PB-responsive element before mixing with the magnetic beads. The peak fractions from the phosphocellulose column were analysed by gel mobility shift assay. Only those fractions that showed binding to the *CYP2B1* PB-responsive element were used for magnetic bead DNA affinity purification using either the NR1 or NR2 site ligated to the tether. Despite many attempts, only very faint protein bands were observed by SDS-PAGE in the elution fraction obtained from the binding of protein to the NR2 sequence (Figure 3.4.2). However, this proved not to be reproducible.

a.

| Protein/ fraction | PB | W 1 | W 2 | E 1 | E 2 | E 3 | E 4 | E 5 | E 6 | E 7 | E 8 | E 9 | E 10 | E 11 | E 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |



b.

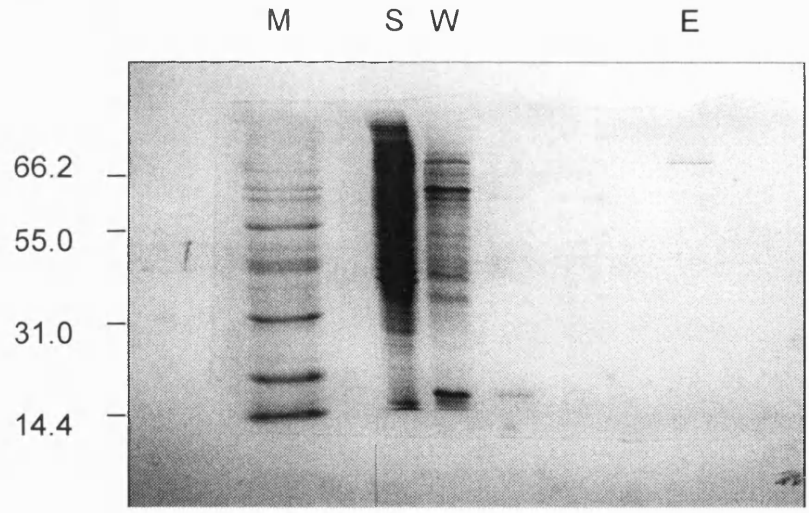



Figure 3.4.2 **Magnetic DNA-affinity purification of proteins that bind to the NR2 sequence** (a) Partial purified crude liver nuclear extracts from the phosphocellulose column were tested for their binding activity to the radiolabelled 159 bp DNA probe, as compared to liver nuclear extracts from PB-treated rats (lane 1), lane 2 and 3 = wash (W1-W2) fractions, lane 4-15 = eluate (E1-E11) fractions. Only the fractions E1-E4 were used for subsequent magnetic DNA-affinity purification. (b) SDS polyacrylamide gel electrophoresis of purified protein binding to the *CYP2B1* NR2 binding site by magnetic DNA-affinity beads, M = molecular weight protein marker, S = supernatant after the binding reaction, W = non-specific proteins from washing step, E = specific protein from elution step.

### 3.4.2 RXRα is one of the proteins that binds to the NR1 and NR2 binding sites of the *CYP2B1* PB-responsive element

After the identification of a 163 bp *Sau3A*I-fragment of the rat *CYP2B2* 5' flanking sequence as the phenobarbital-responsive element (PBRE) (Trottier *et al.*, 1995), another phenobarbital-responsive element was identified in the mouse *Cyp2b10* gene between -2426 to -2250 (132 bp) (Honkakoski *et al.*, 1996). This 132 bp fragment conferred PB-responsiveness (8-10 fold) to a heterologous thymidine kinase (TK) promoter. Moreover, this mouse 132-bp sequence was found to be about 91% similar to the rat *CYP2B2* 163 bp DNA fragment. Subsequent studies from the same group refined this region to a 33-bp core element, which they named the mouse PB-responsive enhancer module (PBREM) (Honkakoski and Negishi, 1997). The core PBREM contains a NF-1 binding site and two nuclear receptor-binding sites (NR1 and NR2) of the type -AGGTCA-. Subsequent deletion studies within the 163-bp PBRE of rat *CYP2B2* gene also verified two nuclear receptor-binding sites (NR1 and NR2), flanking the NF-1 site and a putative glucocorticoid response element (Stoltz *et al.*, 1998). Figure 3.4.3 demonstrates the DNA sequence of the 159-bp fragment and the core PB-responsive units of rat *CYP2B1* and *CYP2B2,* mouse *Cyp2b10*, and human *CYP2B6.* There is substantial sequence similarity between the rat *CYP2B1*, rat *CYP2B2*, mouse *Cyp2b10* and human *CYP2B6* core PB-responsive element. This suggests that these four genes may be activated by a common signalling

pathway involving orthologous proteins from the three species. The NR1 sequence is identical in rodent and human, which suggests a significant role for NR1 in the regulation of the *CYP2B* genes.

Oligonucleotides specifying the NR1 and NR2 sequences were synthesised with a 4 bp-*Sal* I restriction site 5'-overhang. The NR1 and NR2 oligonucleotides were 23 bp and 24 bp, respectively. The 5' to 3' complementary strandsof NR1 and NR2 were phosphorylated. These were then annealed to their corresponding partner. Subsequently, they were radiolabelled with $^{32}$P-α-dCTP and used in the gel mobility shift assay to examine the pattern of protein binding to these nuclear receptor-binding sites.

**a.**


**b.**

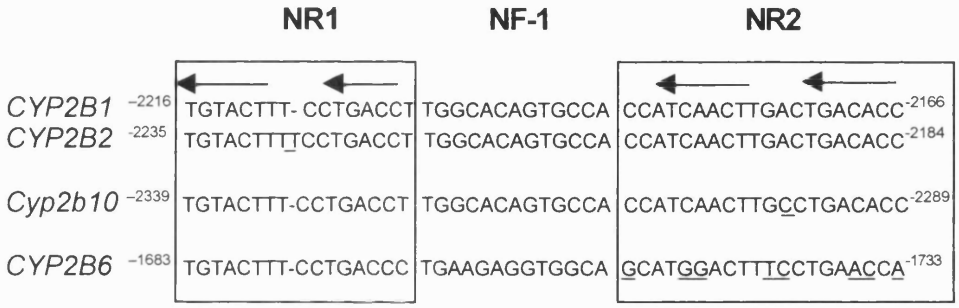



Figure 3.4.3 **The core PB-responsive element contains two nuclear receptor-binding sites separated by a NF-1 site** (a) *CYP2B1* upstream sequence from -2301 to -2142. Restriction sites, two nuclear receptor-binding sites (NR1 and NR2) and nuclear factor-1 binding site (NF-1) are indicated. (b) Comparison of core PB-response elements of *CYP2B1*, *CYP2B2*, *Cyp2b10* and *CYP2B6*. Arrows indicate direct repeats (DR) sequences.

When the NR1 binding site was used as a probe in a gel mobility shift assay, it produced protein-DNA complexes with liver nuclear extracts from both untreated and PB-treated animals that were equally abundant. But the NR1 probe produced a smaller and distinct complex compared to the complexes formed when the 159-bp fragment was used as a probe (see Figure 3.4.4). The lack of difference in both the pattern and abundance of complexes formed with NR1 and nuclear extracts from untreated and PB-treated rats suggests that the NR1 site can be occupied in the absence of any inducer. If the NR1 site is critical for PB induction (see section 3.5), then changes in the conformation/activation of proteins formed to NR1 must occur. Such changes are not detected using an *in vitro* assay.

The retinoid X receptorα (RXRα) has been reported to be a common heterodimerisation partner for several nuclear receptors, which bind to DNA of the direct repeats. Therefore, it seemed very likely that RXRα might bind to the NR1 region. To test this possibility, the RXRα antibody was used in a supershift analysis of liver nuclear proteins. As shown in Figure 3.4.4, the proteins bound to the NR1 binding site from both untreated and PB-treated animals are supershifted by the RXRα antibody. As a negative control, the antibody to NF-1 was included in the binding reaction. This does not supershift any protein-DNA complexes. These results confirm that the RXRα protein can bind to the NR1 binding site in both the untreated and PB-treated condition.

| Probe | NR1 | | | | | |
|---|---|---|---|---|---|---|
| Nuclear extracts | U | PB | U | PB | U | PB |
| Supershift antibody | - | | RXRα | | NF-1 | |
| Lane | 1 | 2 | 3 | 4 | 5 | 6 |

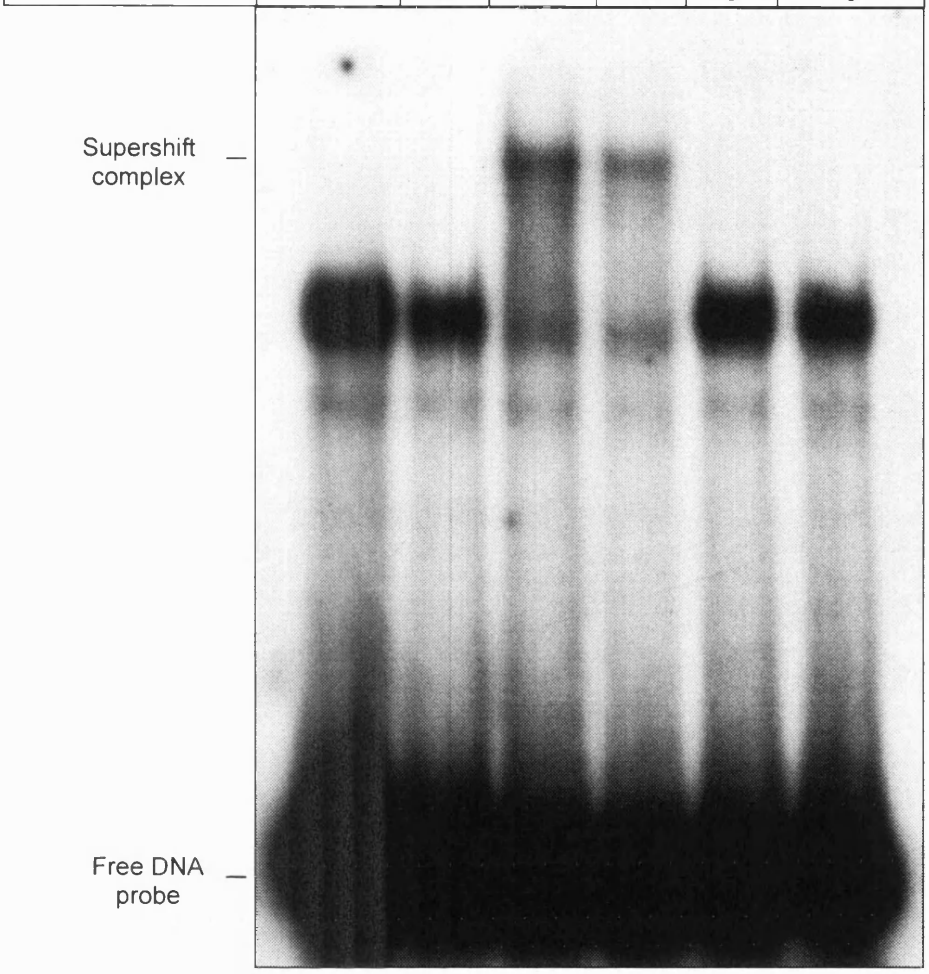



Figure 3.4.4 **Supershift analysis confirms that retinoid X receptor α (RXRα) is one of the nuclear proteins that can bind to nuclear receptor 1 (NR1) binding site.** A gel mobility shift assay was performed with liver nuclear extracts from untreated (U) and phenobarbital-treated (PB) rats and radiolabelled NR1 oligonucleotide. Antibody to RXRα protein was added 15 min after the binding reaction (lane 3 and 4); antibody to nuclear factor-1 (NF-1) protein was included as a non-specific antibody (lane 5 and 6). Note that the amount of nuclear extract in lane 1 is greater than in the other lanes.

The pattern of protein-DNA complexes formed on the NR2 binding site was also examined. No increase in protein binding was observed between the liver nuclear extracts from PB-treated animals as compared to untreated ones. It was also of interest to see what proteins bind to the *CYP2B1* NR2 binding site. We speculated that RXRα might also be able to bind to the NR2 site, as it is similar in sequence to the NR1 binding site. The RXRα antibody was able to supershift complexes formed on the NR2 by liver nuclear proteins (Figure 3.4.5). To compare the protein-DNA complexes formed on the NR2 binding site and NR1 binding site, they were run side by side on the same gel. Similar complexes, regarding pattern and location of the retarded band were observed. However, the intensity of the DNA-protein complexes was greater with the NR2 binding site (Figure 3.4.6). In the same experiment, the RXR consensus oligonucleotide was included as a competitor for protein binding to either the NR1 or NR2 binding sites. The results show that the protein-DNA complexes formed on both the NR1 and NR2 binding sites are competed by RXR consensus oligonucleotide at 100 molar excess. Surprisingly, despite the similarity in sequence between the NR1 and NR2 sites, addition of a 100 molar excess of NR2 did not inhibit the protein-DNA complexes formed on the NR1. Similarly, a 100 molar excess of NR1 could not compete for protein-DNA complexes formed on NR2 (Figure 3.4.6).

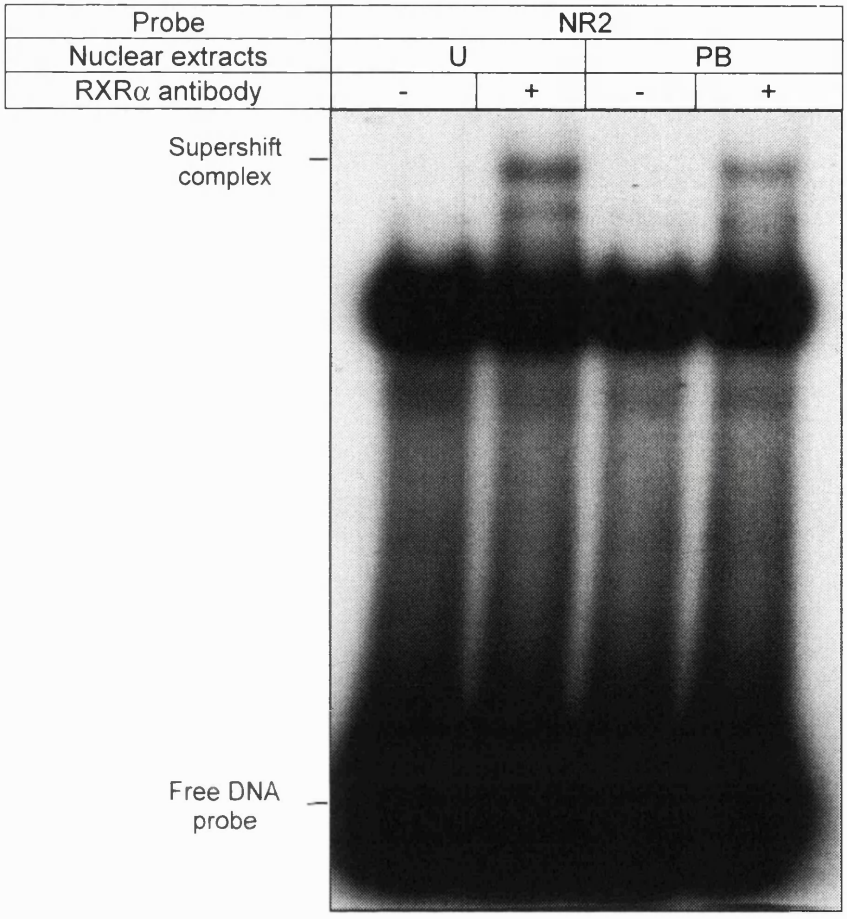



Figure 3.4.5 **RXRα can bind to the nuclear receptor 2 (NR2) binding site.** A supershift assay was performed by adding antibody to RXRα (2 μg) 15 min after the binding reaction was begun. The incubation was continued for further 30 min.

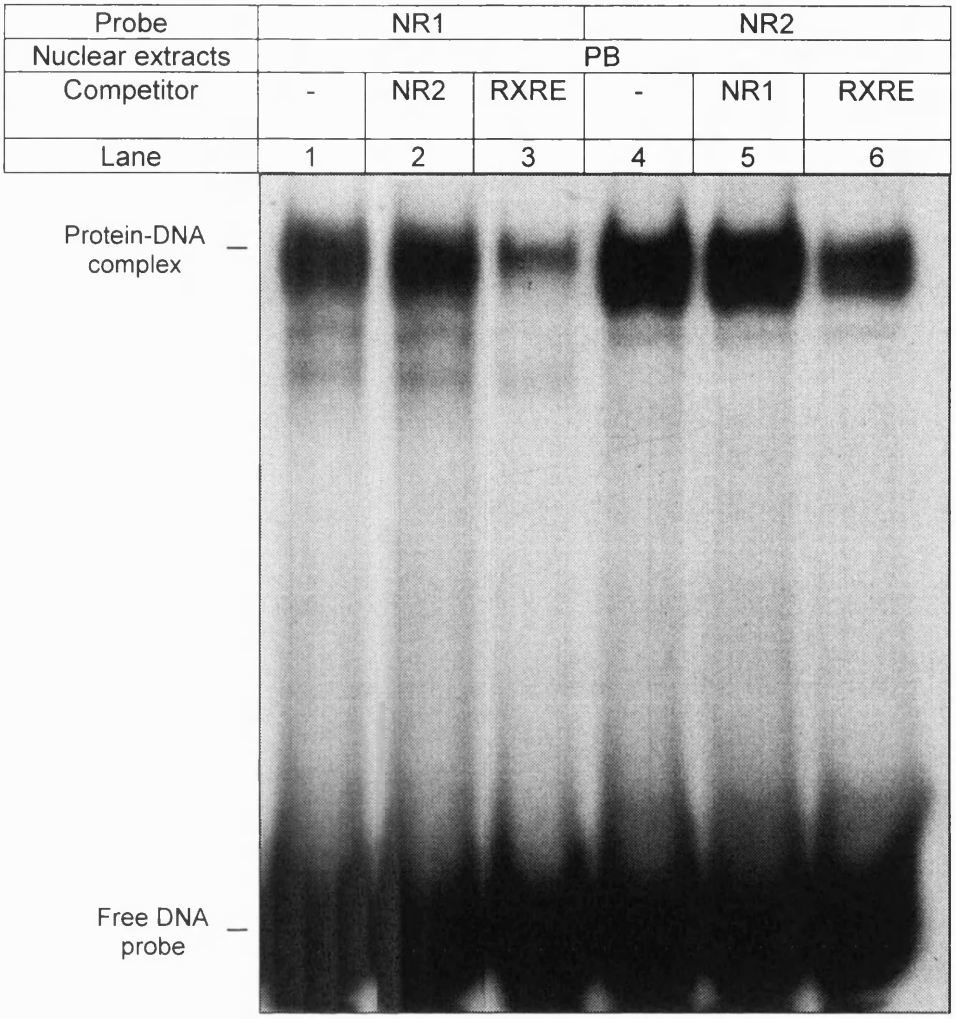



Figure 3.4.6 **RXRα can bind to both NR1 and NR2.** A gel mobility shift assay was performed with liver nuclear extracts from phenobarbital-treated (PB) animals and radiolabelled NR1 or NR2 oligonucleotides. A 100-fold molar excess of, unlabelled NR2 (lane 2), NR1 oligonucleotide (lane 5) or RXRE consensus oligonucleotide (lane 3 and 6) were used as the competitors.

These results show that RXRα can bind to both the NR1 and NR2 sites. However, the heterodimerisation partner for RXRα must be different for the NR1 and NR2 binding sites.

Nuclear proteins from untreated and PB-treated animals bind equally to both nuclear receptor-binding sites. In one experiment (the results of which are not shown here), PB was added to the binding reaction to see if it has any effect on the protein binding, however, no change in the abundance of the protein-DNA complexes was observed. This suggests the activation or modification of pre-bound proteins *in vivo* by PB does not occur *in vitro*. *In vivo* DNase I footprinting by Kim *et al* 1997 demonstrated a 20 bp protected region within the 163 bp PBRE by liver nuclear extracts from untreated animals. PB treatment extends this protected region to 60 bp (Kim and Kemper, 1997). *In vitro* DNase I footprinting, however, shows no change in the protected region by nuclear extracts from PB-treated animals as compared to untreated ones (Honkakoski *et al.*, 1996; Stoltz *et al.*, 1998). The reason for the different results observed between the *in vivo* and *in vitro* system may be due to the lack of chromatin structure *in vitro*, which normally *in vivo* restricts the access of some nuclear proteins, in addition to the pre-bound proteins, to interact with one another or with the DNA target.

### 3.4.3 RXRα heterodimerises with nuclear orphan receptor CAR-β and binds to the *CYP2B1* NR1 region

During the course of this study, Honkakoski *et al* 1998 identified a nuclear orphan receptor, mouse constitutively active receptor-β (CAR-β), which binds to the NR1 region of the mouse *CYP2b10* gene as a heterodimer with RXRα and transactivates mouse PBREM reporter constructs in transfection experiments (Honkakoski *et al.*, 1998b). Mouse CAR-β is 88 % similar to the previously identified human CAR (MB67) in the DNA binding domain. The two receptors have 72 % identity in the ligand-binding domain. Both the human and mouse receptors were discovered by their ability to bind to a DR-5 type of retinoic acid response element (RARE). The receptors can transactivate a reporter gene without addition of any ligand, hence the name, constitutively active receptor. Both mouse CAR-β and human CAR are expressed in greatest abundant in the liver. Interestingly, the rodent NR1 sequences are identical to each other and differ by one nucleotide from human NR1. The NR2 region is also highly similar between species. There is one base pair mismatch between rat and mouse NR2 sequences, but six base pairs difference between rodent and human NR2 elements (referred to Figure 3.4.2).

Despite the isolation and characterisation of CAR cDNAs and protein from human and mouse, the rat CAR cDNA has not been isolated. The experiment was carried out by UV-crosslinking of the protein-DNA complex between either labelled or unlabelled NR1 probe and liver nuclear extract. After polyacrylamide gel electrophoresis, the band was detected by autoradiography and western blot analysis as shown in Figure 3.4.7. An antibody to mouse CAR-β was used to detect the presence of CAR in liver nuclear extracts from untreated and PB-treated rats. Mouse liver nuclear extracts were included on the gel as a positive control. The western blot showed that rat CAR is about the same molecular weight as mouse CAR-β. Liver nuclear extracts from PB-treated and untreated rats contain similar amounts of CAR-β. Unexpectedly, the protein complex formed with the NR1 probe had the same molecular weight as CAR-β, instead of the combined molecular weight of the heterodimers of CAR-β and RXRα. This may suggest that only CAR-β is susceptible to UV-crosslinking to the NR1 probe. If this is the case then RXRα would separate from the DNA-bound CAR-β during SDS-PAGE.

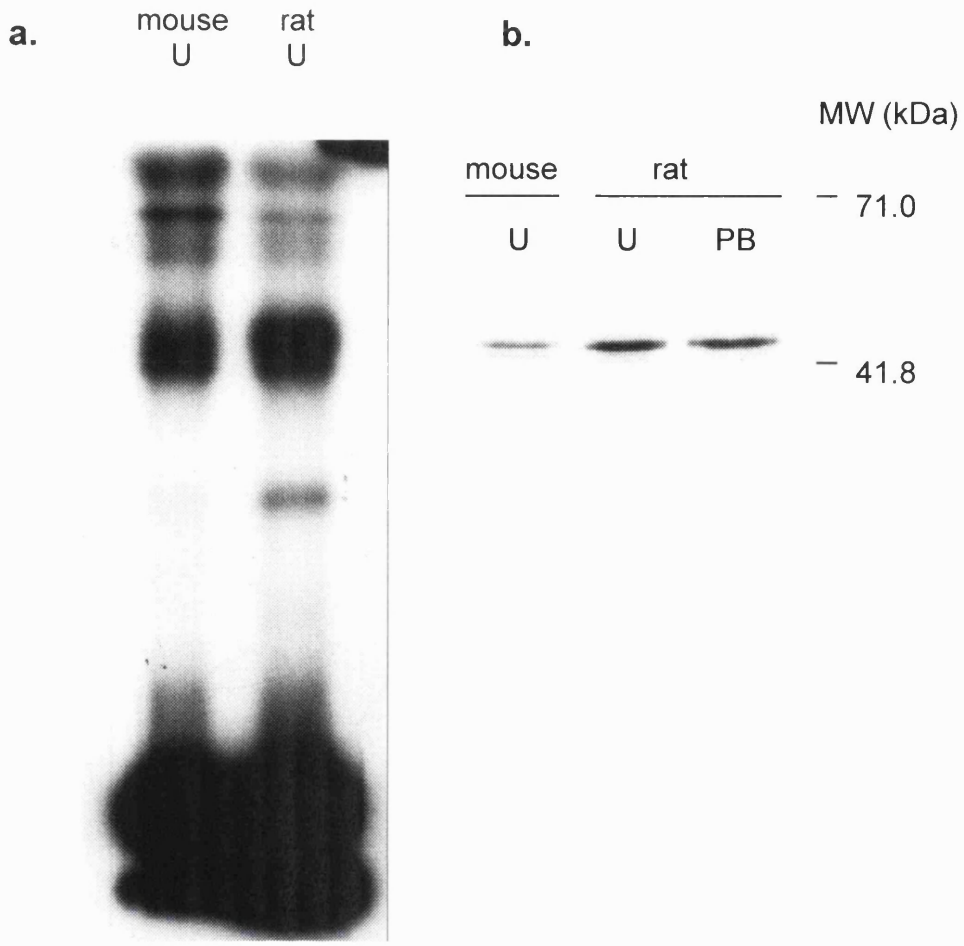



Figure 3.4.7 **Determination of CAR expression in the rat liver.** Rat and mouse liver nuclear extracts of untreated and PB-treated animals were incubated with either radiolabelled or unlabelled NR1 oligonucleotide. The protein-DNA complexes formed were UV-crosslinked and subjected to SDS polyacylamide gel electrophoresis on the same gel. The gel was divided. One half was dried and exposed to X-ray film (a). The other half was blotted and probed with CAR antibody (b).

However, as an expression plasmid for rat CAR is not available, we obtained the expression vectors for mouse CAR-β and human CAR (MB67) from Dr. David Moore to allow us to investigate the role of CAR-β in the regulation of rat *CYP2B1*. CAR-β cDNA is cloned in the CDM8 vector, which contains both the T7 and CMV promoters. The hCAR cDNA is cloned into the pT7 vector, which contains only the T7 promoter. From Prof. Pierre Chambon we obtained the RXRα cDNA cloned into the pSG5 vector, which contains T7 and SV40 promoters. The T7 phage RNA polymerase (T7) promoter is required for the proteins to be synthesised *in vitro* using a reticulocyte lysate coupled transcription/translation system. The CMV and SV40 promoters enable the transcription and translation of the expression plasmids in eukaryotic cells. As a result, the expression plasmids for CAR-β and RXRα can be used for both *in vitro* and *in vivo* studies. The expression plasmid for hCAR can be used only for *in vitro* studies. The expression vectors were first used in the *in vitro* transcription /translation system to synthesise CAR-β, hCAR and RXRα. These proteins were then used in a gel mobility shift assay to confirm their binding to the rat *CYP2B1* NR1 region (see figure 3.4.8). Subsequently, the expression plasmids for CAR-β and RXRα were used for transfection studies *in vivo*, in primary hepatocytes and cell lines (see section 3.5 and 3.6).

Figure 3.4.8 shows the heterodimerisation of CAR-β and RXRα on the rat *CYP2B1* NR1 binding site. The protein-DNA complexes formed with the *in vitro* translated products are the same size as those formed with liver nuclear extracts from PB-treated rats (Lane 1). Without its partner, neither CAR-β nor RXRα is able to bind to the *CYP2B1* NR1. Unprogrammed reticulocyte lysate shows a non-specific band, which is also present in each sample of *in vitro* translated protein. A 100 molar excess of unlabelled NR1 probe was included as a specific competitor to demonstrate specific binding of these two proteins. The RXRα antibody was used in supershift analysis to confirm RXRα binding to NR1. Moreover, a 100 molar excess of unlabelled NR2 probe was not able to compete for protein binding to the *CYP2B1* NR1 binding site. This, in turn, suggests that CAR-β and RXRα heterodimers do not bind to the *CYP2B1* NR2 binding site. In a separate experiment, the binding to radiolabelled NR2, of either CAR-β or RXRα or both together as a heterodimer was also tested. Neither CAR-β/RXRα heterodimers nor CAR-β on its own or RXRα on its own bind to the *CYP2B1* NR2 (Figure 3.4.9). In contrast to these results, Sueyoshi and co-workers 1999 have shown that human CAR and human RXR heterodimerise and bind to both the NR1 and NR2 of human *CYP2B6* PB-responsive element. However, the binding of the heterodimer to the human (h) NR2 site is very weak (Sueyoshi *et al.*, 1999). The sequence similarity between hNR1 and hNR2 is about 87 % (referred to Figure 3.4.2), so the same proteins may still be able to recognise this degree of similarity. On the other hand, the degree of similarity between rat (r) NR1 and rNR2 is only 67%. This may be the

reason why CAR-β and RXRα bind exclusively to the NR1 site, not to the NR2 site of rat *CYP2B1.* In addition, the human CAR (hCAR) and mouse RXRα were tested for the ability to bind to the *CYP2B1* NR1 binding site. The proteins can bind to the *CYP2B1* NR1 binding site as a heterodimer (results not shown). Thus, in rodents and man both CAR-β and RXRα can bind to the NR1 site and this may represent a common set of proteins that regulate *CYP2B* genes in different species. Functional studies on CAR-β and RXRα were carried out *in vivo* and these are described in the following sections.

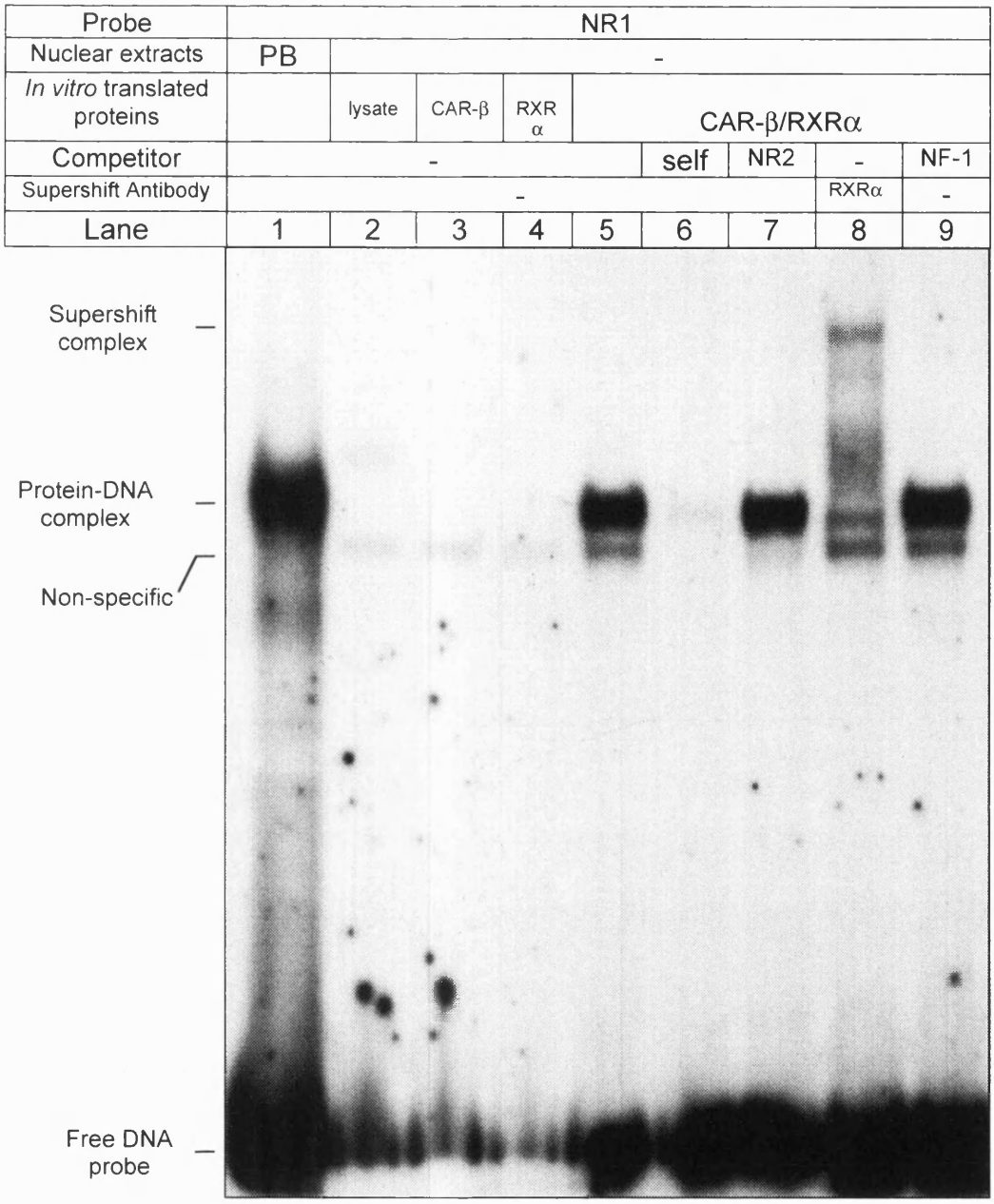



Figure 3.4.8 ***In vitro* translated CAR-β and RXRα heterodimerise and bind to the NR1 site.** A gel mobility shift assay was carried out with liver nuclear extracts from phenobarbital-treated rats (lane 1); unprogrammed rabbit reticulocyte lysate (lane 2); *in vitro* translated CAR-β (lane 3); *in vitro* translated RXRα (lane 4); *in vitro* translated CAR-β and RXRα (lane 5-9). 100-fold molar excess of unlabelled NR1 (lane 6), NR2 (lane 7), and NF-1 (lane 9) oligonucleotides were included as the competitors. Antibody to RXRα was used for supershift analysis (lane 8).

| Probe | NR2 | | | |
|---|---|---|---|---|
| Reticulocyte lysate | + | - | - | - |
| CAR-β | - | + | - | + |
| RXRα | - | - | + | + |
| Lane | 1 | 2 | 3 | 4 |

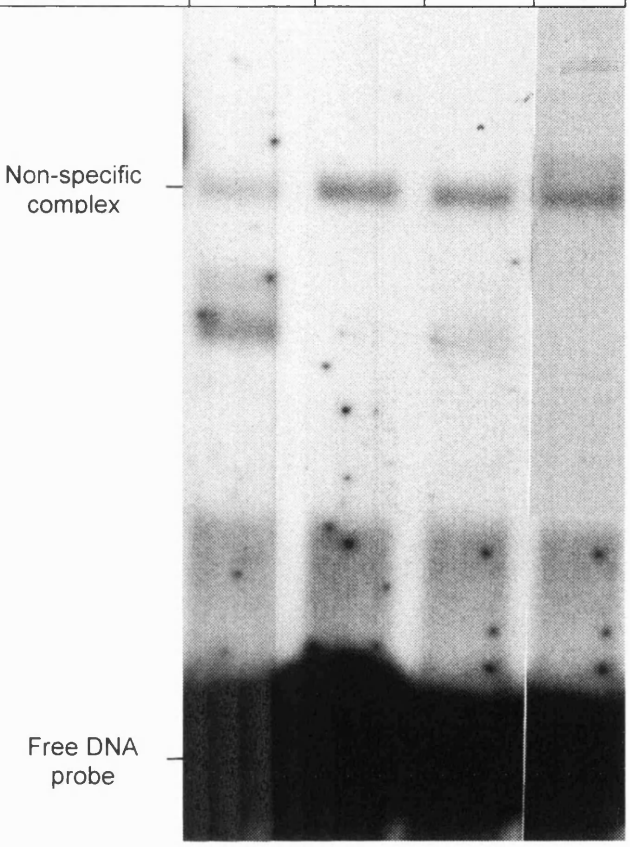



Figure 3.4.9 **CAR-β and RXRα do not bind to *CYP2B1* NR2.** A gel mobility shift assay was performed with unprogrammed reticulocyte lysate (lane 1), *in vitro* translated CAR-β (lane 2), RXRα (lane 3), or both CAR-β and RXRα (lane 4) and radiolabelled NR2.

## 3.5 Transactivation studies by transient transfection of primary rat hepatocyte cultures and human cell lines

Transient transfection using biolistic particle-mediated gene transfer technology proved to be a suitable model for functional studies *in vivo.* However, experiments using cell cultures is an alternative method particularly to reduce the number of animals used per experiment. Moreover, transfection with numerous DNA constructs and xenobiotic treatment makes the biolistic particle delivery method laborious in terms of surgery and animal handling. This is because only up to 3 shots can be made into the liver lobe of one animal. Only the same DNA and same treatment can be used per animal. Therefore, modification of conditions for culturing primary rat hepatocytes was made to maintain the expression of *CYP* genes, and to retain the xenobiotic response for subsequent transfection experiments. The major modification, based on the previously published results in our laboratory (Ciaramella *et al.*, 1994), is to culture the hepatocytes on Matrigel®-coated plates. Some experiments were also carried out on the cell lines, HepG2, Hela, and CV-1 cells.

## 3.5.1 Preparation of cells for transfection

### *3.5.1.1 Primary hepatocyte cultures*

Primary cell cultures refer to cells that are freshly isolated from a tissue and grown *in vitro* without being passaged. Such cell cultures are considered to be more similar to the *in vivo* situation, although specific functions are lost over a period of time. The two-step collagenase liver perfusion method was employed to prepare primary rat hepatocyte cultures. The cells that survive this disaggregation technique subsequently attach to the basement membrane matrix coated culture plates. A number of studies regarding the effect of basement membrane matrices such as Vitrogen® and Matrigel®, on liver-specific gene expression have been reported (Schuetz *et al.*, 1988; Waxman *et al.*, 1990). Vitrogen® is composed of pepsin-solubilised bovine dermal collagen, while Matrigel® (major component is laminin) is extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor rich in extracellular matrix proteins. In our laboratory, either the co-culture of primary rat hepatocytes with rat liver epithelial cells or those cultured on Vitrogen-coated plates maintain the expression of several *CYP* genes and their induction upon exposure to xenobiotics (Akrawi *et al.*, 1993; Ciaramella *et al.*, 1994). However, the co-culture with rat epithelial cells needs the preparation and culture of rat epithelial cells beforehand, which makes it less practical than the use of Vitrogen®-coated plates. A recent study showed a higher PB-induced *CYP2B* gene expression on Matrigel®-coated plates, than that observed with the cells cultured on Vitrogen®-

coated plates (LeCluyse *et al.*, 1999). This result led us to compare the two matrices with regard to CYP2B induction. Under our culture conditions, the level of PB-induced and PCN-induced CYP2B mRNA from the cells cultured on either Vitrogen® or Matrigel® was very similar. Both show very good induction of CYP2B mRNA. Nevertheless, the PB-induction of a reporter gene in transfected DNA in hepatocytes cultured on Matrigel® coated plates, is slightly higher than that cultured on Vitrogen-coated plates (results not shown). In addition, the preparation of Matrigel® coated plates is much quicker and easier than Vitrogen® coated plates. Therefore, the Matrigel® is preferentially used in this study together with the previous conditions for culturing hepatocytes established by our laboratory.

#### *3.5.1.2 Cell lines*

Cell lines were cultured either from a frozen stock or from a secondary subculture. These are immortal and phenotypically stable in long term culture. However, since such cells are derived from malignant cells, some characteristics differ from the normal cell type.

### *3.5.1.3 Transfection protocol*

Initially, several transfection methods were used to deliver DNA into cells. These included calcium phosphate (CellPhect®), and liposome based reagents (TransFast®, Tfx®-20, and Effectene®). The calcium phosphate method offers an easy, inexpensive method for both transient and stable transfection. The protocol involves precipitation of DNA with calcium chloride in a buffered saline/phosphate solution. The precipitate is taken up into the cells via endocytosis or phagocytosis. Liposome DNA delivery was developed in 1980 and offers high transfection efficiency. The synthetic lipid component is composed of cationic lipid and a neutral lipid, L-dioleoyl phosphatidyl-ethanolamine (DOPE). The overall net positive charges of liposome/nucleic acid complex allow close association with negative charges on the cell membrane so that endocytosis readily occurs. Liposome; Tfx-20 reagent, gave good transfection efficiencies and reproducible results, so most of the transfection experiments described were carried out with this reagent.

### 3.5.2 CAR-β transactivates the *CYP2B1* PBRE-*SV40*-Luc reporter gene expression in response to xenobiotics in primary hepatocyte cultures, but not in transformed cell lines

Different types of chemicals previously reported to increase *CYP2B* gene expression were used in this study to investigate the mechanism(s) by which they activate *CYP2B1*. These include the barbiturate; phenobarbital (PB), the natural plant product; picrotoxin, the synthetic glucocorticoid; dexamethasone, the synthetic anti-glucocorticoid; pregnenolone 16α-carbonitrile (PCN). Picrotoxin is a naturally occurring compound composed of picrotoxinin and picrotin. It is a convulsant that acts by blocking the chloride channel of γ-aminobutyric acid (GABA) receptors in brain. It is used as antidote to PB overdose (which also acts on GABA receptor). Administration of picrotoxin to rats, mimics the effect of PB, as it induces CYP2B proteins and other enzymes e.g. glutathione-S-tranferases (Yamada *et al.*, 1993). It was of interest to see whether picrotoxin acts through CAR-β to increase *CYP2B1* expression. The effect of dexamethasone, on *CYP2B* expression is controversial. Yamazoe and co-workers showed that chronic administration of dexamethasone led to a significant induction of CYP2B1/2 (20-100 fold) (Yamazoe *et al.*, 1987). In contrast, two groups demonstrated a slight decrease or no change in expression of these protein using a similar dosing treatment (Meehan *et al.*, 1988; Simmons *et al.*, 1987). In primary rat hepatocytes, treatment with dexamethasone alone does not increase *CYP2B* gene expression.

However, if added together with PB, a low concentration of dexamethasone markedly up regulates *CYP2B* gene expression, whereas higher concentrations of dexamethasone inhibit *CYP2B* gene expression (Sidhu and Omiecinski, 1995). In addition, dexamethasone has been reported to mediate its effect on CYP3A induction through a nuclear receptor, pregnane X receptor (PXR). So dexamethasone was included in this study to see if it activates *CYP2B* gene expression and if it does, does it act via PXR or CAR-β? The *CYP2B1* PBRE-*SV40*-Luc reporter was transfected into primary rat hepatocytes either in the presence or absence of an expression plasmid for CAR-β. Transfected cells were then treated with PB, picrotoxin, dexamethasone, PCN, or vehicle alone (dimethylsulfoxide). Results are shown in Figure 3.5.1. CAR-β constitutively activates a *CYP2B1* PBRE-*SV40*-Luc reporter expression ~5-fold without the addition of xenobiotics. PB and picrotoxin further enhance ~ 17-fold and 45-fold, respectively, the activation of a *CYP2B1* PBRE-*SV40*-Luc reporter expression through CAR-β. Therefore, CAR-β mediates the transactivation of *CYP2B1* PBRE-*SV40*-Luc reporter expression upon exposure to both these xenobiotics in primary rat hepatocytes. However, treatment with PCN only slightly increases the *CYP2B1* PBRE-*SV40*-Luc reporter expression, whereas dexamethasone has no effect on reporter expression. A Northern blot hybridisation analysis was carried out to examine the effect of these chemicals on endogenous primary hepatocyte CYP2B mRNA levels. A 14-fold increase in CYP2B mRNA level is observed in PB-treated hepatocytes, with 5-fold increase in response to picrotoxin. PCN slightly increase CYP2B mRNA level about 2 fold,

however, no increase in CYP2B mRNA was detected in dexamethasone-treated hepatocytes.

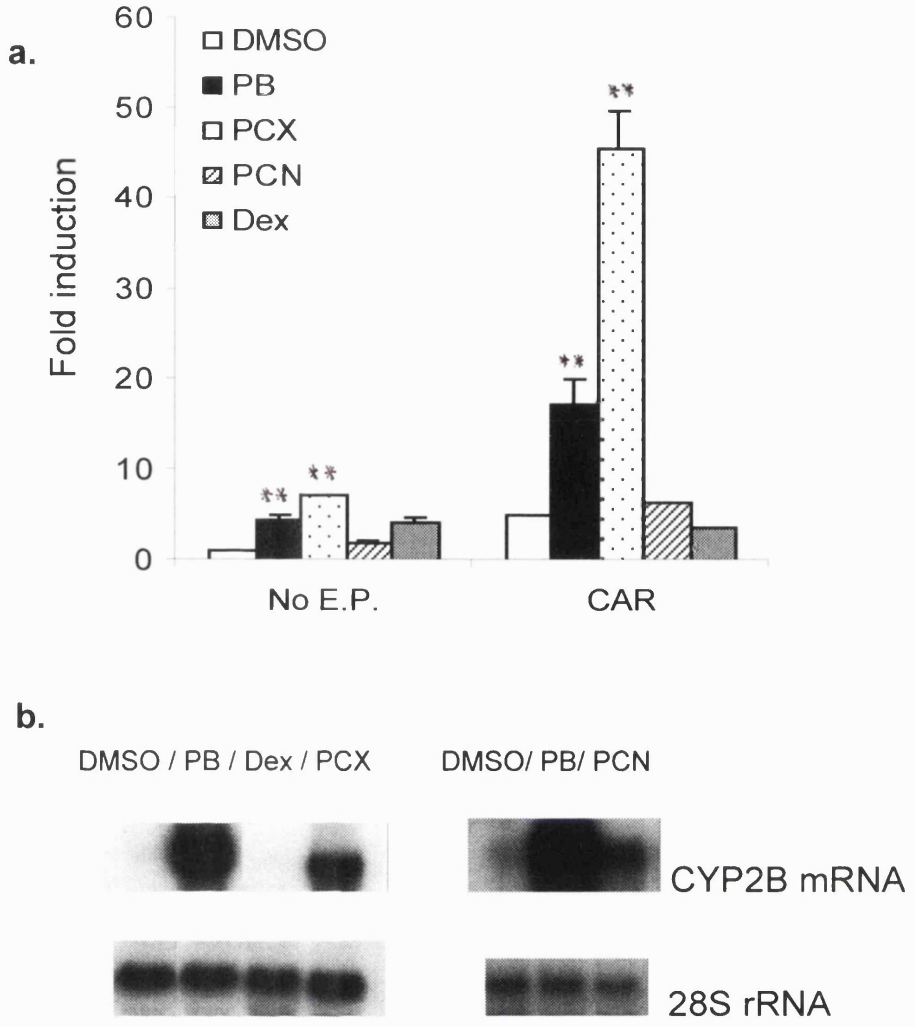



Figure 3.5.1 **CAR-β supports constitutive and xenobiotic activation of reporter gene expression.** (a) Primary rat hepatocytes were transfected with the reporter *CYP2B1* PBRE-*SV40*-Luc either in the absence (No expression plasmid, No E.P.) or presence of the expression plasmid for CAR-β (CAR) as indicated. Transfected cells were treated with either vehicle alone, 0.1 % dimethylsulfoxide (DMSO), 0.1 mM phenobarbital (PB), 0.5 mM picrotoxin (PCX), 10 μM pregnenolone 16α-carbonitrile (PCN), or 20 μM dexamethasone (Dex). Results are expressed as fold induction ± S.D., where 1 represents the relative luciferase activity obtained in cells transfected with the *CYP2B1* PBRE-*SV40*-Luc reporter alone. Significantly different from the corresponding control value, ** $p<0.05$ (two tail, paired Student's *t* test) (b) Northern blot analysis of RNA isolated from primary hepatocytes treated with the chemicals shown in figure. The probe is a CYP2B cDNA and the results are normalised to 28S rRNA.

Transfection of cell lines, HepG2, Hela, and CV-1 cells, was also carried out with the *CYP2B1* PBRE-*SV40*-Luc reporter along with an expression plasmid for CAR-β(Figure3.5.2). These transfected cell lines were either untreated or treated with 0.1 mM PB. In HepG2 cells, CAR-β constitutively activates the expression of reporter ~2 fold. The addition of PB does not further activate luciferase activity. In transfected Hela cells, although CAR-β transactivates *CYP2B1* PBRE-*SV40*-Luc expression up to 19 fold, in the absence of xenobiotic, no further stimulation of reporter expression was seen in transfected cells when exposed to PB. CV-1 cells, which are frequently used in receptor-mediated transactivation studies because they lack endogenous orphan nuclear receptors, supported a constitutive activation (2.5 fold) of a *CYP2B1* PBRE-*SV40*-Luc reporter construct via CAR-β. However, no additional response to PB of CAR-β-mediated transactivation was observed. In our hands, therefore the use of CV-1 cells is not an appropriate model to study CAR-β-mediated *CYP* gene expression in response to PB. Northern blot hybridisation analysis was carried out to monitor the endogenous level of CYP2B mRNA in the three cell lines and upon exposure to PB. CYP2B mRNA was not detected nor was it increased by PB (results not shown). This corresponds to the lack of PB response observed in the transfection experiments described above. It is likely that the intrinsic transcriptional capacity due perhaps to differential regulation of transcription factors is different in transformed cell lines compared to primary untransformed cells. In addition, these results demonstrate direct CAR-β-mediated transactivation via the *CYP2B1* PBRE

of reporter gene expression in non-liver cell types such as Hela and CV-1 cells.

To ensure the activity of CAR-β in transfected HepG2 cells with an expression plasmid for CAR-β, the whole cell extract was prepared from HepG2 cells transfected with CAR-β and RXRα expression vectors and mock-transfected cells. Notably, the amount of an expression vector for RXRα was co-transfected three fold less than expression vector for CAR-β. The whole cell extract from trasfected cells was then tested for the binding activity of the overexpressed CAR-β and RXRα to the *CYP2B1* NR1 probe compared to the mock-trasfected cells. The result as shown in figure 3.5.3 demonstrates that the transfected expression plasmid for CAR-β functionally expressed and endogenous CAR-β is undetectable in HepG2 cells.

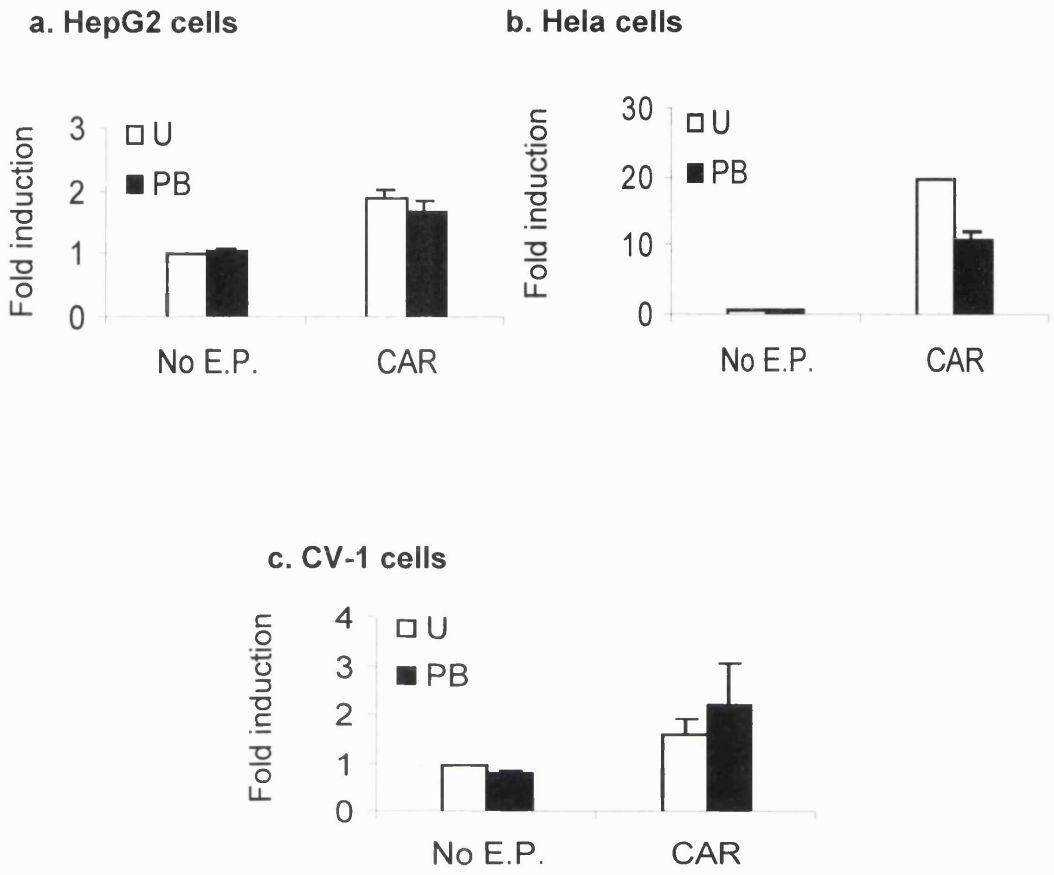



Figure 3.5.2 **CAR-β constitutively activates expression** from the ***CYP2B1* PBRE-*SV40*-Luc reporter plasmid in transfomed cell lines.** The cell lines (a) HepG2, (b) Hela, and (c) CV-1 cells were transfected with the reporter *CYP2B1* PBRE-*SV40*-Luc in the absence (No expression plasmid, No E.P.) or presence of expression plasmid for CAR-β (CAR). Transfected cell lines were untreated (U) or treated with 0.1 mM phenobarbital (PB). Results are expressed as fold induction ± S.D., where 1 represents the relative luciferase activity obtained in cells transfected with the *CYP2B1* PBRE-*SV40*-Luc reporter alone.
The results represent triplicates of two independent experiments.

| Probe | NR1 | | | | | | |
|---|---|---|---|---|---|---|---|
| Sample | M | T | M | T | M | T | - |
| Microcon-10 | + | + | + | + | - | - | - |
| *In vitro* translated RXRα | - | - | + | + | - | - | + |
| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 |

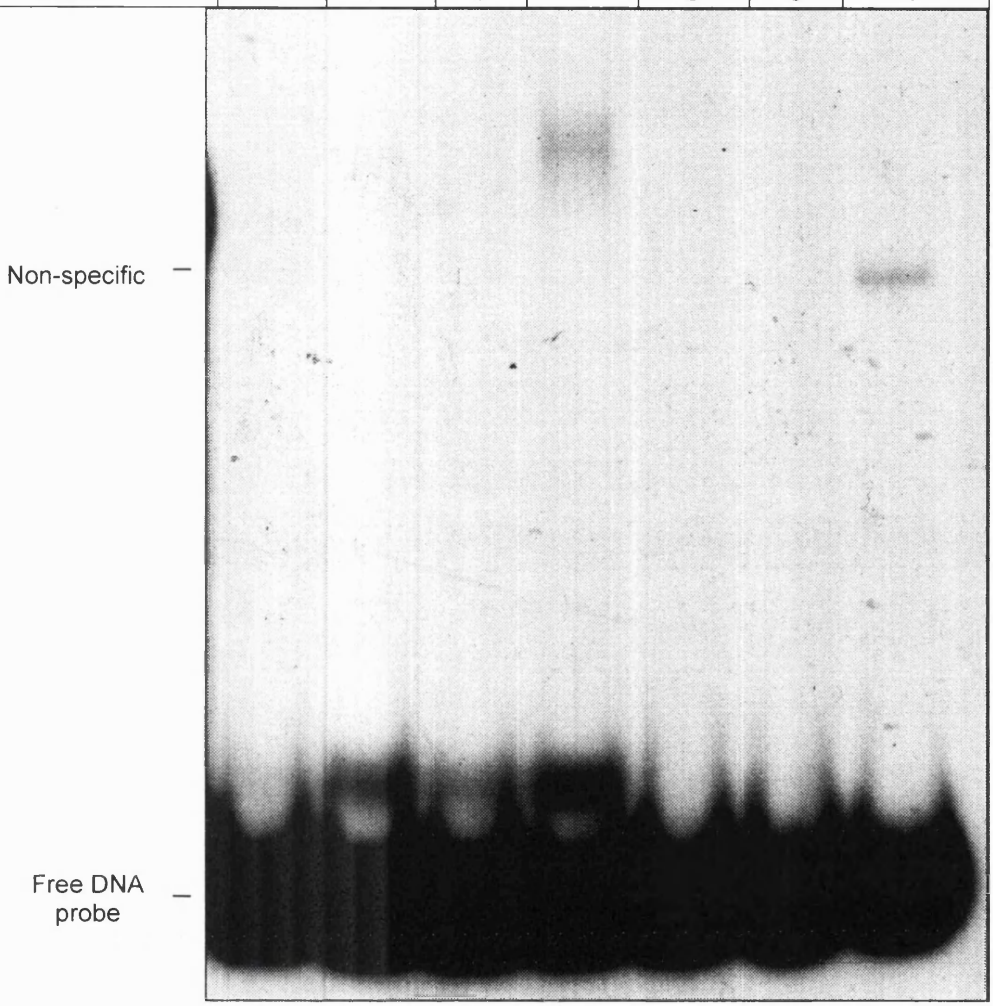



Figure 3.5.3 **CAR-β protein is expressed in a functional form in HepG2 cells.** Whole cell extracts were prepared from mock-transfected (M) and CAR-β/RXRα transfected (T) HepG2 cells and investigated for their binding activity to the *CYP2B1* NR1 region. Lane 3-6 = whole cell extracts were concentrated using Microcon-10 column; lane 3-4 = *in vitro* translated RXRα was added to the gel shift binding reaction; lane 7 = *in vitro* translated RXRα on its own

These results provide the first direct evidence that CAR-β activation of reporter gene expression is enhanced by PB. This response is seen in primary hepatocytes and not in transformed cell lines. A previous study showed only constitutive transactivation of *Cyp2b10* PBRE by hCAR in human HepG2 cells (Honkakoski *et al.*, 1998b).

Kawamoto and colleagues have suggested that a mechanism responsible for CAR-β translocation from the cytoplasm to the nucleus is permanently activated in transformed HepG2 cells and as a result, the addition of PB would have no further effect on transcription (Kawamoto *et al.*, 1999). Therefore, if PB causes the nuclear translocation of CAR-β, we should expect to see a greater abundance of protein binding to the NR1 sequence of PB-treated hepatocytes nuclear extracts. We therefore examined the complexes formed between the *CYP2B1* NR1 binding site and nuclear and cytosolic extracts from primary rat hepatocytes that were untreated and treated with PB for three hours. No difference in the abundance of complexes formed with nuclear extracts from either untreated or PB-treated cells was observed. Moreover, no complexes were observed with NR1 and cytosolic extracts, even though *in vitro* translated RXRα protein was added to the binding mixture to ensure the amount of this protein was not limiting in the cytosolic fraction (Figure 3.5.4). Note that the nuclear extract of PB-treated primary hepatocytes for 6 hr also showed no increase in binding to NR1 probe as compared to untreated ones (results not shown). These results suggest that mechanism(s) other than, or in addition to, xenobiotic induced CAR-β nuclear translocation are

responsible for xenobiotic induction of gene expression via CAR-β. In this case, it is likely that PB may activate the DNA binding activity of CAR-β/RXRα heterodimer via the ligand-activated DNA binding mechanism (DeFranco, 1999) rather than ligand-induced nuclear translocation of CAR-β.

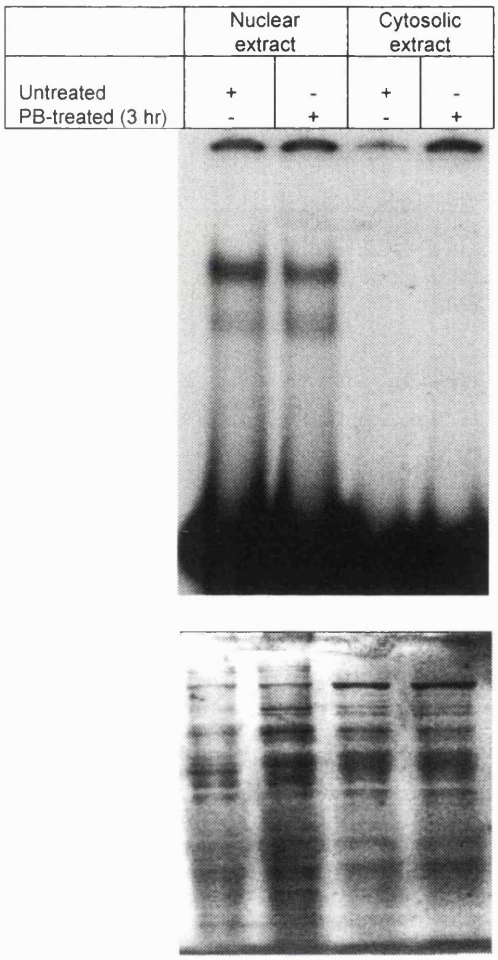



Figure 3.5.4 **PB-treatment does not increase the abundance of protein binding to the *CYP2B1* NR1.** Gel mobility shift assays were performed with radiolabelled NR1 oligonucleotide and nuclear (10 μg) and cytosolic (10 μg) protein extracts from primary rat hepatocytes which were untreated or treated with phenobarbital (PB) for 3 hours (top panel). *In vitro* translated RXRα (1 μl) was added to the cytosolic fraction. SDS polyacrylamide gel electrophoresis of 5 μg of the corresponding nuclear- and cytosolic extracts (lower panel).

### 3.5.3 Transactivation of a *CYP2B1* $(NR1)_2$-*SV40*-Luc reporter gene expression via CAR-β upon treatment of xenobiotics

The results presented so far represent the transactivation activity of CAR-β in response to xenobiotics on a reporter construct containing the 159 bp-PB responsive element. This experiment was carried out to investigate a direct transactivation, by CAR-β, of a reporter gene expression through the *CYP2B1* NR1 site. Two copies of the *CYP2B1* NR1 binding site were cloned into the *Sma*I site upstream of the SV40 promoter of the pGL3-promoter reporter vector. The plasmid was designated *CYP2B1* $(NR1)_2$-*SV40*-Luc. This plasmid was transfected into primary hepatocytes alone, or together with, the expression plasmid for CAR-β. Transfected cells were treated with PB, picrotoxin, PCN, dexamethasone or vehicle alone. The results are as shown in Figure 3.5.5. In *CYP2B1* $(NR1)_2$-*SV40*-Luc reporter-transfected cells, PB, picrotoxin, and PCN caused a 3-fold increase in reporter activity. No increase of luciferase activity was observed in cells treated with dexamethasone. Overexpressed CAR-β constitutively activates reporter expression about 4 fold. PB, picrotoxin, and PCN further enhance *CYP2B1* $(NR1)_2$-*SV40*-Luc expression about 3, 2, and 1.5 fold, respectively, above the constitutive activity of CAR-β. Dexamethasone had no effect on reporter gene expression. The increase in reporter gene expression in response to xenobiotics is lower in cells transfected with the *CYP2B1* $(NR1)_2$-*SV40*-Luc compared to that observed when the *CYP2B1* PBRE-*SV40*-Luc is used as the reporter construct. Other sequences within

the full PBRE such as the NF-1 and NR2 sites may be required for full xenobiotic responsiveness. Transient transfection studies, in primary hepatocytes, have shown that mutations in the NF-1 site reduce PB responsiveness (Honkakoski and Negishi, 1997; Liu *et al.*, 1998; Stoltz *et al.*, 1998). However, mutations in the NF-1 site of the *CYP2B2* PBRE , in transgenic mice, did not alter the responsiveness to PB (Ramsden, et al., 1999). Within the PBRE, a putative glucocorticoid element is also present, mutation of this sequence dramatically reduces PB responsiveness of a reporter gene (Stoltz *et al.*, 1998). Our results, and those of others, indicate that efficient transactivation of *CYP2B* gene expression in response to PB or to other xenobiotics require the interaction between several regulatory factors and sequences in addition to NR1 site (see also section 3.7).

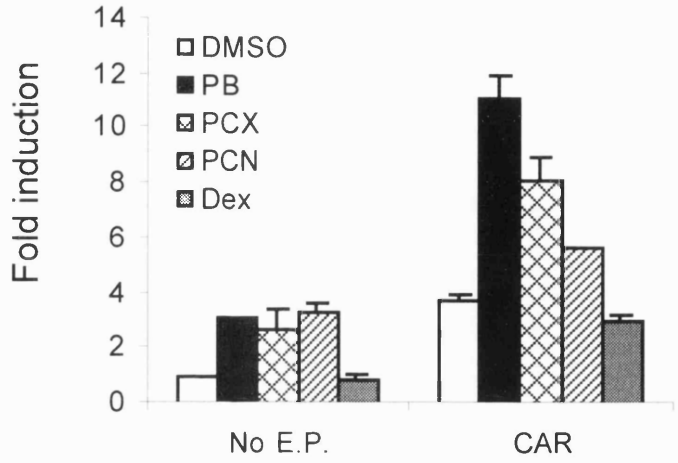



Figure 3.5.5 **CAR-β transactivates via the *CYP2B1* NR1 element.** Primary rat hepatocytes were transfected with the reporter *CYP2B1* $(NR1)_2$-*SV40*-Luc either in the absence (No expression plasmid; No E.P.) or presence of co-transfected expression plasmid for CAR-β (CAR) as indicated. Transfected cells were treated with either vehicle alone, 0.1% dimethylsulfoxide (DMSO), 0.1 mM phenobarbital (PB), 0.5 mM picrotoxin (PCX), 10 μM pregnenolone 16α-carbonitrile (PCN), or 20 μM dexamethasone (Dex). Results are expressed as fold induction ± S.D., where 1 represents the relative luciferase activity of cells transfected with *CYP2B1* $(NR1)_2$-*SV40*-Luc reporter alone.

The results represent duplicates of two independent experiments.

### 3.5.5 Overexpressed RXR α and 9-cis retinoic acid down-regulate PB-induced *CYP2B1* PBRE-*SV40*-Luc activity

As mentioned previously, co-transfection of an expression plasmid for CAR-β into the rat liver does not require the co-expression plasmid of the partner, RXRα, to achieve transactivation activity. This is in contrast to the *in vitro* results by gel mobility shift assay, whereby CAR-β is unable to bind to the *CYP2B1* NR1 element without its partner, RXRα. These results are explained by sufficient amounts of endogenous RXRα in the liver. This experiment was carried out to see if added exogenous RXRα and treatment with a specific ligand further increased the transactivation of reporter gene expression. Primary rat hepatocytes were transfected with a *CYP2B1* PBRE-*SV40*-Luc reporter together with the expression plasmids for CAR-β or both CAR-β and RXRα. Cells were then treated with PB, 9-cis retinoic acid (9-cis RA) or both chemicals. Results are shown in Figure 3.5.6. CAR-β alone showed some constitutive activity on a *CYP2B1* PBRE-*SV40*-Luc reporter without any chemical treatment. In the presence of PB, CAR-βtransactivates a *CYP2B1* PBRE-*SV40*-Luc reporter expression up to 23 fold. Surprisingly, when both RXRα and CAR-β are overexpressed, the expression of the *CYP2B1* PBRE-*SV40*-Luc reporter is down regulated, either in the presence or absence of PB. At high concentration, partially purified recombinant RXR has been reported to bind the retinoid X receptor response element (RXRE) as a homodimer (Mader *et al.*, 1993). Overexpressed of RXRα might lead to the formation of RXRα homodimers

and hence cause reduction in the formation of CAR-β/RXRα heterodimers. Or overexpressed RXRα may oversaturate the cells in such a way to disturb signalling pathways. The reason for the down-regulation of RXR is however not clear. In contrast, in HepG2 cells, that overexpressed hRXR, synergistic enhancment of hCAR transactivation of a *CYP2B6* reporter plasmid was observed (Honkakoski *et al.*, 1998b). The variation in the amounts of endogenous factors might account for the different effect of added RXRα seen in primary and transformed cells. The addition of 9-cis RA does not change the expression of a *CYP2B1* PBRE-*SV40*-Luc reporter. Moreover, in the presence of both 9-cis RA and PB, the expression of a *CYP2B1* PBRE-*SV40*-Luc reporter was down regulated. The transcription activity of RXR has been reported to be influenced by its partner, as non-permissive and permissive heterodimers. The formation of heterodimers such as $T_3R$/RXR and RAR/RXR inhibit RXR binding to its ligand, 9-cis RA. The inhibition is released only when RAR or $T_3R$ are ligand bound, then RXR is able to bind its ligand and transcription is activated (Forman *et al.*, 1995b). In contrast to this, when RXR heterodimerises with PPAR, both receptors are independently responsive to their own ligand. Furthermore, they are synergistically activated in the presence of both ligands (Kliewer *et al.*, 1992). CAR-β/RXRα heterodimers are of the non-permissive type because they are not responsive to 9-cis RA. In addition, PB has not yet been proved to bind CAR-β as a ligand. In this study, the presence of PB does not permit the independent activation of RXRα by 9-cis RA.

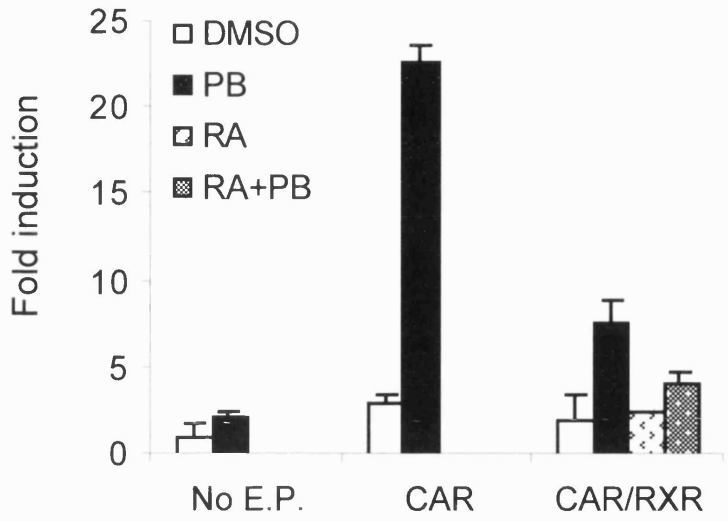



Figure 3.5.6 **Overexpressed RXRα and 9-cis retinoic acid down regulate the *CYP2B1* PBRE-*SV40*-Luc expression.** A *CYP2B1* PBRE-*SV40*-Luc reporter was co-transfected into primary rat hepatocytes either in the absence (No expression plasmid, No E.P.) or presence of expression plasmids for CAR-β (CAR) or RXRα (RXR) or both. Transfected cells were treated with 0.1 mM phenobarbital (PB), 1.0 μM 9-cis retinoic acid (RA), or both, or a vehicle alone (0.1 % dimethylsulfoxide, DMSO) as indicated. Results are expressed as fold induction ± S.D., where 1 represents the relative luciferase activity of a *CYP2B1* PBRE-*SV40*-Luc reporter alone.

The results represent duplicates of two independent experiments.

## 3.6 Cross-talk between promiscuous receptors and cytochrome P450 gene expression

The versatility of cytochrome P450 in the detoxification of structurally diverse xenobiotics is well recognised. However, the molecular mechanisms whereby different classes of chemicals increase the expression of the same CYP or how the same chemical induces more than one CYP are not fully understood. The structurally unrelated barbiturate phenobarbital (PB) and the synthetic steroid, pregnenolone 16α-carbonitrile (PCN) increase the expression of both the *CYP2B1* and *CYP3A1* genes (Bertilsson *et al.*, 1998; Blumberg *et al.*, 1998; Heuman *et al.*, 1982). *In vivo* CYP3A1 is less PB-inducible (3 fold) than is CYP2B1 (50-100 fold). Similarly, PCN induces CYP2B1 (3 fold) less than of CYP3A1 (10-20 fold). Kliewer (1998) identified the mouse pregnane X receptor (PXR), by its binding to the DR-3 motif in the promoter region of the rat *CYP3A1* gene and subsequent activation of a *CYP3A1* reporter vector in response to PCN. In an earlier section, when primary hepatocytes were transfected with a reporter gene containing a *CYP2B1* PBRE, PCN increased the expression of luciferase reporter activity ~2-fold (see figure 3.5.1). This suggested the possibility that PXR might bind to the nuclear receptor sites, NR1 or NR2 or to both sites, within the PBRE. We speculated the binding capacity of CAR-β and PXR1 to the same DNA motifs either *CYP2B1* NR1 or *CYP3A1* PXRE. In addition, mouse PXR1 has been demonstrated to bind as a heterodimer with RXRα to an ER-6

motif of human *CYP3A4* gene, although the ER-6 motif is different from the DR-3 motif of the *CYP3A1* gene. ER-6 is an everted repeat of hexanucleotides motif with a 6-bp spacer, whereas the DR-3 is a direct repeat of a hexanucleotide motif separated by 3 bp (Figure 3.6.1). The *CYP3A4* ER-6 and the *CYP3A1* DR-3 are referred to as PXRE, pregnane X receptor response element.

### 3.6.1 Both CAR-β and PXR1 bind to the *CYP2B1* NR1 and *CYP3A1* PXRE motifs

The expression plasmid for mouse PXR (PXR1) is composed of the PXR1 cDNA cloned into a pSG5 vector harbouring T7 and SV40 promoters. The expression plasmids for CAR-β, PXR1 and RXRα were used for synthesising the respective protein products *in vitro*. The oligonucleotides specifying the rat *CYP2B1* NR1 binding site and the rat *CYP3A1* PXRE are shown in figure 3.6.1. These were radiolabelled and incubated with *in vitro* translated CAR-β, PXR1,or RXRα and the complexes formed analysed by the gel mobility shift assay.

Neither CAR-β, PXR1 nor RXRα alone could bind to the *CYP2B1* NR1 element or the *CYP3A1* PXRE. The CAR-β/RXRα and PXR1/RXRα heterodimers bound to both the *CYP2B1* NR1 element and *CYP3A1* PXRE. CAR-β/RXRα heterodimers preferentially bind to *CYP2B1* NR1 than *CYP3A1* PXRE (Figure 3.6.2). The abundance of the PXR1/RXRα complex formed on the *CYP3A1* PXRE was greater than that formed on the *CYP2B1* NR1 element (Figure 3.6.3). In the same experiment, antibodies against PXR1 and RXRα proteins were included to confirm the binding of these two proteins to the receptor binding sites. Both the antibodies supershifted the protein-DNA complexes formed on the PXRE. The RXRα antibody shifted the complex to a greater degree than did the PXR1 antibody.

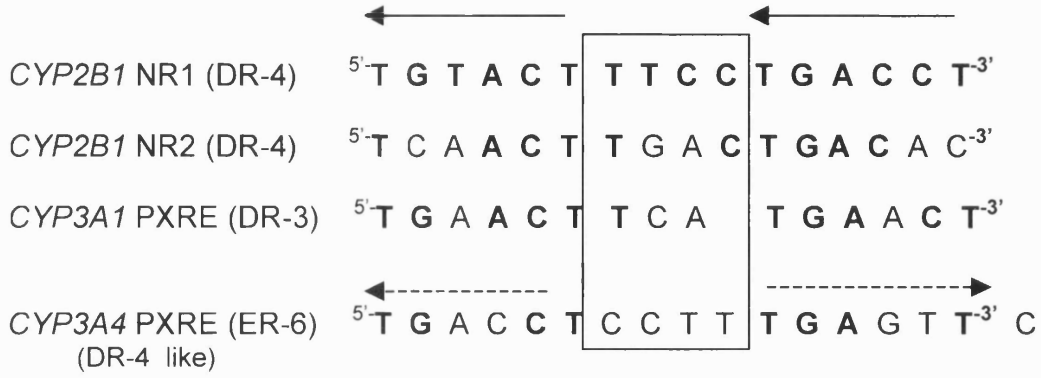



Figure 3.6.1 **Sequence of the *CYP2B1* NR1 and the *CYP3A1* PXRE.** Rat *CYP2B1* NR1 sequence is compared to rat *CYP2B1* NR2, rat *CYP3A1* PXRE, and human *CYP3A4* PXRE sequence. Arrows indicate direct repeats (DR), dashed arrows represent everted repeats (ER), in box represents spacer bases.

| Probe | NR1 | | PXRE | |
|---|---|---|---|---|
| *In vitro* translated CAR-β | + | | + | |
| *In vitro* translated RXRα | + | - | + | - |
| Lane | 1 | 2 | 3 | 4 |

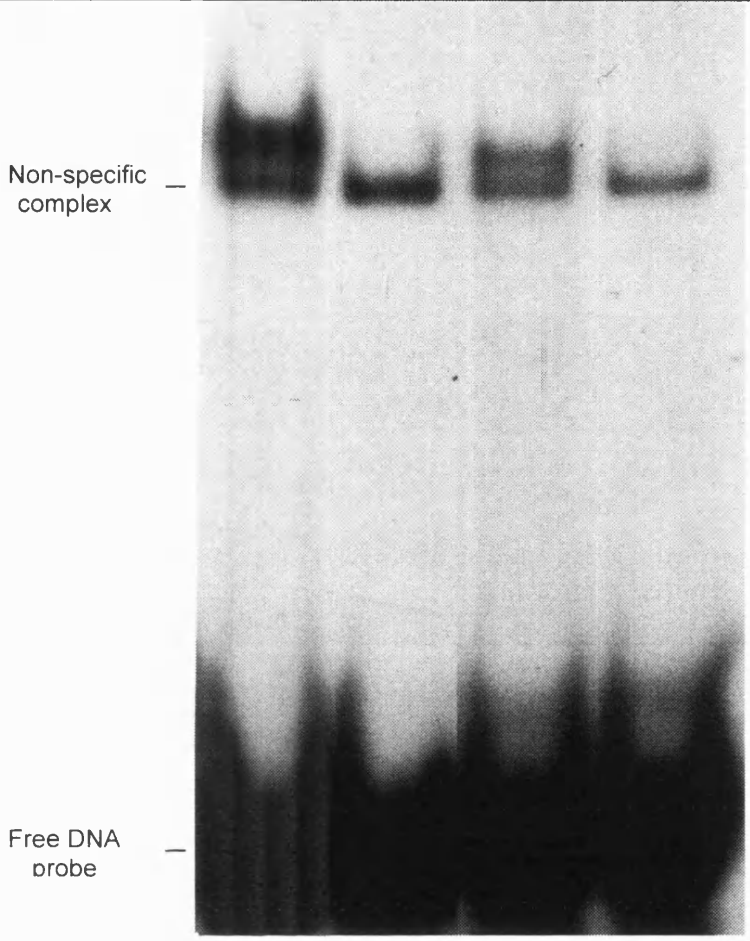



Figure 3.6.2 **Binding of *in vitro* translated CAR-β and RXRα to *CYP2B1* NR1 and *CYP3A1* PXRE.** *In vitro* translated CAR-β and RXRα were included (lane 1 and 3), whereas only *in vitro* translated CAR-β was used in the binding reaction (lane 2 and 4).

| Probe | PXRE | | | | | NR1 | NR2 |
|---|---|---|---|---|---|---|---|
| PXR1 | - | + | + | + | + | + | + |
| RXRα | - | + | + | + | + | + | + |
| Competitor | - | - | - | - | VDRE | - | - |
| Rabbit reticulocyte lysate | + | - | - | - | - | - | - |
| Supershift antibody | - | - | PXR1 | RXRα | - | - | - |
| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 |

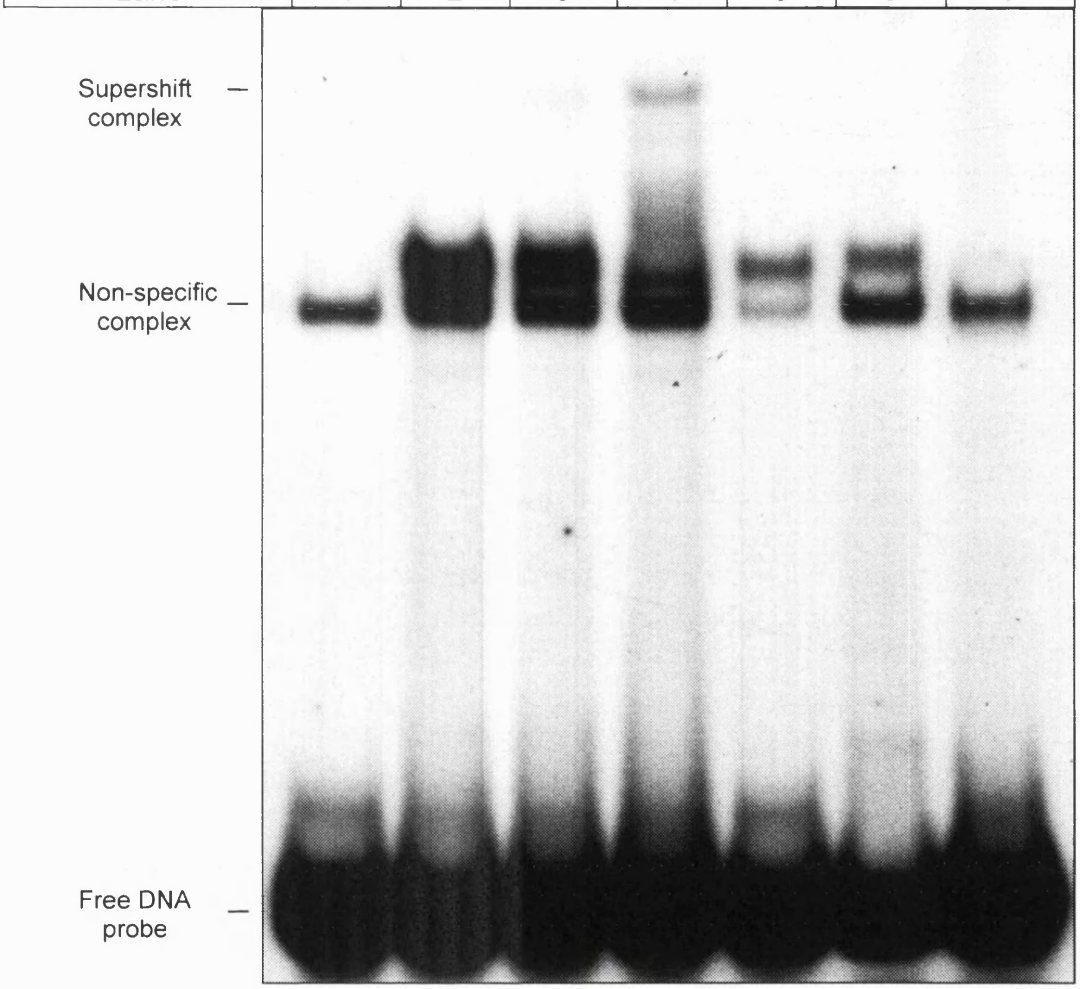



Figure 3.6.3 **Binding of *in vitro* translated PXR and RXRα to PXRE and NR1.** Gel mobility shift assay of $^{32}$P-labelled PXRE, NR1, and NR2 and *in vitro* translated PXR1 and RXRα. Lane1 = 2 μl rabbit reticulocyte lysate; lane 3 and 4 = 1 μl of antibody to PXR1 or RXRα were added after the binding reaction, respectively; lane 5 = 100-fold molar excess of vitamin D response element (VDRE) consensus oligonucleotide were added as competitors.

These results show the binding of two nuclear receptors to the same DNA motif. This suggests that the protein-DNA complexes formed between the radiolabelled NR1 oligonucleotide and liver nuclear extracts might represent more than one nuclear receptor/RXRα heterodimer, which migrate at similar rates. Sometimes the two complexes were noticed when the gel electrophoresed for a long time or in the competition assays, which partially inhibited the formation of protein-DNA complexes. This was further confirmed when the PXR1 antibody was included in the binding reaction. This antibody supershifts the complexes of liver nuclear extracts formed on the *CYP3A1* PXRE and on the *CYP2B1* NR1 element (results not shown). Because an antibody to CAR-β was not available, supershift assay of the complexes formed on the PXRE and NR1 elements by CAR-β antibody was not examined. Competition gel shift assays were carried out using liver nuclear extracts and radiolabelled NR1 or PXRE oligonucleotides. 100-fold molar excess of the NR1 oligonucleotide reduced the protein-DNA complexes formed on the *CYP3A1* PXRE. On the other hand, a 100-fold molar excess of the PXRE oligonucleotide reduced the protein complexes formed on the *CYP2B1* NR1 element (Figure 3.6.4). Taken together, these results show the capacity of two nuclear receptors, who are members of the same family, to bind to two DNA motifs; *CYP2B1* NR1 (DR-4) and *CYP3A1* PXRE (DR-3). The vitamin $D_3$ receptor (VDR) is classified in the same family (namely NR1I) of the nuclear receptor superfamily as are CAR-β and PXR1. It seemed possible therefore that the VDR could be another candidate able to bind to these two DNA motifs. When the VDRE oligonucleotides were used as a

competitor in the gel shift assay, it was able to partially inhibit protein binding to the *CYP2B1* NR1 element and the *CYP3A1* PXRE (Figure 3.6.4). In Figure 3.6.3, it was demonstrated that the VDRE oligonucleotide at 100-fold molar excess reduced the protein-DNA complexes formed by the *in vitro* translated PXR1 and RXRα on the PXRE.

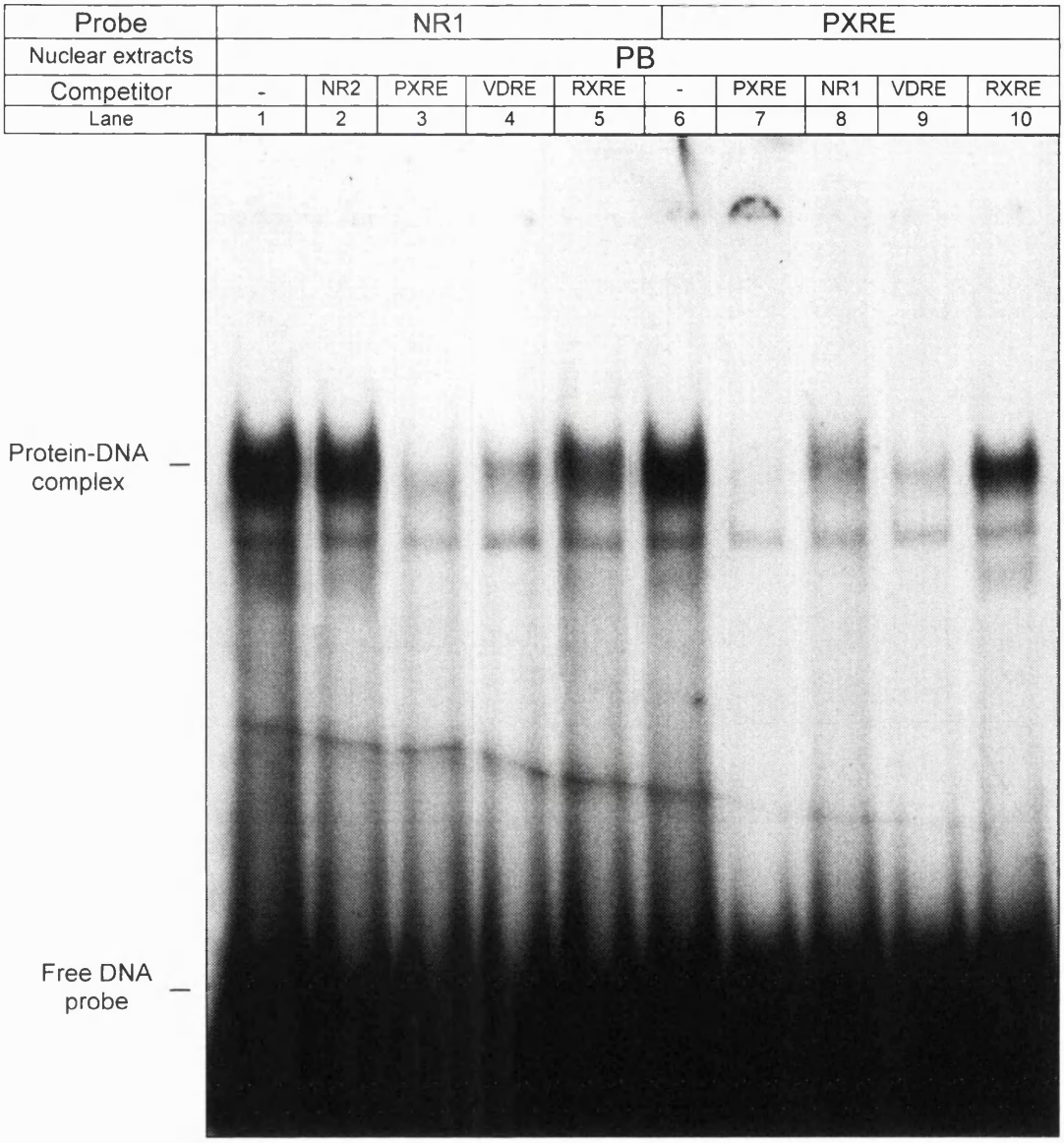



Figure 3.6.4 **Competition assay for the nuclear proteins bound to NR1 and PXRE.** Gel mobility shift assay of liver nuclear extract from PB-treated rats and radiolabelled *CYP2B1* NR1 and *CYP3A1* PXRE oligonucleotides. 100-fold molar excess of unlabelled oligonucleotides for NR2 (lane 2), NR1 (lane 8), PXRE (lane 3 and 7), VDRE (lane 4 and 9) and RXRE consensus (lane 5 and 10) were added as competitors in the gel mobility shift assay.

### 3.6.2 PXR1 and CAR-β transactivate gene expression via the *CYP2B1* PBRE

As shown in section 3.6.1, PXR1, as a heterodimer with RXRα , is able to bind the *CYP2B1* NR1 in a gel mobility shift assay. We next investigated the functional significance of this by examining whether PXR1 could transactivate the expression of a reporter gene through the *CYP2B1* NR1 upon treatment of cells with PCN and dexamethasone. The *CYP2B1* $(NR1)_2$-*SV40*-Luc reporter vector was transfected into primary hepatocytes alone or together with the expression plasmid for PXR1. Transfected cells were treated with PCN, dexamethasone or vehicle alone. The results are as shown in Figure 3.6.5. PCN increased *CYP2B1* $(NR1)_2$-*SV40*-Luc reporter gene expression ~ 3 fold. No induction was observed in the transfected cells treated with dexamethasone. Overexpressed PXR1 show little constitutive activity. PCN and dexamethasone further enhanced the activation of *CYP2B1* $(NR1)_2$-*SV40*-Luc reporter via PXR1 about 5 and 3.8 fold, respectively. Treatment of cells with PB had no effect on the PXR1-mediated transactivation (results not shown).

Transfection with the reporter construct *CYP2B1* PBRE-*SV40*-Luc and an expression plasmid for PXR1 were also carried out in primary hepatocytes. The transfected cells were treated with PCN or dexamethasone and luciferase activity measured. The results are shown in Figure 3.6.6. PCN and dexamethasone increased reporter expression about 2 and 4 fold, respectively, in the absence of added PXR1 expression

vector and 7 and 8 fold respectively when the PXR1 plasmid was co-transfected together with the reporter plasmid. Treatment with PB or picrotoxin was included as negative controls. These chemicals activate *CYP2B1* PBRE-*SV40*-Luc reporter expression over vehicle-treated transfected cells about 4, and 7 fold, respectively. In the presence of overexpressed PXR1, the transactivation of *CYP2B1* PBRE-*SV40*-Luc reporter expression upon exposure to PB and picrotoxin, is reduced to 2 and 5 fold (results not shown). The reason for the decreased induction by PB and picrotoxin of reporter expression in the presence of overexpressed PXR1 as compared to reporter-transfected cells without PXR1 may be due to the competition of overexpressed PXR1 with activated endogenous CAR-β for the same DNA-binding site. Notably, PXR1-mediated transactivation of the *CYP2B1* PBRE containing reporter is lower than that observed in response to CAR-β. This might result from the lower binding ability of PXR1 for the PBRE as demonstrated in the gel mobility shift assay as compared with CAR-β. These results support the PXR1-mediated transactivation of *CYP2B1* PBRE-*SV40*-Luc reporter expression in response to PCN, and dexamethasone.

Thus, PXR1 and CAR-β are able to bind to and transactivate gene expression via the same DNA motif, *CYP2B1* PBRE, but their effects are xenobiotic-dependent.

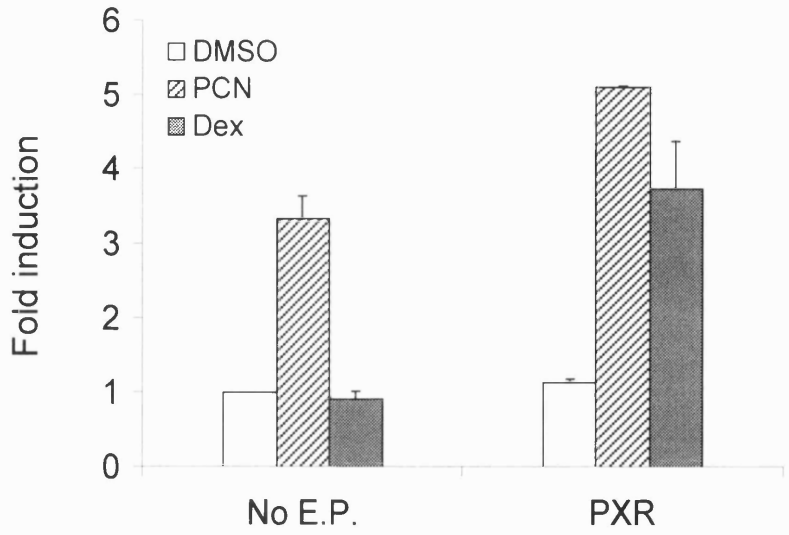



Figure 3.6.5 **PXR1 transactivates via the *CYP2B1* NR1 element in response to steroid treatment.** Primary rat hepatocytes were transfected with the reporter *CYP2B1* $(NR1)_2$-*SV40*-Luc either in the absence (No expression plasmid; No E.P.) or presence of co-transfected expression plasmid for mPXR1 as indicated. Transfected cells were treated with either vehicle alone, 0.1% dimethylsulfoxide (DMSO), 10 μM pregnenolone 16α-carbonitrile (PCN), or 20 μM dexamethasone (Dex). Results are expressed as fold induction ± S.D., where 1 represents the relative luciferase activity of the *CYP2B1* $(NR1)_2$-*SV40*-Luc reporter alone.

The results represent duplicates of two independent experiments.

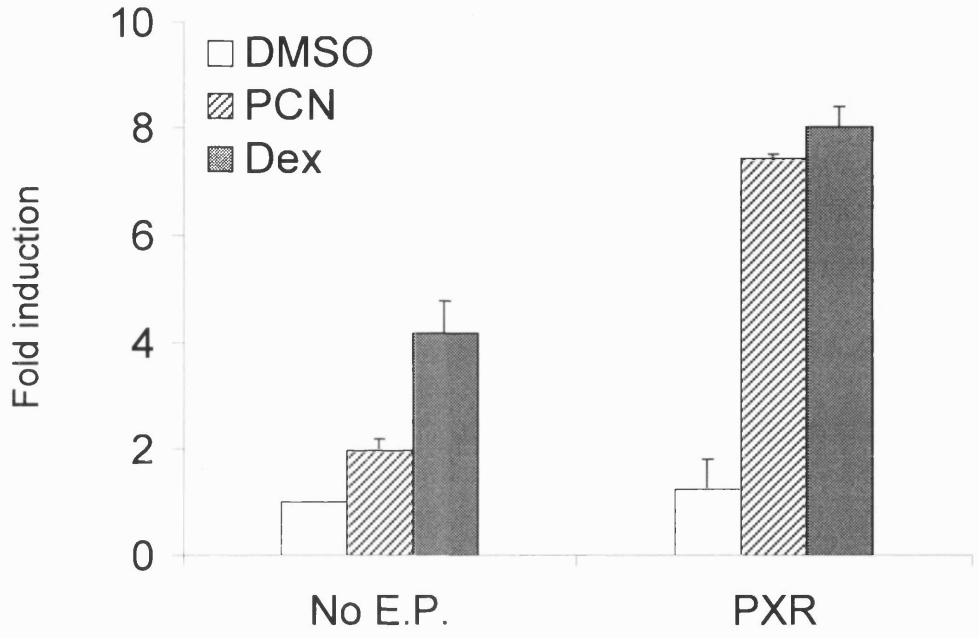



Figure 3.6.6 **PCN and dexamethasone activate *CYP2B1* PBRE-*SV40*-Luc gene expression through PXR1.** Primary rat hepatocytes were transfected with the reporter *CYP2B1* PBRE-*SV40*-Luc either in the absence (No expression plasmid, No E.P.) or presence of an expression plasmid for mPXR1 as indicated. Transfected cells were treated with either vehicle, 0.1% dimethylsulfoxide (DMSO), 10 μM pregnenolone 16α-carbonitrile (PCN) or 20 μM dexamethasone (Dex). Results are expressed as fold induction ± S.D., where 1 represents the relative luciferase activity obtained in cells transfected with a *CYP2B1* PBRE-*SV40*-Luc reporter alone.

The results represent duplicates of three independent experiments.

### 3.6.3 CAR-β and PXR1 transactivate *CYP2B1* PBRE-*SV40*-Luc reporter gene expression *in vivo*

To ensure that PB- and PCN-mediated transactivation of the *CYP2B1 PBRE* by CAR-β and PXR1 also occurred *in vivo* and is not simply the result of factors present in the cell culture medium, the transfection by biolistic particle delivery was carried out in rat liver. A *CYP2B1* PBRE-*SV40*-Luc reporter was co-transfected either in the absence or presence of the expression plasmid for CAR-β or PXR1 using the hand-held gene gun. Then, the animals were injected i.p. with either vehicle alone (0.1 % DMSO) or 100 mg/kg of PB or PCN. As shown in figure 3.6.7, without any expression plasmid, the induction of *CYP2B1* PBRE-*SV40*-Luc reporter gene expression increased 6 fold in response to PB and 2 fold in response to PCN. This is likely to be the result of transactivation via rat endogenous nuclear receptors. When CAR-β was co-expressed together with the reporter plasmid, *CYP2B1* PBRE-*SV40*-Luc, reporter activity exhibited ~ 4 fold induction above that of cells transfected with *CYP2B1* PBRE-*SV40*-Luc reporter alone. This demonstrates the constitutive transactivation activity of CAR-β in the absence of any ligand. In co-transfected cells treated with PB, CAR-β mediated reporter gene expression was increased 9 fold compared with untreated cells transfected with reporter plasmid alone. This result strongly suggests that CAR-β mediate PB induction of *CYP2B1* gene expression. The effect of CAR-β on reporter gene expression was accomplished without addition of exogenous RXRα. PCN

did not increase the CAR-β transactivation of reporter gene expression. But in the presence of co-transfected PXR1 expression plasmid, the receptor increased the expression of the reporter gene in response to PCN. As expected, PXR1 did not mediate PB-induced activation of reporter gene expression. These results, therefore, confirm those obtained from the transfection experiments carried out in primary hepatocytes culture.

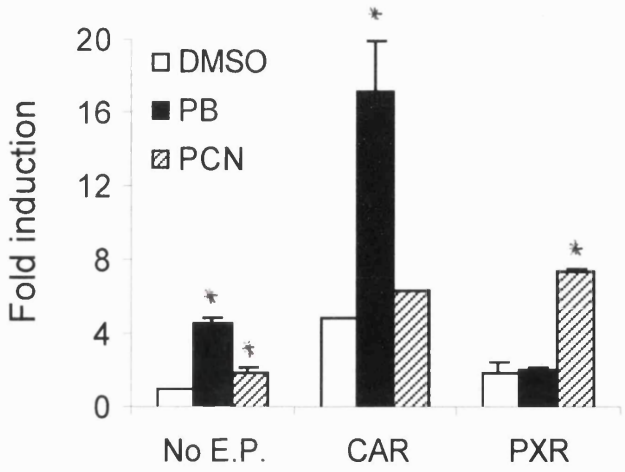



Figure 3.6.7 **Both CAR-β and PXR1 transactivate *CYP2B1* PBRE-*SV40*-Luc reporter gene expression *in vivo*.** A *CYP2B1* PBRE-*SV40*-Luc reporter was delivered to the liver of SD rats using the hand-held gene gun either in the absence of expression plasmid (No E.P.) or presence of the expression plasmids for CAR-β (CAR) or PXR1 (PXR) as indicated. The animals were injected i.p. with 100 mg/kg bodyweight of phenobarbital (PB) or pregnenolone 16α-carbonitrile (PCN) or vehicle alone, 0.1 % dimethylsulfoxide (DMSO). Results are expressed as fold induction ± S.D., where 1 represents the relative luciferase activity obtained in cells transfected with the *CYP2B1* PBRE-*SV40*-Luc reporter alone. Significantly different from the corresponding control value, $*p<0.01$ (two tail, paired Student's *t* test).

### 3.6.4 Transactivation of *CYP3A1* $(PXRE)_4$-*tk*-CAT reporter gene expression via PXR1 and CAR-β in response to xenobiotics

As both CAR-β and PXR1 increase *CYP2B1* gene expression by binding to the same site of the *CYP2B1* promoter (PBRE), we next investigated whether these two receptors activate *CYP3A1* gene expression by binding to the same site, the PXRE, within the *CYP3A1* promoter. A reporter construct, *CYP3A1* $(PXRE)_4$-*tk*-CAT, containing four copies of PXRE upstream of the thymidine kinase (tk) promoter and the chloramphenicol acetyl transferase (CAT) reporter gene was used to investigate transactivation by the nuclear receptors, PXR1 and CAR-β, in primary rat hepatocytes. The results are shown in Figure 3.6.8. In cells transfected without the expression plasmid, PCN activates *CYP3A1* $(PXRE)_4$-*tk*-CAT reporter expression about 8 fold whereas PB had no effect. When PXR1 is overexpressed, constitutive activity of reporter gene expression was observed. This may be due to the presence of endogenous steroid ligands in the culture medium. As expected, treatment of transfected cells with PCN, in the presence of overexpressed PXR1, resulted in a 20 fold, over that seen in vehicle treated cells increase in reporter expression. PB had little effect on the activation of *CYP3A1* $(PXRE)_4$-*tk*-CAT reporter via PXR1. When CAR-β is overexpressed, reporter gene activity is constitutive (2 fold). Upon treatment with PB or PCN, *CYP3A1* $(PXRE)_4$-*tk*-CAT reporter expression increased up to 20 and 5 fold, which is about 10 and 2.5 fold, respectively, above the constitutive activity of CAR-β.

Taken together, these results show that PXR1 and CAR-β bind to the same DNA motif in the *CYP3A1* promoter to activate gene expression and that CAR-β plays a role in the PB-mediated activation of *CYP3A1.*

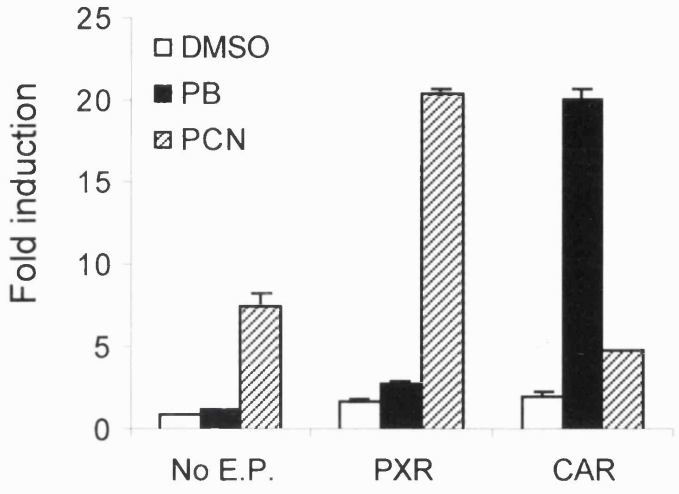



Figure 3.6.8 **PCN induces *CYP3A1* $(PXRE)_4$-*tk*-CAT expression via PXR.** Primary rat hepatocytes were transfected with a *CYP3A1* $(PXRE)_4$-*tk*-CAT reporter either in the absence (No expression plasmid, No E.P.) or presence of expression plasmids for PXR1 (PXR) or CAR-β(CAR). Transfected cells were treated with 0.1 mM phenobarbital (PB), 10 μM pregnenolone 16α-carbonitrile (PCN), or vehicle, 0.1% dimethylsulfoxide (DMSO). Results are expressed as fold induction ± S.D., where 1 represents the relative luciferase activity of cells transfected with a *CYP3A1* $(PXRE)_4$-*tk*-CAT reporter alone. The results represent duplicates of two independent experiments.

## 3.7 The effect of the steroid receptor co-activator-1 (SRC-1) in the transactivation of gene expression by the nuclear receptors, CAR-β and PXR1

Additional cofactors essential for transcriptional activation have been termed co-activators. Transcriptional interference as a result of the interaction between nuclear receptors and a common, but limiting, co-activator has been demonstrated in the case of steroid receptors such as estrogen receptor (ER), glucocorticoid receptor (GR), progesterone receptor (PR) and thyroid hormone receptor (TR) (Meyer *et al.*, 1989; Zhang *et al.*, 1996). A number of co-activators have been characterised and classified into a family of steroid receptor co-activators (SRCs). These include SRC-1, SRC-2 e.g. TIF2, GRIP1, and SRC-3 e.g. ACTR, p/CIP. The finding that SRC-1 interacts *in vitro* with the general transcription machinery factors such as TFIIB and TBP as well as with other co-activators such as CBP/p300 and p/CIP suggests that co-activators function presumably by bridging the activated nuclear receptors to the basal transcription machinery. Onate and co-workers have also shown that SRC-1 interacts with the activation domains, AF-1 and AF-2, of the steroid receptors (Onate *et al.*, 1998). Additional evidence that SRC-1 possesses intrinsic histone acetyltransferase activity suggests that activated nuclear receptors may recruit these co-activators to remodel chromatin structure,

allowing the formation of the preinitiation complex at the gene promoter (Spencer *et al.*, 1997).

In this study, the expression plasmid for SRC-1 was co-transfected into primary rat hepatocytes along with the expression plasmids for the nuclear receptors CAR-β and PXR1 and the reporter constructs containing the *CYP2B1* PBRE or the *CYP3A1* PXRE to determine if the co-activator plays a role in xenobiotic activation of *CYP* gene expression.

### 3.7.1 SRC-1 enhances the transactivation of *CYP2B1* PBRE-*SV40*-Luc reporter via CAR-β, but not via PXR1

The cDNA for SRC-1, a gift from Dr. M. Parker, was cloned into the pSG5 vector under the control of T7 and SV40 promoter. Transfection of primary rat hepatocytes was performed with a *CYP2B1* PBRE-*SV40*-Luc reporter, either in the absence or the presence of expression plasmids for SRC-1 or CAR-β, or both. Transfected cells were then treated with PB or picrotoxin. As shown in figure 3.7.1, SRC-1 increases *CYP2B1* PBRE-*SV40*-Luc reporter expression, in response to PB and picrotoxin, about 4 and 6 fold, respectively. CAR-β constitutively activates *CYP2B1* PBRE-*SV40*-Luc reporter expression 5 fold. Upon exposure to PB and picrotoxin, the transactivation by CAR-β is up to 17 and 46 fold, respectively. When both SRC-1 and CAR-βwere co-transfected, the transactivation of *CYP2B1* PBRE-*SV40*-Luc reporter in response to PB and picrotoxin is enhanced to 40 and 60 fold, respectively. But the SRC-1 effect on CAR-β-mediated transactivation is only 2 fold above that without added SRC-1, by both PB and picrotoxin. Possibly sufficient endogenous SRC-1 is present in the cells, therefore addition of exogenous SRC-1 cannot greatly enhance the transactivation effect of CAR-β.

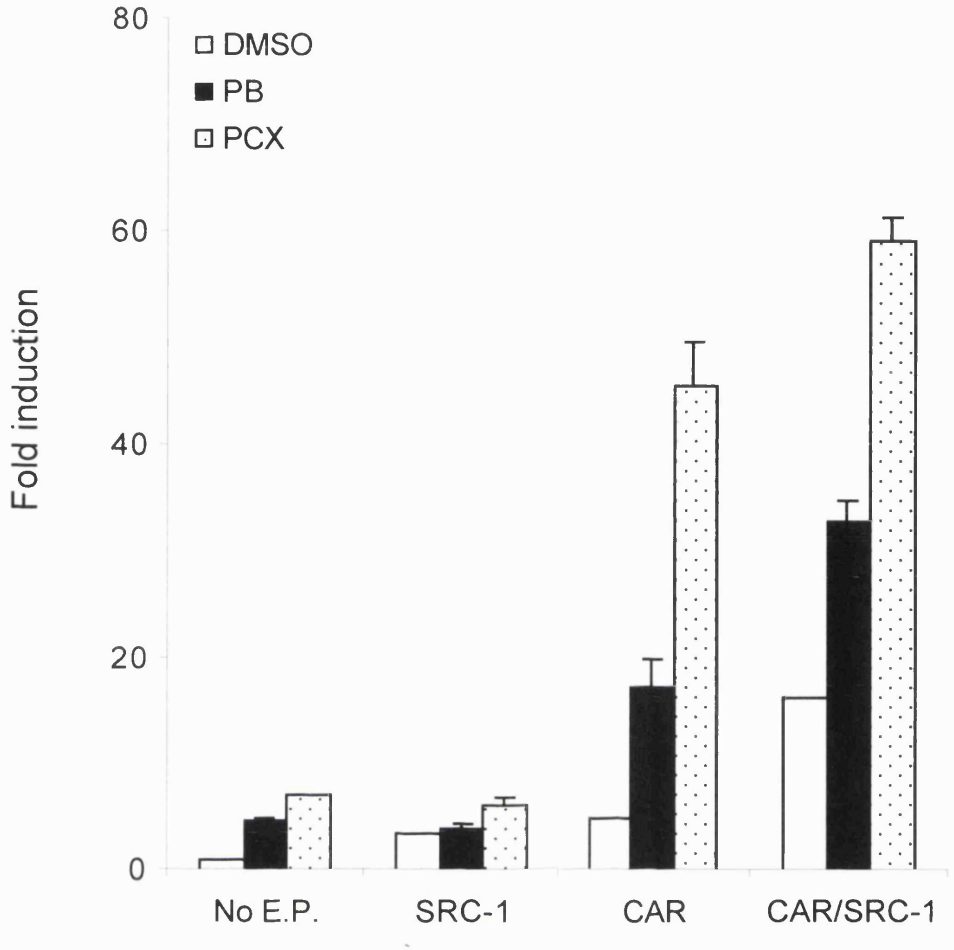



Figure 3.7.1 **SRC-1 increases CAR-β transactivation of *CYP2B1* PBRE-*SV40*-luc reporter gene expression.** Primary hepatocytes were transfected with the reporter *CYP2B1* PBRE-*SV40*-luc in the absence (No expression plasmid; No E.P.) or presence of the expression plasmids for SRC-1, CAR-β, or CAR-β and SRC-1 as indicated. Transfected cells were treated with vehicle, 0.1 % dimethylsulfoxide (DMSO), 0.1 mM sodium phenobarbital (PB), or 0.5 mM picrotoxin (PCX). Results are expressed as fold induction ± S.D., where 1 represents the relative luciferase activity of a *CYP2B1* PBRE-*SV40*-Luc reporter alone.

The results represent duplicates of three independent experiments.

Similar experiment were performed in primary rat hepatocytes with the *CYP2B1* PBRE-*SV40*-Luc reporter, in the presence of an expression plasmid for SRC-1, PXR1 or the combination of SRC-1 and PXR1. Transfected cells were treated with PCN or dexamethasone. Results are shown in figure 3.7.2. When an expression plasmid for SRC-1 was co-transfected with a *CYP2B1* PBRE-*SV40*-Luc reporter, reporter gene expression increased about 4 and 3 fold in response to PCN and dexamethasone treatment, respectively. In the presence of overexpressed PXR1, the activation of *CYP2B1* PBRE-*SV40*-Luc reporter in response to PCN and dexamethasone is increased to 7 and 8 fold, respectively. However, the overexpressed SRC-1 did not significantly enhance the activation of *CYP2B1* PBRE-*SV40*-Luc reporter via PXR1.

The results suggest that SRC-1 enhance the transactivation of *CYP2B1* expression via CAR-β, but not via PXR1. CAR-β, unlike conventional nuclear receptors, is constitutively active. The binding of the naturally occurring inverse antagonist, androstane, keeps CAR-β in an inactive state by promoting dissociation of the CAR-β and SRC-1 interaction (Forman *et al.*, 1998). When CAR-β is overexpressed and if an inverse antagonist is not sufficient to repress CAR-β, upon treatment with xenobiotics, endogenous SRC-1 as well as exogenous SRC-1 further enhances the transactivation. Our results confirm the positive effect of SRC-1 on CAR-β. Nevertheless, PXR1 does not show an interaction with SRC-1 in this study. Two contradictory evidences have been reported contradictly. In the presence of inducers, the association of PXR and SRC-

1 was demonstrated (Lehmann *et al.*, 1998). In contrast, Blumberg *et al* failed to show any association of PXR with coactivators or dissociation of co-repressors in the presence of inducers (Blumberg *et al.*, 1998).

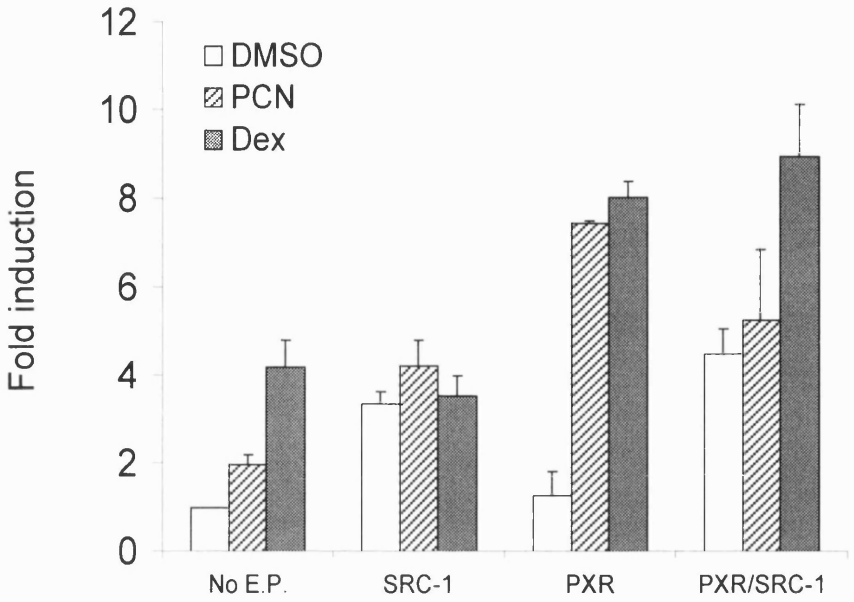



Figure 3.7.2 **SRC-1 does not effect PXR1-mediated transactivation of *CYP2B1* PBRE-*SV40*-Luc reporter in response to PCN and Dex.** Primary hepatocytes were transfected with a reporter *CYP2B1* PBRE-*SV40*-Luc either in the absence (No expression plasmid; No E.P.) or presence of the expression plasmids for SRC-1, PXR1, or PXR1 and SRC-1 as indicated. Transfected cells were treated with a vehicle alone, 0.1 % dimethylsulfoxide (DMSO), 10 μM pregnenolone 16α-carbonitrile, or 20 μM dexamethasone. Results are expressed as fold induction ± S.D., where 1 represents the relative luciferase activity of a *CYP2B1* PBRE-*SV40*-Luc reporter alone.

The results represent duplicates of three independent experiments.

### 3.7.2 SRC-1 does not enhance the transactivation of the *CYP2B1* $(NR1)_2$-*SV40*-Luc reporter

Primary rat hepatocytes were co-transfected with *CYP2B1* $(NR1)_2$-*SV40*-Luc reporter construct with the expression plasmid for CAR-β or expression plasmids for both CAR-β and SRC-1. Transfected cells were then treated with PB and picrotoxin. As shown in Figure 3.7.3, in the presence of overexpressed CAR-β, *CYP2B1* $(NR1)_2$-*SV40*-Luc reporter expression is increased in response to PB and picrotoxin about 11- and 8-fold, respectively, which is about 3- and 2-fold above constitutive activation by CAR-β. In contrast to the results obtained when the *CYP2B1* PBRE is included in the reporter construct, SRC-1 does not further enhance the transactivation of *CYP2B1* $(NR1)_2$-*SV40*-Luc by CAR-β.

Primary rat hepatocytes were co-transfected with the *CYP2B1* $(NR1)_2$-*SV40*-Luc reporter and the expression plasmids for either PXR1 or PXR1 and SRC-1. Transfected cells were treated with PCN and dexamethasone. Results are shown in Figure 3.7.4. Overexpressed PXR1 shows no constitutive activity. Upon exposure to PCN and dexamethasone, the transactivation of *CYP2B1* $(NR1)_2$-*SV40*-Luc expression is increased 5- and 4-fold, respectively. However, in the presence of overexpressed SRC-1, the PXR1-transactivation of *CYP2B1* $(NR1)_2$-*SV40*-Luc expression is not further enhanced upon treatment of PCN or dexamethasone.

These results suggest that the association of SRC-1 with CAR-β require sequences other than NR1, to stabilise the complex formed, and the recruitment of other transcription factors. PXR1 shows little, if any, interaction with SRC-1

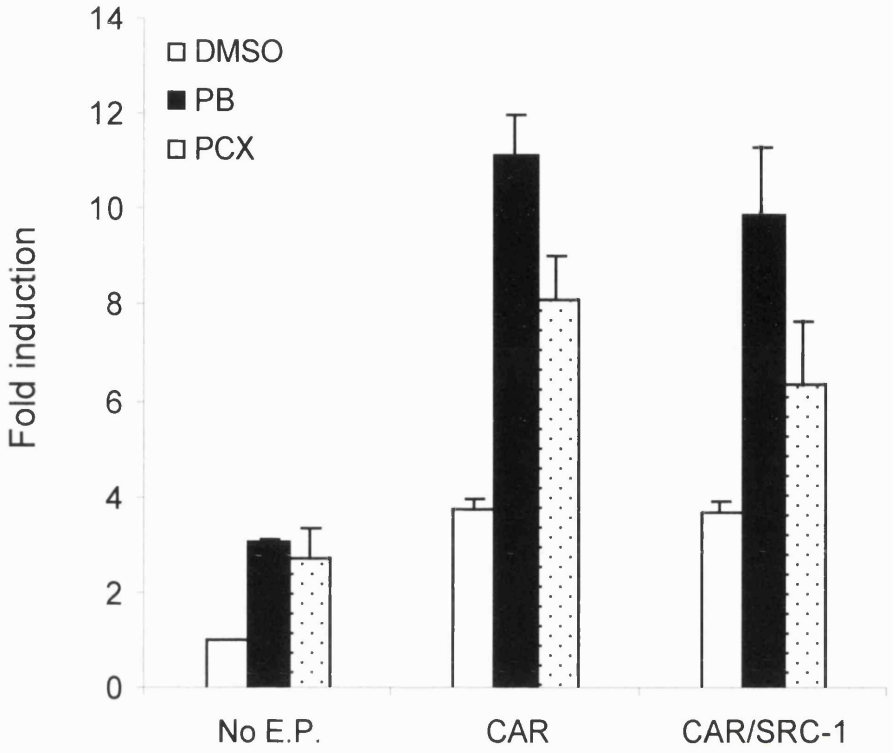



Figure 3.7.3 **SRC-1 does not increase CAR-β transactivation of the reporter plasmid *CYP2B1* $(NR1)_2$-*SV40*-luc.** Primary hepatocytes were transfected with the reporter *CYP2B1* $(NR-1)_2$-*SV40*-Luc either in the absence (No expression plasmid; No E.P.) or presence of the expression plasmids for CAR-β(CAR) or CAR-β and SRC-1 (CAR/SRC-1) as indicated. Transfected cells were treated with a vehicle alone, 0.1 % dimethylsulfoxide (DMSO), 0.1 mM phenobarbital (PB), or 0.5 mM picrotoxin (PCX). Results are expressed as fold induction ± S.D., where 1 represents the relative luciferase activity of a *CYP2B1* $(NR1)_2$-*SV40*-Luc reporter alone.

The results represent duplicates of two independent experiments.

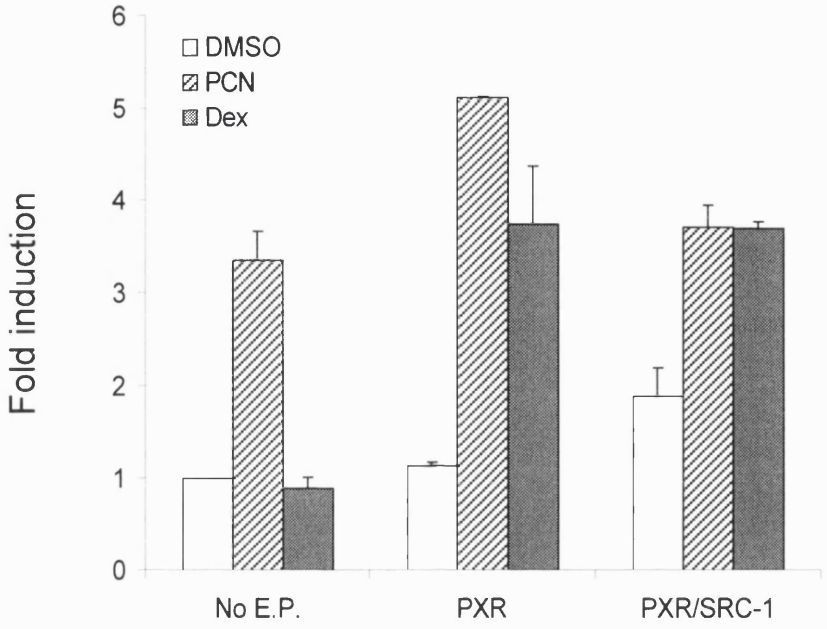



Figure 3.7.4 **SRC-1 does not further enhance *CYP2B1* $(NR1)_2$-*SV40*-Luc reporter activation by PXR.** Primary hepatocytes were transfected with a *CYP2B1* $(NR1)_2$-*SV40*-Luc reporter either in the absence (No expression plasmid; No E.P.) or presence of co-transfected expression plasmid for mPXR1 or mPXR1 and SRC-1. Transfected cells were treated with vehicle, 0.1 % dimethylsulfoxide (DMSO), 10 μM pregnenolone 16α carbonitrile (PCN), or 20 μM dexamethasone (Dex). Results are expressed as fold induction ± S.D., where 1 represents the relative luciferase activity of a *CYP2B1* $(NR1)_2$-*SV40*-Luc reporter alone.

The results represent duplicates of two independent experiments.

### 3.7.3 SRC-1 interacts with CAR-β but not PXR1, in the transactivation of the *CYP3A1* $(PXRE)_4$-*tk*-CAT reporter

The effect of SRC-1 on the transactivation of *CYP3A1* $(PXRE)_4$-*tk*-CAT reporter by PXR1 and CAR-β in primary hepatocytes was investigated. Cells were co-transfected with *CYP3A1* $(PXRE)_4$-*tk*-CAT reporter and the expression plasmids for either PXR1, SRC-1, or both PXR1 and SRC-1. Transfected cells were then treated with PB or PCN. Results are shown in Figure 3.7.5. Overexpressed SRC-1 has little effect on the transactivation of *CYP3A1* $(PXRE)_4$-*tk*-CAT reporter in response to PB or PCN, as compared to the cells transfected with a reporter alone. In the presence of overexpressed PXR1, *CYP3A1* $(PXRE)_4$-*tk*-CAT expression is activated, in response to PCN, up to 20 fold. However, overexpressed SRC-1 does not further enhance the transactivation of *CYP3A1* $(PXRE)_4$-*tk*-CAT via PXR1 by PCN. Neither PB nor SRC-1 activates *CYP3A1* $(PXRE)_4$-*tk*-CAT expression via PXR1.

The effect of SRC-1 on CAR-β-mediated transactivation of *CYP3A1* $(PXRE)_4$-*tk*-CAT expression in response to PB or PCN was also investigated. As shown in Figure 3.7.6, overexpressed SRC-1 has little effect on the transactivation of *CYP3A1* $(PXRE)_4$-*tk*-CAT reporter in response to PB or PCN, as compared to the cells transfected with reporter alone. In the presence of overexpressed CAR-β, the transactivation of *CYP3A1* $(PXRE)_4$-*tk*-CAT is increased 20 fold in response to PB. In the

presence of both overexpressed SRC-1 and CAR-β, transactivation of *CYP3A1* $(PXRE)_4$-*tk*-CAT is enhanced 2 fold.

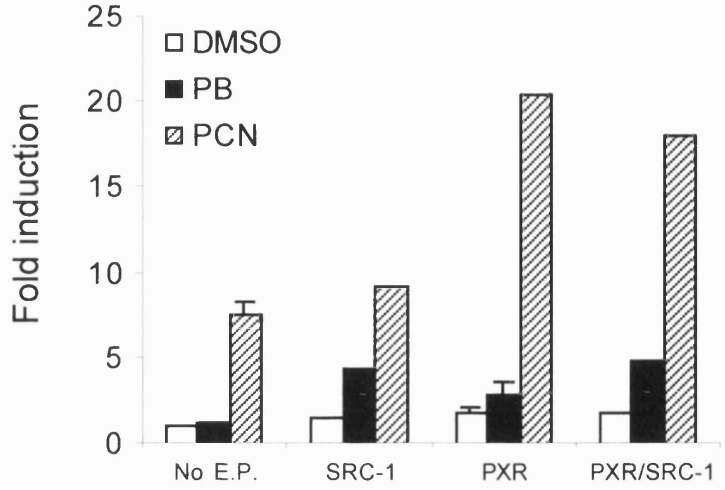



Figure 3.7.5 **SRC-1 has no effect on PXR-mediated transactivation of *CYP3A1* (PXRE)$_4$-*tk*-CAT reporter in response to PCN.** Primary hepatocytes were transfected with a reporter *CYP3A1* (PXRE)$_4$-*tk*-CAT either in the absence (No expression plasmid; No E.P.) or presence of the expression plasmids for SRC-1, PXR1 (PXR), or PXR1 and SRC-1 as indicated. Transfected cells were treated with vehicle, 0.1 % dimethylsulfoxide (DMSO), 0.1 mM phenobarbital (PB), or 10 μM pregnenolone 16α-carbonitrile. Results are expressed as fold induction ± S.D., where 1 represents the relative luciferase activity of cells transfected with the *CYP3A1* (PXRE)$_4$-*tk*-CAT reporter alone. The results represent at least duplicates of two independent experiments.

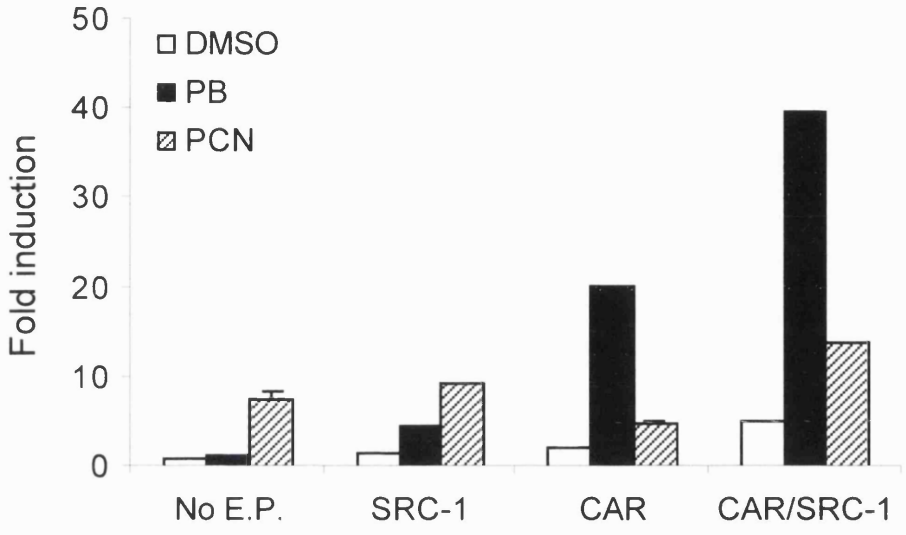



Figure 3.7.6 **SRC-1 further enhances CAR-β-mediated trans-activation of *CYP3A1* $(PXRE)_4$-*tk*-CAT reporter in response to PB.** Primary hepatocytes were transfected with a reporter *CYP3A1* $(PXRE)_4$-*tk*-CAT either in the absence (No expression plasmid; No E.P.) or presence of the expression plasmids for SRC-1, CAR-β, or CAR-β and SRC-1 as indicated. Transfected cells were treated with vehicle, 0.1 % dimethylsulfoxide (DMSO), 0.1 mM phenobarbital (PB) or 10 μM pregnenolone 16α-carbonitrile (PCN). Results are expressed as fold induction ± S.D., where 1 represents the relative luciferase activity of the *CYP3A1* $(PXRE)_4$-*tk*-CAT reporter alone. The results represent at least duplicates of two independent experiments.

# CHAPTER 4

# General discussion

Studies of the mechanism(s) underlying the regulation of gene expression have made great progress. Gene expression is an ordered and regulated process in response to an enormous variety of signals. Xenobiotic-induced cytochrome P450 gene expression provides a useful model to study mechanistic details of how genes are regulated at the transcriptional level by the incoming signals. This study supports the role of nuclear receptors in mediating transcriptional activation of *CYP* genes. Xenobiotic-responsive elements upstream of the *CYP* genes in families 2, 3 and 4, that are involved in the metabolism of foreign compounds are organised as repeats of two hexameric half-sites. This type of DNA element is typical of nuclear receptor binding sites, which in turn, determines the nuclear receptor assembly as dimers (Umesono *et al.*, 1991). Despite the large number of nuclear receptors, over 150 identified to date, only two types of consensus hexameric half-sites are present. They are 5'-AGAACA-3' and 5'-AGGTCA-3'. The binding of specific nuclear receptors, either homodimers or heterodimers, is determined by the sequence, orientation and spacing of these types of two hexameric half-sites. For example, receptors such as GR, MR, PR and AR bind to palindromic repeats of the former type, whereas ER, RXR, TR, VDR, RAR, PPAR, COUP-TF and NGFI-B preferentially bind to direct repeats of the latter type (Kliewer *et al.*, 1992; Perlmann *et al.*, 1993; Umesono *et al.*, 1991). The transcriptional activation

by these nuclear receptors shares a common mechanism in that ligand binding is required to convert an inactive receptor to an active form. By interaction with a specific response element, the activated receptor mediates its signal through a conformational change of the activation function (AF) domains, which results in the recruitment of co-activators and the basal transcription machinery. Point mutations within a 17-bp core region of AF-2 domain have been reported to decrease or abolish ligand-dependent activation, even though the ligand binding, DNA binding, and dimerisation property remained unaffected (Danielian *et al.*, 1992). A number of laboratories have identified the targets that AF-1 and AF-2 domains interact with, by either using a yeast two-hybrid assay or *in vitro* protein-protein interaction between the recombinant affinity-tagged nuclear receptors and radiolabelled basal initiation factors and vice versa. The results, including the interaction of the AF-2 of RXR specifically with the TATA binding protein (TBP) (Schulman *et al.*, 1995) and of both AF-1 and AF-2 of ER with TBP (Sadovsky *et al.*, 1995) reveal direct contact between nuclear receptors and basal initiation factors. However, the identification of many co-activators strongly support a role for these proteins as mediators or bridging molecules that facilitate the interaction between nuclear receptor complexes formed on upstream regions and the basal initiation factors on the proximal promoter (Halachmi *et al.*, 1994; Onate *et al.*, 1995).

The direct repeat type with a 4 bp-spacer of a core PB-responsive element (PBRE) of the *CYP2B1* gene has been shown, by the gel mobility

shift assay, to accommodate heterodimers formed between CAR-β and RXRα, with presumably RXRα occupying the 5' half site and CAR-β the 3' half site. Notably, this binding occurs even though no ligand is supplied. Moreover, in an *in vitro* gel shift experiment, although the on and off rate is slow, protein-DNA complex formation is driven under conditions of excess nuclear receptors and DNA binding site. In contrast, *in vivo* a few DNA response elements are present amongst the large excess of genomic DNA, which may prevent the binding of nuclear receptors to the DNA target. Addition of ligand, which activates the receptor, may result in enhanced on and off rates and a scanning process of genomic DNA for a cognate binding site (Beato *et al.*, 1991).

This study provides evidence that PB treatment directly enhances CAR-β mediated transactivation in primary rat hepatocytes and *in vivo.* In HepG2 cells, although CAR-β displayed constitutive activity on reporter gene expression, the direct effect of PB on CAR's activity was not shown. Honkakoski *et al.* showed, using a green fluorescent protein, (GFP)-CAR-β expression plasmid transfected into HepG2 cells, that CAR-β was localised to the nucleus in the absence of PB. In contrast, by immunostaining of liver tissue from a PB-treated mouse using a CAR-β antibody, the receptor was shown to reside in the cytoplasm, and to translocate to the nucleus upon exposure to PB (Kawamoto *et al.*, 1999). These workers suggested that the mechanisms for regulating *CYP2B* genes differ between HepG2 cells and primary hepatocytes (Honkakoski *et al.*, 1998b). In the present study, constitutive activity of CAR-β on a PBRE-containing reporter gene was

observed in transfected primary hepatocytes, which implies the presence of CAR-β in the nucleus. The sub-cellular localisation of many unliganded nuclear receptors proves to be controversial depending on the method used for analysis. VDR, which belongs to the same family as CAR-β, has been shown, in the absence of ligand, to localise predominantly in the nucleus but with a significant amount of the receptor in the cytoplasm. The presence of its ligand, 1,25-hydroxyvitamin D3, promotes VDR nuclear translocation (Michigami *et al.*, 1999). Other evidence argues that nuclear receptors shuttle between the nucleus and cytoplasm to maintain an equilibrium (DeFranco, 1998). One possible explanation for our transfection results is that overexpressed CAR-β might shift the equilibrium toward the nuclear import after being synthesised in the cytoplasm. Upon PB treatment, some of the CAR-β, which is retained in the cytoplasm, might translocate to the nucleus. Another possibility is that overexpressed CAR-β might titrate out any inhibitory factor(s) that keep CAR-β inactive. However, our gel shift results, which showed no increase in protein binding with nuclear extracts from PB-treated hepatocytes as compared to untreated ones, do not support the model of PB-induced CAR-β translocation to the nucleus. Mechanism(s) other than, or in addition to, PB-induced nuclear translocation of CAR-β may exist to regulate *CYP2B1* expression.

The constitutive activity of CAR-β is inhibited by the inverse agonist ligand, androstane, which promotes dissociation of co-activators from

CAR-β(Forman *et al.*, 1998). It is possible therefore that PB either displaces or prevents the formation of, an inhibitory ligand that would normally repress CAR-β. The $IC_{50}$ of 3α-androstenol required to inhibit CAR's activity is much higher than the physiological level of this compound (Gower and Ruparelia, 1993). Therefore, a related, more potent steroid, which is possibly a metabolite of CYP metabolism, may be a true inverse agonist ligand for CAR-β. It will be of interest to determine whether compounds such as PB and picrotoxin or other PB-like inducers, bind directly to CAR-β, either at an allosteric site that would cause dissociation of the inhibitor, or if these chemicals displace the CAR-β inhibitory compound from the ligand binding pocket. It will also be interesting to investigate if the pleitropic effects of PB in the liver, including the induction of many other genes, are mediated by CAR-β.

*In vivo*, the *CYP* genes are embedded in a complex structure of chromatin in such a way that their expression is suppressed. Also sequences, either upstream or downstream of *CYP* genes, may contribute to the negative control of *CYP* gene expression. In transfection experiments, a reporter vector is not constrained by correct chromatin formation and the reporter DNA construct lacks other upstream or downstream DNA sequences that may influence gene expression. This could explain the high basal expression observed of a reporter gene under the control of the PBRE, which is in contrast to the *in vivo* situation where *CYP2B1* expression is barely detectable in the liver. The consequence of high basal expression is the low PB induction observed in primary

hepatocytes (5-8 fold) as compared to 20-100 fold in animals. In addition, the transfection efficiency and the nature and quality of transfected DNA affect the results obtained. Addition of more DNA does not necessarily improve transfection efficiency (results not shown). It may depend on how much of the DNA remains supercoiled after transfection into the cells. Studies by Pina and colleagues showed that only 2-3% of transfected DNA is in the supercoiled form, most of the transfected DNA is either nicked or linearised promptly after transfection (Pina *et al.*, 1990).

In this study, the co-activator SRC-1 is shown to stimulate CAR-β-mediated transactivation of gene expression via the PBRE. Although the NR1 site of the PBRE is sufficient to support transactivation by CAR-β, additional sequences within the PBRE are required for the stimulation of this activity by SRC-1, and thus for the maximal induction of CYP2B1 in response to xenobiotics. That sequences other than NR1 may be important for the function of the PBRE has been suggested by the results of *in vivo* DNase I footprinting experiments (Kim and Kemper, 1997). These showed that, in rat liver, a footprint centered on the NF-1 site of the PBRE expanded, in response to treatment of animals with PB, to encompass the whole of the adjacent NR1 and NR2 sites. In this study, the NR2 site of the PBRE is shown to bind RXRα as a heterodimer with an unidentified nuclear recptor. This unknown receptor may facilitate the interaction between CAR-β, bound to the NR1 site, and SRC-1. Mutations of the NF-1 site of the PBRE reduce responsiveness to PB of a reporter gene in primary hepatocytes (Liu *et al.*, 1998; Stoltz *et al.*, 1998) but not in

transgenic mice (Ramsden *et al.*, 1999). The contribution of the NF-1 site to the function of the PBRE is thus unclear.

Based on the evidence obtained so far, Kemper has proposed two models for PB induction of mammalian *CYP* genes (Kemper, 1998). Model 1 is based on the finding of the DNase I footprint of the PBRE and proximal promoter in untreated animals. These pre-bound factors are suppressed, possibly by negative regulators or by chromatin structure. Kemper proposes that PB alter proteins bound, possibly by changing the binding affinity of the pre-bound proteins to the PBRE, resulting in changes in chromatin structure and as a consequence recruitment of the basal transcription machinery. In the model 2, chromatin is in a closed conformation, which allows no binding of factors in both proximal and distal regions. PB then causes a change in chromatin structure at the PBRE, which allows the binding of factors such as NF-1 and other regulatory proteins, resulting in the recruitment of basal transcription factors to the proximal promoter. A model showing the factors we have identified in playing a role in *CYP2B1* gene regulation is shown in Figure 4.1. A recent study showed no protein binding in the native chromatin structure of the kidney where CYP2B1/2 is not expressed, as compared to changes of protein binding to the PBRE and the proximal promoter in the liver chromatin upon PB treatment (Kim *et al.*, 2000). This emphasises the PBRE as a key element for PB responsiveness; as remodelling the chromatin structure facilitates communication between the PBRE and the proximal promoter of the *CYP2B* gene, hence causing gene activation. In

this study we used biolistic particle mediated gene transfer to show the functional importance of the PBRE *in vivo* and the role played by CAR-β and PXR1 in the regulation of *CYP2B1* gene expression. The technology of transgenic mice will provide the reassurance of the PBRE's functional importance in CYP2B1 induction by PB. However, there may be some other elements further upstream of the PBRE (or downstream of the gene) that might play a role in supporting and co-operating to produce the maximal PB-response.

Finally, in this study we have shown that two different nuclear receptors are able to bind to and mediate gene activation via the same DNA element. This provides information that may explain how different chemicals induce the same CYP or how a single chemical induces more than one CYP, to different extents. It seems that organisms have evolved not only CYPs that can metabolise a tremendous range of xenobiotics, but also promiscuous nuclear receptors that regulate *CYP* gene expression in response to foreign chemicals.

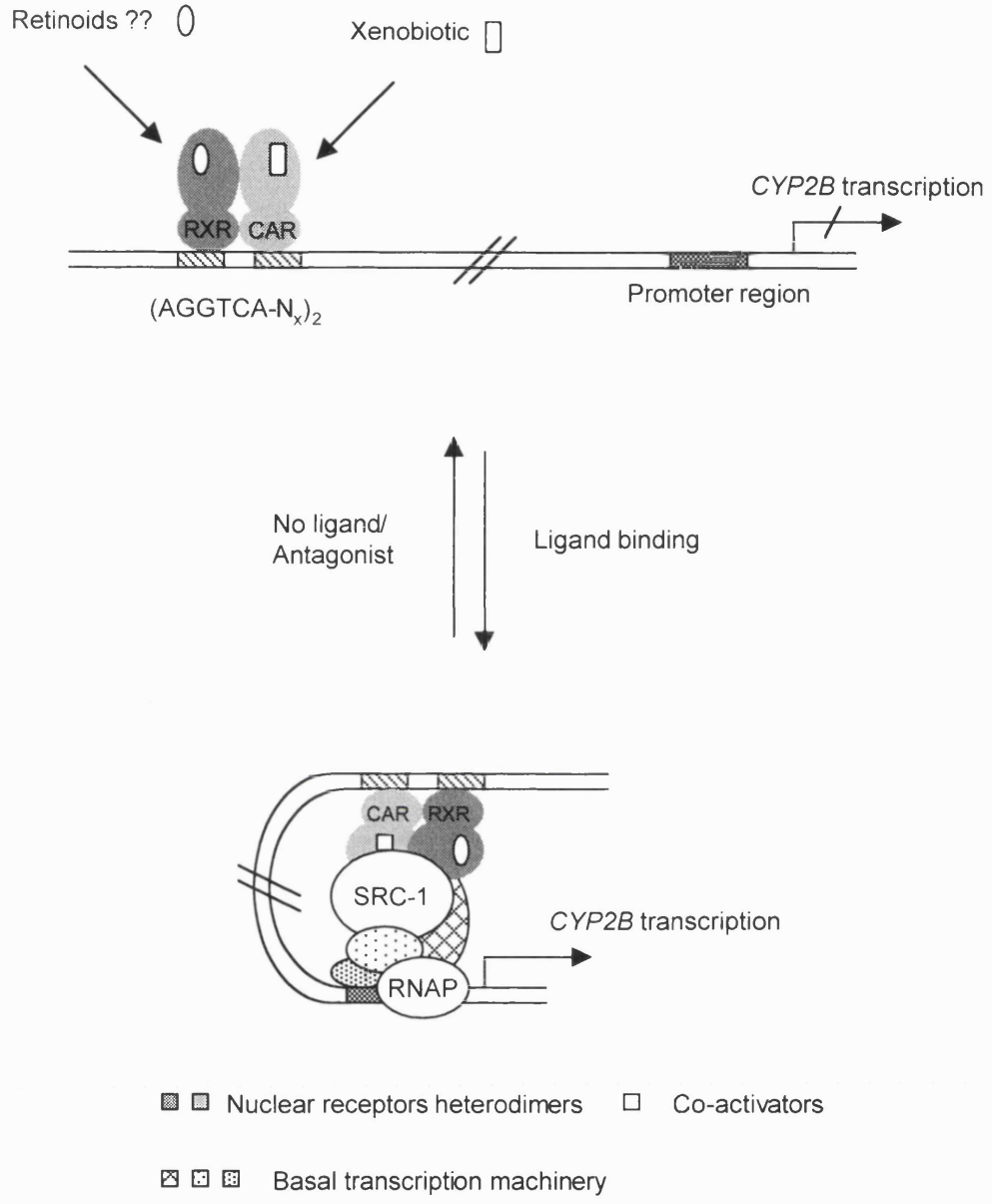



Figure 4.1 **Model of nuclear receptors-mediated *CYP2B* gene expression in response to xenobiotics.** Ligand binding induces conformational change of the receptors, followed by recruitment of the co-activators, which serve as a bridge to interact with the basal transcription machinery at the proximal promoter. RNAP = RNA polymerase (Adapted from Kemper, 1998)

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